Inhibition of Vascular Endothelial Growth Factor-induced Receptor Activation with Anti-Kinase Insert Domain-containing Receptor Single-Chain Antibodies from a Phage Display Library

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Abstract

A single-chain antibody phage display library was constructed from spleen cells of mice immunized with a soluble form of a human vascular endothelial growth factor (VEGF) receptor, kinase insert domain-containing receptor (KDR). After two rounds of biopanning, >90% of the clones recovered were specifically reactive to KDR. Subsequent selection identified two clones that blocked VEGF binding to KDR. The clones were expressed in Escherichia coli and purified as soluble single-chain Fv (scFv) antibodies. The affinities of the scFv for binding to KDR were determined by BIACore analysis (2.1 × 10^{-5}-5.9 × 10^{-7} M). One scFv, p1C11, was shown to inhibit VEGF-induced KDR phosphorylation and VEGF-stimulated DNA synthesis in human umbilical vein endothelial cells. There is much experimental evidence to suggest that the VEGF/KDR/Flk-1 pathway plays an important role in tumor angiogenesis, a process that is essential for tumor growth and metastasis. The antibodies described here, which block VEGF binding to KDR, have potential clinical application in the treatment of cancer and other diseases where pathological angiogenesis is involved.

Introduction

Angiogenesis, a multistep process that results in new blood vessel formation from preexisting vasculature (see Ref. 1 for review), is essential for both the growth of solid tumors beyond the size of 2 mm³ and for tumor metastasis (2, 3). A number of growth factors, e.g., VEGF, bFGF, and angiogenin have been implicated as possible regulators in this process (4, 5). VEGF is distinct among these factors in that it acts as an endothelial cell-specific mitogen during angiogenesis (6–8). Its receptor, KDR/Flk-1 (also known as VEGF receptor 2), is expressed on endothelial cells and hematopoietic cells (9, 10). The level of the receptor has been shown to be up-regulated by VEGF in several tumors and confined to the vascular endothelial cells in close proximity to the tumors (11). Endothelial cells transfected with a dominant-negative mutant of the KDR/Flk-1 receptor inhibited glioblastoma growth in vivo (12). Taken together, these findings suggest that the VEGF/KDR/Flk-1 pathway plays an important role in tumor angiogenesis (6, 7, 13). This role is further supported by studies showing inhibition of tumor growth in nude mice by anti-VEGF antibodies (14), anti-Flk-1 antibodies (15, 16), anti-VEGF antisense RNA expression (17), and VEGF-diphtheria toxin conjugate (18).

We previously reported the production of a rat anti-Flk-1 Mab, DC101, by hybridoma technology and demonstrated that this antibody could specifically inhibit VEGF stimulation of Flk-1 receptor expression on transfected 3T3 cells (19). Furthermore, DC101 strongly inhibited growth of human tumor xenografts in nude mice by blocking the tumor-associated angiogenesis (15, 16). DC101 does not, however, react with KDR, the human homologue of murine Flk-1; therefore, it is not a suitable candidate for potential human therapy. Here, we describe the production and characterization of scFv anti-KDR antibodies isolated from an antibody phage display library. One scFv, p1C11, binds to KDR (but not to Flk-1) with high affinity (2.1 nm), blocks VEGF binding to KDR, and shows potent inhibition of VEGF-induced receptor phosphorylation and DNA synthesis in HUVECs.

Materials and Methods

Cell Lines and Proteins. Primary-cultured HUVECs were obtained from Dr. S. Rafii at Cornell Medical Center (New York, NY) and maintained in EBM-2 medium (Clonetics, Walkersville, MD) at 37°C and 5% CO₂. Cells were used between passages 2 and 5 for all assays. VEGF165 protein was expressed in baculovirus and purified following the procedures described (20).

cDNA encoding the extracellular domain of KDR was isolated by RT-PCR from human fetal kidney mRNA (Clontech, Palo Alto, CA) and subcloned into the BglII and BapEl sites of the vector AP-Tag (21). In this plasmid, the cDNA for KDR extracellular domain is fused in-frame with the cDNA for human placental AP. The plasmid was electroplated into NIH 3T3 cells together with the neomycin expression vector pSV-Neo, and stable cell clones were selected with G418. The soluble fusion protein KDR-AP was purified from cell culture supernatant by affinity chromatography using immobilized Mab to AP (Medix Biotech, Inc., Foster City, CA).

Immunization of Mice and Construction of a scFv Antibody Phage Display Library. Female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were given two i.p. injections of 10 μg of KDR-AP in 200 μl of RIBI Adjuvant System (RIBI Immunochem Research, Inc., Hamilton, MT), followed by one i.p. injection without RIBI adjuvant over a period of 2 months. The mice were also given a s.c. injection of 10 μg of KDR-AP in 200 μl of RIBI solution at the time of the first immunization. The mice were boosted i.p. with 20 μg of KDR-AP 3 days before euthanasia. Spleens from donor mice were removed and the cells were isolated. Total splenocyte RNA was first extracted with a kit from Ambion (Austin, TX), and mRNA was purified from total RNA with the Quickprep mRNA kit (Pharmacia Biotech, Piscataway, NJ). A scFv phage display library was constructed using the mRNA with the use of the Recombinant Phage Antibody System (Pharmacia Biotech) following the manufacturer’s protocol. The assembled scFv DNA was ligated into the pCANTAB 5E vector using SfiI/Nool restriction sites and electroporated into TG1 cells. The transformed TG1 cells were plated onto 2YTAG plates (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter medium, plus 100 μg/ml ampicillin and 2% glucose) and incubated overnight at 30°C.
colonies were scraped into 10 ml of 2YT medium, mixed with 5 ml 50% glycerol, and stored at -70°C as the library stock.

**Biopanning.** The library stock was grown to logarithmic phase, reseeded with M13K07 helper phage, and amplified overnight in 2YTAK medium (2YT containing 100 μg/ml ampicillin and 50 μg/ml kanamycin) at 30°C. The phage preparation was precipitated in 4% PEG/0.5 M NaCl, resuspended in 3% fat-free milk/PBS containing 500 μg/ml AP protein, and incubated at 37°C for 1 h to capture phage displaying anti-AP scFv and to block other non-specific binding.

KDR-AP (10 μg/ml)-coated Maxiisorp Star tubes (Nunc, Roskilde, Denmark) were first blocked with 3% milk-PBS at 37°C for 1 h and then incubated with the phage preparation at RT for 1 h. The tubes were washed 10 times with PBST, followed by 10 washes with PBS. The bound phage was eluted at RT for 5 min with 1 ml of a freshly prepared solution of 0.5 M triethylamine (Sigma Chemical Co., St. Louis, MO). The eluted phage were incubated with 10 ml of mid-logarithmic-phase TG1 cells at 37°C, first stationary (30 min) and then shaking (30 min). The infected TG1 cells were then plated onto 2YTAG plates and incubated overnight at 30°C.

**Phage ELISA.** Individual TG1 clones were grown at 37°C in 96-well plates and rescued with M13K07 helper phage as described above. The amplified phage preparation was blocked with 1/6 volume of 18% milk-PBS at RT for 1 h and added to Maxi-sorp 96-well microtiter plates (Nunc) coated with KDR-AP or AP (1 μg/ml × 100 μl). After incubation at RT for 1 h, the plates were washed three times with PBST and incubated with a rabbit anti-M13 phage antibody-HRP conjugate (Pharmacia Biotech). The plates were washed five times, TMB peroxidase substrate (KPL, Gaithersburg, MD) was added, and the A450 read using a microplate reader (Molecular Devices, Sunnyvale, CA).

DNA BstNI Pattern Analysis and Nucleotide Sequencing. The diversity of the KDR binders after each round of selection was analyzed by restriction enzyme digestion patterns (i.e., DNA fingerprinting). The scFv insert of representative clones from each digestion pattern were determined by dideoxynucleotide sequencing.

**Preparation of Soluble scFv.** Phage of individual clones were used to infect a nonsuppressor Escherichia coli host HB2151, and the infectant was selected on 2YTAG-N plates (2YTAG containing 100 μg/ml nalidixic acid; Sigma). Expression of scFv in HB2151 cells was induced by culturing the cells in 2YT medium containing 1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) at 30°C. A periplasmic extract of the cells was prepared by resuspending the cell pellet in 25 mM Tris (pH 7.5) containing 20% (w/v) sucrose, 200 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, followed by incubation at 4°C with gentle shaking for 1 h. After centrifugation at 15,000 rpm for 15 min, the soluble scFv was purified from the supernatant by affinity chromatography using the RPAS Purification Module (Pharmacia Biotech).

**Quantitative KDR Binding Assay.** Two assays were used to examine quantitatively the binding of purified soluble scFv to KDR. In the direct binding assay, various amounts of soluble scFv were added to KDR-coated 96-well Maxi-sorp microtiter plates and incubated at RT for 1 h, after which the plates were washed three times with PBST. The plates were then incubated at RT for 1 h with 100 μl of mouse anti-E tag antibody (Pharmacia Biotech), followed by incubation with 100 μl of rabbit anti-mouse antibody-HRP conjugate (Biosource, Camarillo, CA). The plates were washed and developed following the procedure described above for the phage ELISA. In another assay, the competitive VEGF blocking assay, various amounts of soluble scFv were mixed with a fixed amount of KDR-AP (50 ng) and incubated at RT for 1 h. The mixture were then transferred to 96-well microtiter plates coated with VEGF165 (200 ng/ml) and incubated at RT for an additional 2 h, after which the plates were washed five times and the substrate for AP (p-nitrophenyl phosphate; Sigma) was added to quantify the bound KDR-AP molecules. IC50, i.e., the scFv concentration required for 50% inhibition of KDR binding to VEGF, was then calculated.

**Phosphorylation Assay.** Subconfluent HUVECs were grown in growth factor-depleted EBM-2 medium for 24-48 h prior to experimentation. After pretreatment with 50 nM sodium orthovanadate for 30 min, the cells were incubated in the presence or absence of antibodies (5 μg/ml) for 15 min, followed by stimulation with 20 ng/ml VEGF165 or 10 ng/ml bFGF (R&D Systems, Minneapolis, MN) at RT for an additional 15 min. The cells were then lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 0.25% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin; pH 7.5), and the cell lysate was used for both the KDR and MAP kinase phosphorylation assays. The KDR was immunoprecipitated from the cell lysates with Protein A-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) coupled to an anti-KDR antibody, Mab 4.13 (ImClone Systems). Proteins were resolved with SDS-PAGE and subjected to Western blot analysis. To detect KDR phosphorylation, blots were probed with an anti-phosphotyrosine Mab, PY20 (ICN Biomedicals, Aurora, OH). For the MAP kinase activity assay, cell lysates were resolved with SDS-PAGE followed by Western blot analysis using a phophospecific MAP kinase antibody (New England Biolabs, Beverly, MA). All signals were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL). In both assays, the blots were reprobed with a polyclonal anti-KDR antibody (ImClone Systems) to assure that equal amount of protein was loaded in each lane of SDS-polyacrylamide gels.

**Antimigratory Assay.** HUVECs (5 × 104 cells/well) were plated onto 96-well tissue culture plates (Wallac, Gaithersburg, MD) in 200 μl of EBM-2 medium without VEGF, bFGF, or epidermal growth factor and incubated at 37°C for 72 h. Various amounts of soluble scFv were added to duplicate wells and preincubated at 37°C for 1 h, after which VEGF was added to a final concentration of 16 ng/ml. In a parallel assay, bFGF (4 ng/ml) was added to the cells to evaluate the specificity of anti-KDR scFv on ligand-induced DNA synthesis in HUVECs. After 18 h of incubation, 0.25 μCi of [3H]thymidine (Amersham) was added to each well and incubated for an additional 4 h. The cells were then placed on ice and washed twice with serum-containing medium, followed by a 10-min incubation at 4°C with 10% trichloroacetic acid. The cells were then washed once with water and solubilized in 25 μl of 2% SDS. Scintillation fluid (150 μl/well) was added, and DNA-incorporated radioactivity was determined on a scintillation counter (Wallac; model 1450 Microbeta Scintillation Counter).

**Results**

**Library Construction and Biopanning.** mRNA was isolated from mice immunized with KDR-AP soluble protein and used to construct a combinatorial scFv library, which was displayed on the surface of the filamentous phage M13. In displaying the scFv on filamentous phage surface, antibody V4 and V1 domains are joined together by a 15-amino acid linker (GGGGS3) and fused to the NH2-terminus of phage protein III. A 15-amino acid E tag, which is followed by an amber codon (TAG), was inserted between the COOH-terminus of V1, and the protein III for detection and other analytic purposes. The amber codon, positioned between the E tag and the protein III, enables the construct to make scFv in surface-displaying format when transformed into a suppressor host (such as TG1 cells) and to make scFv in soluble form when transformed into a nonsuppressor host (such as HB2151 cells).

After ligation of the scFv fragments into the pCANTAB 5 E vector and electropresentation into TG1 cells, a library of 2.7 × 108 clones was obtained. Anti-KDR scFv were selected from the library by two to three rounds of biopanning. A saturating concentration of AP protein was added to phage preparation in all rounds of panning as a counterselection to remove phage-displaying anti-AP scFv. A total of 270 clones were randomly picked after the second round of biopanning and screened for binding to KDR-AP or AP, and 243 clones (90%) of them were found to bind to KDR-AP. None of the 270 clones bound
to AP protein, indicating the high efficiency of the counter selection used during the biopanning process. Further selection was achieved with a competitive VEGF-binding assay in which the binding of soluble KDR to immobilized VEGF in the presence or absence of scFv was determined (see "Materials and Methods"). The assay identified 53 clones (20% of all KDR binders) as being capable of blocking the binding between VEGF and its receptor, KDR. DNA BstNI fingerprinting of these 53 clones indicated the presence of four different digestion patterns (with one pattern dominant, 79%), whereas 26 randomly picked VEGF nonblockers yielded six different patterns (also with one pattern dominant, 46%).

Ninety-nine % (185 of 186) of clones screened after the third round of panning were found to be specific KDR blockers. However, only 15 (8%) of these binders could block KDR binding to immobilized VEGF. DNA BstNI fingerprinting of these 15 clones indicated the presence of two different digestion patterns, whereas 21 randomly picked VEGF nonblockers yielded four different patterns. All of the digestion patterns were also seen in clones identified after the second round of panning. Representative clones of each digestion pattern were picked from clones recovered after the second round of panning and subjected to DNA sequencing. Of 15 clones sequenced, 2 unique VEGF blockers and 3 nonblockers were identified (Fig. 1). One scFv, p2A7, which neither binds to KDR nor blocks VEGF binding to KDR, was selected as a negative control for all studies.

KDR Binding Assay of Purified scFv. Four different clones, including the two VEGF blockers (p1C11 and p1F12), one nonblocker (the dominant clone p2A6), and the nonbinder p2A7, were expressed in shaker flasks using a nonsuppressor host E. coli HB2151 cells. The soluble scFv was purified from the periplasmic extracts of E. coli by anti-E tag affinity chromatography. The yield of purified scFv of these clones ranged from 100-400 μg/liter culture.

Fig. 2A shows the dose-dependent binding of the KDR-binding scFv to immobilized KDR, as assayed by a direct-binding ELISA. Clones p1C11 and p1F12 but not p2A6 also block KDR binding to immobilized VEGF (also see Fig. 2B). Determined by BIAcore analysis (see "Materials and Methods"). 

![Fig. 2. Binding of scFv antibodies to KDR. A. direct binding of scFv to immobilized KDR. Various amounts of scFv antibodies were added to 96-well plates coated with KDR-AP and incubated at RT for 1 h, after which the plates were incubated with a mouse anti-E tag antibody followed by a rabbit antimouse antibody-HRP conjugate. The plates were washed, peroxidase was substrate added, and ß445U nmwas read (see "Materials and Methods" for details). B. inhibition of binding of KDR to immobilized VEGF165 by scFv. Various amounts of scFv antibodies were incubated with a fixed amount of KDR-AP in solution at RT for 1 h, after which the mixtures were transferred to 96-well plates coated with VEGF165. The amounts of KDR-AP that bound to VEGF165 were quantified by incubation of the plates with AP substrate and reading of ß445 nm.Data points, means of triplicate determinations; bars, SD.](https://cancerres.aacrjournals.org/article-pdf/58/9/3211/27945826/cancerres-58-09-3211.pdf)

**Fig. 1. Deduced amino acid sequences [according to the hypervariable sequence definition of Kabat et al. (39)] of complementarity-determining regions (CDR1–CDR3) of the VH and VL of the scFv antibodies isolated from the phage display library.**

<table>
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<th>Heavy chain</th>
<th>CDR1</th>
<th>CDR2</th>
<th>CDR3</th>
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<tr>
<td>p1C11</td>
<td>GFNIKDYFYMH</td>
<td>WIDPENGDSYAPFKQQ</td>
<td>YGYDYEYGY</td>
</tr>
<tr>
<td>p1F12</td>
<td>GYSFTGYNNM</td>
<td>IDPPYGGTYYNNKFKQ</td>
<td>SGNVGGYFDY</td>
</tr>
<tr>
<td>p2A6</td>
<td>GYTTEITYMH</td>
<td>GINPNITGGTSYNNKFKQ</td>
<td>YRYDAMDY</td>
</tr>
<tr>
<td>p3A1</td>
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<td>QIYGPDGDTPKNFQ</td>
<td>EYGDSDFDY</td>
</tr>
<tr>
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<td>GYTTEITYMH</td>
<td>GINPNITGGTYNNPFQ</td>
<td>SKYGSGLDY</td>
</tr>
<tr>
<td>p2A7</td>
<td>GYPFSSYWMN</td>
<td>QIYGPDGDTPNNGFQ</td>
<td>FYGNYDPMYNY</td>
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<table>
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<th>Light chain</th>
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<th>CDR2</th>
<th>CDR3</th>
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<tr>
<td>p1C11</td>
<td>SASSSSVSYM</td>
<td>STSNLAS</td>
<td>QRRSSYPFT</td>
</tr>
<tr>
<td>p1F12</td>
<td>SASSSSSSSYLH</td>
<td>RTSNLAS</td>
<td>QQQSDSYFPT</td>
</tr>
<tr>
<td>p2A6</td>
<td>RASSSSVYVH</td>
<td>DTKLAS</td>
<td>QQQSDYFPT</td>
</tr>
<tr>
<td>p3A1</td>
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<td>DTKNAS</td>
<td>QQQSSNPWT</td>
</tr>
<tr>
<td>p2A7</td>
<td>RATSSSVNM</td>
<td>DTKNAS</td>
<td>QQQHGESPLT</td>
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**Table 1** KDR binding analysis of anti-KDR scFv antibodies

<table>
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<tr>
<th>scFv clone</th>
<th>KDR binding* (ED, nm)</th>
<th>VEGF blocking** (IC, nm)</th>
<th>k&lt;sub&gt;on&lt;/sub&gt; (10&lt;sup&gt;7&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;off&lt;/sub&gt; (10&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (10&lt;sup&gt;-7&lt;/sup&gt; M)</th>
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<tr>
<td>p1C11</td>
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<td>Yes (0.3)</td>
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<td>2.1</td>
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<td>Yes (1.0)</td>
<td>Yes (15)</td>
<td>0.24</td>
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<td>11.2</td>
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<tr>
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<td>No (&gt;300)</td>
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<td>NA</td>
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</tbody>
</table>

* Determined by competitive VEGF-blocking ELISA. Numbers in the parenthesis represent the scFv concentrations that give 50% of maximum binding (see also Fig. 2A).

** Determined by competitive VEGF-blocking ELISA. Numbers in the parenthesis represent the scFv concentrations required for 50% inhibition of KDR binding to immobilized VEGF (also see Fig. 2B).

* Determined by ELISA analysis (see "Materials and Methods").
immobilized VEGF (Fig. 2B). The negative control clone, p2A7, did not bind to KDR or block KDR binding to VEGF (Fig. 2). Clone p1C11, the dominant clone after each round of panning, showed the highest KDR binding capacity and the highest potency in blocking VEGF binding to KDR (Table 1). The antibody concentrations of clone p1C11 required for 50% of maximum binding to KDR (Fig. 2A) and for 50% of inhibition of KDR binding to VEGF (Fig. 2B) were 0.3 nM and 3 nM, respectively (Table 1). Fluorescence-activated cell sorting analysis demonstrated that p1C11, p1F12, and p2A6 were also able to bind to cell surface expressed receptor on HUVECs (data not shown).

The kinetics of scFv binding to KDR was determined by surface plasmon resonance on a BIAcore instrument (Table 1). The VEGF-blocking scFv antibodies, p1C11 and p1F12, bound to immobilized KDR with $K_d$s of 2.1 and 5.9 nM, respectively. The nonblocking scFv p2A6 bound to KDR with an ~6-fold weaker affinity ($K_d$, 11.2 nM) than the best binder p1C11, mainly due to a much faster dissociation rate. As anticipated, p2A7 did not bind to the immobilized KDR on the BIAcore.

**Phosphorylation and Mitogenesis Assays.** The biological effects of the anti-KDR scFv antibodies on VEGF-induced KDR phosphorylation (Fig. 3) and cell mitogenesis (Fig. 4) were determined on HUVECs. In the phosphorylation assay, the levels of KDR phosphorylation stimulated by VEGF were assayed in the presence and absence of scFv antibodies. Results showed that VEGF-blocking scFv p1C11 but not the nonblocking scFv p2A6 was able to inhibit KDR phosphorylation stimulated by VEGF (Fig. 3, A and B). Furthermore, p1C11 also effectively inhibited VEGF-stimulated activation of MAP kinases p44/p42 (Fig. 3C). In contrast, neither p1C11 nor p2A6 inhibited bFGF-stimulated activation of MAP kinases p44/p42 (Fig. 3D).

The ability of scFv antibodies to block VEGF-stimulated mitogenic activity on HUVECs is shown in Fig. 4. The VEGF-blocking scFv p1C11 strongly inhibited VEGF induced DNA synthesis in HUVECs with an EC$_{50}$, i.e., the antibody concentration that inhibited 50% of VEGF-stimulated mitogenesis of HUVECs, of ~5 nM. The nonblocking scFv p2A6 showed no inhibitory effect on the mitogenic activity of VEGF. Neither p1C11 nor p2A6 inhibited bFGF-induced DNA synthesis in HUVECs (data not shown).

**Discussion**

High-affinity anti-KDR scFv antibodies, which block VEGF binding to KDR, were isolated from a phage display library constructed from mice immunized with a soluble form of the human VEGF receptor. The following are noteworthy in this study: first, over 90% of recovered clones after two rounds of selection are specific to KDR. The binding affinities for KDR of these scFv are in the nanomolar range, which are as high as those of several bivalent anti-KDR Mabs produced using hybridoma technology. These results indicate high efficiency of the immunization and/or biopanning process, plus a relatively large size of the library. Second, we show that a scFv antibody, p1C11, is able to block VEGF-KDR interaction and inhibit VEGF-stimulated receptor phosphorylation and mitogenesis of HUVECs. To our knowledge, this is the first report on the production of high-affinity anti-KDR antibodies and the first demonstration that such antibodies can specifically inhibit VEGF-stimulated activation of KDR and mitogenesis of endothelial cells.

Several results presented in this study suggest that the mechanism of endothelial cell growth inhibition mediated by p1C11 scFv is due to a direct block of VEGF-KDR interaction, resulting in the inhibition of VEGF-induced KDR activation. This is based on the observation that p1C11 scFv specifically binds to both soluble KDR and cell surface-expressed KDR on HUVECs; once bound, it blocks VEGF binding to KDR (Fig. 2). Furthermore, p1C11 blocks VEGF-induced KDR phosphorylation (Fig. 3A) at saturating levels of VEGF (19). The scFv also blocks VEGF (but not bFGF)-stimulated activation of MAP kinases p44/p42 (Fig. 3, C and D). These MAP kinases are
known to be associated with cell proliferation induced by a number of mitogens, including VEGF and bFGF (22). Finally, p1C11 specifically inhibits VEGF-induced but not bFGF-induced DNA synthesis in HUVECs. On the other hand, p2A6, which binds KDR but does not block VEGF-KDR interaction, has no effect on VEGF-induced DNA synthesis in HUVECs (Fig. 4).

A growing list of inhibitors of angiogenesis are currently under evaluation as potential cancer therapeutics (23), including small molecule inhibitors such as linomide (24), AGM-1470 (also known as linomide) (24), and thalidomide (26). More recently, several small molecule Flk-1 inhibitors, including SU1433 and SU1498, were found to inhibit VEGF-induced endothelial cell proliferation and angiogenesis in the chorioallantoic membrane of chicken embryo (27). Angiostatin and endostatin were found to suppress tumor growth and metastasis by inhibiting angiogenesis (28, 29), and reintroduction of wild-type p53 into glioblastoma cells was found to release an inhibitor of vessel formation (30). Additionally, Mabs against VEGF (14) and αvβ3 integrin (31) have been shown to inhibit tumor neoangio-genesis. While there are several theoretical advantages for using antibodies targeting KDR/Flk-1 on endothelial cells as cancer therapeutics. First, KDR/Flk-1 is expressed exclusively on proliferating endothelial cells at tumor sites (6, 7, 11, 13); therefore, antibodies against the receptor may offer high specificity compared to other agents. Because tumor vessel endothelial cells are in direct contact with the blood, antibodies against KDR/Flk-1 have greater accessibility to their targets on endothelial cells compared to antibodies against markers expressed on individual tumor cells. Furthermore, conventional targeting therapies with antibodies and/or their conjugates requires targeting individual tumor cells, and their efficacy is greatly limited by tumor cell heterogeneity. On the contrary, local interruption of tumor vasculature by targeting molecules expressed on endothelial cells may produce an avalanche of tumor cell death (32). Finally, because endothelial cells possess a normal complement of chromosomes and are relatively genetically stable, they should be far less prone to develop resistance to therapy than tumor cells themselves (29).

We have previously shown that a rat Mab against the mouse Flk-1 receptor, DC101, effectively inhibits human tumor growth in nude mice by blocking the tumor-associated angiogenesis (15, 16). It is important to note that, despite high sequence homology between mouse Flk-1 and its human homologue KDR, none of the VEGF-blocking anti-KDR scFv antibodies produced in our laboratory cross-react with Flk-1. Consequently, human tumors grown in mice, which recruit the mouse vasculature, are not appropriate models to evaluate the antiangiogenesis therapy in vivo of the anti-KDR antibodies described here, because these antibodies do not react with mouse Flk-1 receptor.

Apart from cancer, the VEGF/KDR/Flk-1 pathway has been shown to also play a role in diabetic retinopathy (33), psoriasis (34), hemangioma (35), rheumatoid arthritis (36), and Kaposi’s sarcomas in AIDS patients (37). Thus, identification of KDR/Flk-1 inhibitors may have potential application in the treatment of a variety of human diseases in which pathological angiogenesis is involved. Here, we demonstrate that antibodies against KDR can specifically block VEGF-KDR interaction and inhibit VEGF-induced KDR activation and DNA synthesis in human endothelial cells. Our data, together with previous results with anti-Flik-1 antibodies (15, 16), lend support to further evaluation of these antibodies as antitumor agents (38).

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References


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