Vascular endothelial growth factor (VEGF) and its receptors have been implicated in promoting solid tumor growth and metastasis via stimulating tumor-associated angiogenesis. We previously identified several fully human neutralizing anti-VEGF receptor 2 (or kinase insert domain-containing receptor (KDR)) antibodies from a large antibody phage display library. These antibodies bind specifically to KDR, block VEGF/KDR interaction, and inhibit VEGF-induced proliferation of human endothelial cells and migration of KDR\(^+\) leukemia cells. Three of these antibodies, Interestingly, share an identical heavy chain variable (VH) sequence. In this report, we constructed a new library comprising the single VH paired with the variable light chain (VL) repertoire obtained from the original naïve human library. Initial in vitro selection revealed that the single VH could pair with a number of different VL while retaining its specificity for KDR. However, a consensus VH/VL pair, clone 1121, was identified after three or four rounds of selection by tailoring the stringency of the panning conditions. Clone 1121 showed a >50-fold higher binding affinity to KDR \((K_D = 100 \text{ ps})\) because of improvement on both association and dissociation constants and blocked VEGF/KDR interaction with an IC\(_{50}\) of \(-1 \text{ nM}\), compared with that of 3–4 nM for the parent Fab fragments. Further, clone 1121 was more potent in inhibiting VEGF-stimulated KDR phosphorylation in endothelial cells. A binding epitope mapping study on clone 1121 and one of the parent clones, 2C6, demonstrated that both antibodies interacted with the third immunoglobulin domain within the extracellular region of KDR. Several peptide phage display libraries were utilized to further examine the fine binding specificities of the two antibodies. All of the 2C6-binding peptides are cysteine-constrained, whereas clone 1121 binds to both cysteine-constrained and linear peptides. It is noteworthy that most of the 2C6-binding peptides also cross-react with clone 1121, but none of the clone 1121-specific peptides binds to 2C6, indicating that clone 1121 retained part of the original binding epitope(s) of 2C6 while gaining new binding specificity. Taken together, our observations suggest that clone 1121 may have great clinical potential in anti-angiogenesis therapy. It further underscores the efforts to identify antibodies of high affinity for enhanced biological activities.

Vascular endothelial growth factor (VEGF)\(^1\) and its receptors (VEGFR) have been implicated in promoting solid tumor growth and metastasis via stimulating tumor-associated angiogenesis (1–3). VEGF, the primary endothelial-specific mitogen, exerts its effects via two high affinity tyrosine kinase receptor, VEGFR1 (or fms-like tyrosine kinase, Flt-1), and VEGFR2 (or kinase insert domain-containing receptor, KDR) (1–3). Among these two receptors, KDR appears to be the major transducer of VEGF signals in endothelial cells that result in cell proliferation, migration, differentiation, tube formation, increase of vascular permeability, and maintenance of vascular integrity (3–5). Inhibition of KDR-mediated signal transduction, therefore, represents an excellent approach for anti-angiogenic intervention. In fact, inhibition of angiogenesis and tumor inhibition has been achieved by using agents that either interrupt VEGF/KDR interaction and/or block the KDR signal transduction pathway (4–6), including antibodies to VEGF (7–9) or KDR (10–12), anti-VEGF immunotoxins (13), ribozyme (14), soluble receptors (15), and small molecule tyrosine kinase inhibitors (16, 17).

Monoclonal antibodies (mAb), because of their high specificity toward a given target, represent a unique class of novel therapeutics as angiogenesis inhibitors (18, 19). With the recent progresses in molecular engineering techniques and the availability of human antibody transgenic mice and human antibody phage display libraries, chimeric, humanized, and fully human mAb with desired specificities and properties, e.g., low immunogenicity and high affinity, can be readily produced (20, 21). We previously produced a chimeric and several fully human anti-KDR antibodies and demonstrated that these antibodies were capable of blocking KDR/VEGF interaction and inhibiting VEGF-stimulated receptor activation and mitogenesis of human endothelial cells (10, 22, 23). In addition, these antibodies inhibited VEGF-induced proliferation of human leukemia cells in vitro and prolonged survival of nonobese diabetic-severe combined immunodeficient mice inoculated with human leukemia cells (11, 24, 25). We observed that three of the neutralizing human anti-KDR antibodies identified from a phage display library share an identical heavy chain variable (VH) sequence (23). In this report, we constructed a new library

\(^1\) The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; AP, alkaline phosphatase; CDR, complementarity determining region; ECD, extracellular domain; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; KDR, kinase insert domain-containing receptor (or VEGFR2); mAb, monoclonal antibody; PAF, porcine aortic endothelial cells; VH, variable domain of antibody heavy chain; VL, variable domain of antibody light chain; PEG, polyethylene glycol; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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comprising the single VH paired with the light chain variable (VL) repertoire obtained from the original phage library and, together with a tailored stringent in vitro selection process, identified a consensus VH/VL pair with ~30-fold affinity improvement. The high affinity variant was more potent in blocking KDR/VEGF interaction and in inhibiting VEGF-stimulated receptor activation. Epitope mapping studies using peptide phage display libraries revealed that the high affinity variant gained novel epitope(s) on KDR while retaining part of the original epitope(s) to which the parent antibodies interact.

EXPERIMENTAL PROCEDURES

Cell Lines, Proteins, and Phage Display Libraries—Primary cultured HUVEC were maintained in EBM-2 medium (Clonetics, Walkersville, MD) at 37 °C and 5% CO 2 as described previously (10). Porcine aortic endothelial cells transfected with KDR (PAE-KDR) was generated at ImClone Systems Incorporated (New York, NY) and maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. PhageDisplayWebsiteIndex.html). The soluble KDR-alkaline phosphatase fusion protein (KDR-AP), its extracellular Ig domain (ECD) deletion variant-AP fusions, and VEGF 165 proteins were produced as previously described (26). IMC-1C11 and MAB664, two mAb directed against KDR, were generated at ImClone Systems Incorporated as previously described (10, 12, 27). The human Fab phage libraries, clones 2C6, 1H4, and 2H2, were isolated from a human Fab phage display library (containing 3.7 × 10 10 clones) (28) by several rounds of selection against immobilized recombinant KDR protein as previously described (23). All of the peptide phage display libraries and the Escherichia coli strain R91 were kind gifts from Dr. G. P. Smith of University of Missouri-Columbia (for details see www.biochemistry.missouri.edu/smithgp/PhageDisplayWebsite/PhageDisplayWebsiteIndex.html).

Generation of the Light Chain-shuffled Phage Display Library—The original human Fab phage display library (28), from which the anti-KDR antibodies 2C6, 1H4, and 2H2 were identified, was used as the source of the VL repertoire in the shuffled library. The phagemid preparation from the original library was ligated with SfiI and NotI followed by electrophoresis on an agarose gel to separate the VH gene fragments from the antibody light chain-containing backbone vector to delete the entire VH repertoire. The gene encoding the VH domain of clone 2C6/1H4/2H2 was generated by digestion of 2C6-coding phagemid with the same restriction enzymes and purified as described above. The 2C6 VH-coding gene was then ligated into the purified backbone vector to create the VL-shuffled Fab repertoire. E. coli TG1 cells were transformed with the ligation mixtures via electroporation (Electro Cell Manipulator 600, BTX Electroporation System, San Diego, CA). The transformed TG1 cells were grown with shaking in 2YT medium at 37 °C for 80 min and plated onto several 150-mm 2YT agar plates. The plates were grown to log phase in 20 ml of 2YT agar (2YT plates). All of the colonies grown on the plates were scraped into 5 ml of 2YTAG medium, mixed with 1.2 ml of 20% glycerol (final concentration: 10%), aliquoted, and stored at −70 °C as the library stock.

Selection of the VL-shuffled Library against KDR—The library stock (100 μl) was grown to log phase in 20 ml of 2YTAG medium, rescued with M13K07 helper phage, and amplified overnight in 2YTAK medium (2YT containing 100 μg/ml of ampicillin and 50 μg/ml of kanamycin) at 30 °C. The phage preparation was precipitated in 4% PEG, 0.5 mM NaCl, and resuspended in 1 ml of 3% fat-free milk/PBS containing 500 μg/ml of AP protein and incubated at 37 °C for 1 h to block nonspecific binding. For first round selection, KDR-AP-coated Microtiter tubes (Nunc, 2YT containing 100 μg/ml of AP and 2% glucose (2YTAG plates). All of the colonies grown on the plates were washed five times with PBST. The plates were then incubated at room temperature for 1 h, after which the plates were washed three times with PBST and incubated with a mouse anti-M13 phage-HRP conjugate (Amersham Biosciences). The plates were washed five times, TMB peroxidase substrate (KPL, Gaithersburg, MD) was added, and the absorbance at 450 nm was read using a microplate reader (Molecular Device, Sunnyvale, CA).

Phage-based KDR Binding and Blocking ELISA—Individual TG1 clones recovered after each round of selection were randomly picked and grown at 37 °C in 96-well plates and rescued with M13K07 helper phage as described above. The amplified phage preparation was blocked with 1/4 volume of 18% milk/PBS at room temperature for 1 h and added to Maxi-sorp 96-well microtiter plates (Nunc) coated with KDR-AP or AP (1 μg/ml × 100 μl at 4 °C overnight). After incubation at room temperature for 1 h, the plates were washed three times with PBST and incubated with a mouse anti-M13 phage-HRP conjugate (Amersham Biosciences). The plates were washed five times, TMB peroxidase substrate (KPL, Gaithersburg, MD) was added, and the absorbance at 450 nm was read using a microplate reader (Molecular Device, Sunnyvale, CA). For KDR/VEGF blocking ELISA, 100 μl of the blocked phage were mixed with 100 ng of KDR-AP and incubated at room temperature for 1 h, after which the mixture were transferred to 96-well plates coated with VEGF (200 ng/well) at incubated for additional 2 h. The plates were washed five times with PBST, and the substrate for AP (p-nitrophenyl phosphate; Sigma) was added, followed by reading the absorbance at 405 nm to quantify the bound KDR-AP molecules (10).

DNA Pattern Analysis and Nucleotide Sequencing—The diversity of the VL-shuffled library and the anti-KDR Fab clones recovered after each round of selection was analyzed by restriction enzyme digestion patterns using a frequent cutting enzyme, BstNI (10, 23). DNA sequences of representative clones from each digestion pattern were determined by dideoxynucleotide sequencing. Classification and alignments of the VH and the VL genes were performed using NCBI IgBlast (www.ncbi.nlm.nih.gov/igblast) and V base (www. nrc-pce.cam.ac.uk).

Expression and Purification of the Soluble Fab Fragments and the Full-length IgG—Phagemids of the individual selected clones were used to transform a nonsuppressor E. coli host HB2151. Expression of the Fab fragments in HB2151 and purification of the soluble Fab proteins from the periplasmic extracts of the E. coli were carried out as previously described (10, 25). The IgG1 clones of clones 2C6, 1H4, and 1121 (IMC-1H4, IMC-1121, respectively) were expressed and produced in N50 cells under serum-free conditions following procedures previously described (11).

Quantitative KDR Binding and Blocking Assay—In the direct binding assay, various amounts of anti-KDR antibodies were added to KDR-coated 96-well Maxi-sorp microtiter plates and incubated at room temperature for 1 h, after which the plates were washed five times with PBST. The plates were then incubated at room temperature for 1 h with 100 μl of a rabbit anti-human Fab antibody-HRP conjugate (Jackson Immunoresearch Laboratory Inc., West Grove, PA). The plates were washed and developed, and the absorbance at 450 nm was read following the procedure described above for the phage ELISA. In the competition KDR/VEGF blocking assay, various amounts of VEGF (200 ng/well) and anti-KDR antibodies were mixed with a fixed amount of KDR-AP (100 ng) and incubated at room temperature for 1 h. The mixture were then transferred to 96-well microtiter plates precoated with VEGF165 (200 ng/well) and incubated at room temperature for an additional 2 h, after which the plates were washed five times, and the substrate for AP was added, followed by reading the absorbance at 405 nm to quantify the bound KDR-AP molecules (10). IC 50 , i.e. the antibody concentration required for 50% inhibition of KDR binding to VEGF, was then calculated.

Antibody Affinity Maturation by Tailoring Selection

Antibody Affinity Determination by BIACore Analysis—The binding kinetics of various antibodies to KDR was measured using a BIACore 3000 biosensor system (Biacore, Uppsala, Sweden). The affinity constant, K D , was calculated from the ratio of dissociation rate (k off)/association rate (k on). K D Phosphorylation Assay—The assay was carried out on both HUVEC and PAE-KDR cells following a protocol previously described (10, 22). Briefly, the serum-starved cells were incubated with various concentration of antibodies for 30 min at 37 °C (for 30 min at 4 °C). The cells were lysed, and KDR protein was precipitated from the cell lysates with a polyclonal anti-KDR antibody (ImmClone Systems) followed by protein A-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were resolved with SDS-PAGE and subjected to immunoblotting with a polyclonal anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were detected using enhanced chemiluminescence (Amersham Biosciences).

Antibody Epitope Mapping Using KDR Ig Domain Deletion Variants—Full-length KDR (KDR(Ig1–7)) and KDR Ig domain deletion vari-
Antibody Affinity Maturation by Tailoring Selection

Table I
Selection and identification of anti-KDR antibodies from the V_L-shuffled library under various conditions

| Round | 1 | 2 | 2 | 3 | 3 | 4 | 4 |
|-------|---|---|---|---|---|---|---|
| KDR Coated: | | | | | | | |
| Concentration (µg/mL) | 1.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| Volume (µL) | 1.0 | 1.0 | 0.2 | 0.2 | 0.2 | 0.2 |
| Input Phage (pfu) | 1 x 10^{12} | 1 x 10^{11} | 2 x 10^{10} | 2 x 10^{10} | | |
| Period of Binding (h) | 1.0 | 0.5 | 0.5 | 0.5 | | |
| Initial Washes: PBS + PBS (Times) | 15 + 15 | 15 + 15 | 15 + 15 | 15 + 15 | | |
| Extensive Washes: | | | | | | | |
| Time (h) | 0.0 | 0.5 | 3.0 | 24.0 | | |
| Competition none | PBS | KDR (0.1 µM) | PBS | KDR (2 µM) | PBS | KDR (1 µM) |
| Phage Recovered (pfu) | 5 x 10^{7} | 3.8 x 10^{7} | 2 x 10^{7} | 1.3 x 10^{8} | 6 x 10^{7} | 6.5 x 10^{8} | 5.5 x 10^{9} |
| KDR - Binder (%) | 58.9 | 34.4 | 32.2 | 21.1 | 22.2 | 81.1 | 63.3 |

(A) Deduced amino acid sequences of the various anti-KDR V_l domains

(B) Deduced amino acid sequence of the V_l domain shared by all the above anti-KDR antibodies

Fig. 1. Deduced amino acid sequences of the single VH and the various VL domains of the anti-KDR clones recovered after three to four rounds of selection under different conditions described in Table I. All of the CDRs are underlined according to the definition of Kabat et al. (29).
In 2001, after three rounds of selection, individual phage clones were inoculated to identify peptide phage clones that bind to IMC-2C6 or IMC-1121 antibodies were used to coat the microwells of the 96-well plate. Second and third round selections, 300 and 150 ng, respectively, of IMC-1121 antibody were added to the plate at 1 μl/well and grown at 37°C overnight. Following the procedure described above, starting at 1:10 phage dilution (in 3% milk/PBS) and followed by another 15 times washes, from 0.5 h in the second round, 3 h in the third round to 0.2 h in the fourth round. Further, a process aiming to select clones with improved dissociation rate (or off rate, koff), the number of input phages were reduced in each subsequent selections, and the duration of initial binding processes between the phages and the immobilized antigen was shortened from 1 h in the first round to 0.5 h in the second and the third rounds to 0.2 h in the fourth round. Ten to eight clones were recovered after the fourth round selection; only one of ten sequenced after the third round selection were clone 1121, repeated once (Table I). Five different sequences were identified, with one sequence, designated as clone 1121, one of the parent clones. A total of seven new sequences were between different clones and between these clones and the three parent clones, indicating an excellent diversity of the shuffled library.

### Selection of High Affinity Anti-KDR Clones

Two different selection processes were carried out in parallel using conditions described in Table I. A total of four rounds of selection were performed: the first two in Maxi-sorp star tubes and the last two in 96-well plates. After initial phage binding to immobilized KDR, the tubes or wells were washed 15 times with PBST (PBS containing 0.1% Tween 20) followed by another 15 times with PBS. Approximately 59% of the phage clones recovered after the first round selection were specific KDR binders. In the subsequent selections, conditions of varying stringency were applied. First, to select anti-KDR clones with fast association rate (or on rate, kon), the number of input phages were reduced in each subsequent selections, and the duration of initial binding processes between the phages and the immobilized antigen was shortened from 1 h in the first round to 0.5 h in the second and the third rounds to 0.2 h in the fourth round. Further, a process aiming to select clones with improved dissociation rate (or off rate, koff) was also applied; after the initial binding, the KDR-bound phages were subjected to extensive period of washes, from 0.5 h in the second round, 3 h in the third round to 24 h in the fourth round selection, with PBS or various amounts of KDR (from 100 to 2000 nM) in solution as a competitor (see Table I for details).

### Antibody Affinity Maturation by Tailoring Selection

| V-gene | Human V-gene family | No. of mutations from germline | Closest human-germline gene |
|--------|---------------------|-------------------------------|-----------------------------|
|        |                     | Nucleotides | Amino acids |
| Clone 2C6 | V(H)III | 13 | 8 | Vg/38K |
| Clone 2H2 | V(H)I | 6 | 4 | DPK6/Vb/Vb' |
| Clone 1H4 | V(H)I | 29 | 18 | DPL10/V1-7 |
| Clone 1121 | V(I) | 26 | 14 | DPL8/V1-13 |
| Clone 3A1 | V(I) | 21 | 14 | DPL8/V1-13 |
| Clone 3B5 | V(I) | 20 | 11 | DPL8/V1-13 |
| Clone 3B10 | V(I) | 44 | 22 | DPL11/V1-4 |
| Clone 3D2 | V(I) | 14 | 10 | DPK5/Vb/Vb' |
| Clone 3D5 | V(I) | 26 | 13 | DPL8/V1-13 |
| Clone 3F2 | V(I) | 21 | 12 | DPL8/V1-13 |
| Clone 3F7 | V(I) | 32 | 16 | DPL8/V1-13 |
| Clone 4A3 | V(I) | 24 | 12 | HK137 |
| Clone 4C7 | V(I) | 12 | 9 | DPK5/Vb/Vb' |
| VH | VH3 | 1 | 1 | DP-77 |

* V-gene families and the closest human germlines were deduced from NCBI IgBlast at www.ncbi.nlm.nih.gov/igblast and V-Base at www.mrc-cpe.cam.ac.uk.

Germline gene used for comparison is the closest human germline gene to the individual variable domain sequence.

### RESULTS

#### Construction of the VL-shuffled Library

In constructing the VL-shuffled library, the single VH gene segment shared by the three anti-KDR antibodies, 2C6, 1H4, and 2H2, was subcloned into the large Fab phage display library replacing the original diverse VH repertoire. After electroporating into TG1 cells, a library of 1.5 × 10^10 plaque-forming units was obtained. In this library, the single VH gene is randomly paired with, in theory, 1.5 × 10^9 different VL genes, thus creating the same numbers of independent Fab binding specificities. DNA fingerprinting and nucleotide sequencing of several dozen randomly picked colonies revealed no identical patterns and nucleotide sequences between different clones and between these clones and the three parent clones, indicating an excellent diversity of the shuffled library.
tified from the VL-shuffled library after three or four rounds of selection (Fig. 1 and Table I).

Sequence alignments using NCBI IgBlast and V Base of the single VH domain showed that the gene has the highest homology with the germline DP77 segment of the human VH3 family, with only one amino acid substitution from the germline sequence (Table II). Interestingly, alignments of the VL domains revealed both VH9260 and VH9261 gene families; two of the original clones, 2C6 and 2H2, along with four newly identified clones including 1121 belong to VH9260, whereas the other clones, including the original clone 1H4 and six new clones, are from the VH9261 family. It is noteworthy that all the six VH genes have the same canonical structure for L1, L2, and L3 (2-1-1), whereas the six newly selected VH genes share identical length of both CDR1 and CDR2 (14 and 7 amino acids, respectively) (30, 31). Taken together, these observations underscore the structure requirement on VL genes for pairing with the fixed VH segment to maintain KDR binding specificity.

KDR binding and blocking activities of all ten positive clones were examined using phage-based ELISA. Compared with the parent clone 2C6, six new clones demonstrated moderate to strong enhancement in KDR-binding activity, with clone 1121 being the best binder (Fig. 2A). However, only three clones, 1121, 3A1, and 4C7, were better blockers to KDR/VEGF interaction than clone 2C6 (Fig. 2B). Clone 1121 was therefore selected as the leading candidate for further studies.

KDR Binding and KDR/VEGF Blocking by the Anti-KDR Antibodies—Soluble Fab fragments of all the three parent anti-KDR clones, 2C6, 1H4, and 2H2, and the consensus clone 1121 were produced and compared quantitatively in their antigen...
binding efficiency and potency in blocking KDR/VEGF interaction. Consistent with the phage binding result (Fig. 2A), clone 1121 Fab binds much more efficiently to KDR than all three parent Fab (Fig. 3A). It is also a much stronger blocker to KDR/VEGF interaction (Fig. 3B), with an IC₅₀ value of 0.8 nM, compared with that of 3–5 nM for 2C6, 1H4, and 2H2 Fab. Three Fab fragments, 2C6, 1H4, and 1121, were converted into the full-length bivalent IgG1 format. IMC-1121 again is the strongest KDR-binder and the most potent blocker of KDR/VEGF interaction (Fig. 4). The IC₅₀ values for blocking KDR binding to VEGF were 0.8, 1.2, and 6.2 nM, for IMC-1121, IMC-2C6, and IMC-1H4, respectively (Fig. 4B).

The binding kinetics of various anti-KDR antibodies were determined by surface plasmon resonance on a BIAcore instrument (Table III). As seen previously, the three parent Fab fragments bind to immobilized KDR with a similar overall affinity (Kₐ) of 3.1–4.2 nM, although each individual Fab showed different kₐ on and kₐ off rates; clone 2C6 has the fastest kₐ on and the fastest kₐ off, whereas clone 1H4 possesses the slowest kₐ on and also the slowest kₐ off among the three fragments. Clone 1121 Fab possesses an affinity of 100 ps, representing ~30-fold improvement over the parent clones, because of improvement on both kₐ on and, more remarkably, kₐ off. As a bivalent IgG, IMC-1121 showed an overall affinity of 50 ps, representing a ~4-fold improvement over IMC-2C6, and 12-fold improvement over IMC-1H4 (Table III).

Inhibition of VEGF-stimulated KDR Phosphorylation—The biological activity of IMC-1121 and IMC-2C6 was evaluated in a VEGF-stimulated KDR phosphorylation assay using both HUVEC and PAE-KDR cells. VEGF stimulation resulted in significant KDR phosphorylation in both HUVEC and PAE-KDR cells (Fig. 5). Neither antibody alone had any effects on KDR phosphorylation. In the presence of VEGF, both antibodies neutralized KDR phosphorylation in a dose-dependent manner. Consistent with its potent blocking activity, IMC-1121 was a much more stronger (>5–25-fold) inhibitor in both cell assays than IMC-2C6 (Fig. 5).

Binding Epitope Mapping for IMC-2C6 and IMC-1121—We previously showed that all of the neutralizing anti-KDR antibodies we produced, via both the hybridoma technique and the phage display libraries, block KDR/VEGF interaction by binding to epitope(s) that are located within KDR ECD Ig domain 1–3 (e.g. IMC-1C11) or domain 3 alone (e.g. MAB664) (26, 27). Both IMC-2C6 and IMC-1121 bind equally well to full-length
KDR ECD, KDR(Ig1–3), and KDR(Ig3), thus locating their binding epitope(s) within the single Ig domain 3 of KDR ECD (Fig. 6).

BIAcore analysis was employed to examine whether IMC-2C6 and IMC-1121 competes with each other or with other neutralizing anti-KDR antibodies for binding to KDR ECD. In this assay, an antibody was first injected onto a KDR-coated chip at high concentrations to saturate all of the receptor immobilized on the chip, which was followed by injection of a second antibody. An increase in binding density (as measured by reference units) to the KDR-coated chip upon the injection of the second antibody indicates no competition for binding between the two antibodies, i.e. the two antibodies are most likely bind to different, nonoverlapping epitope(s) on KDR, as seen in the case between MAB664 and IMC-1C11 (Table IV; also see Ref. 26). Binding of IMC-2C6 to the KDR-coated chip blocked further binding by IMC-1121 and vice versa, suggesting the two antibodies interact with either the same or overlapping epitope(s) within KDR ECD Ig domain 3. Both IMC-2C6 and IMC-1121 also blocked subsequent binding by IMC-1C11 but not by MAB664. Because IMC-1C11 requires KDR ECD Ig domain 1–3 for binding, this observation suggests that IMC-1C11 may share an overlapping epitope(s) with both IMC-2C6 and IMC-1121 within Ig domain 3. It is noteworthy that the overall KDR binding reference units increased when IMC-1C11 was followed by either IMC-2C6 or IMC-1121, but there was no such increase when IMC-2C6 or IMC-1121 was followed by IMC-1C11. This phenomenon is most likely a reflection that both IMC-2C6 and IMC-1121 are stronger KDR binder and that they replace KDR chip-bound IMC-1C11 when injected over the chip at high concentration.

We then used a panel of peptide phage display libraries to

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**TABLE III**

| Antibody     | $k_{on}$ ($\times 10^4$ M$^{-1}$ s$^{-1}$) | $k_{off}$ ($\times 10^{-4}$ s$^{-1}$) | $K_d$ (nM) |
|--------------|------------------------------------------|-------------------------------------|-------------|
| 2C6 Fab      | 17.1 ± 5.7$^a$                           | 5.5 ± 0.76                          | 3.6 ± 1.7   |
| 1H4 Fab      | 5.6 ± 0.59                               | 1.5 ± 0.22                          | 3.1 ± 0.86  |
| 2H2 Fab      | 12.4 ± 2.9                               | 4.9 ± 0.18                          | 4.2 ± 0.62  |
| 1121 Fab     | 29.6 ± 7.3                               | 0.30 ± 0.06                         | 0.10 ± 0.02 |
| IMC-2C6 IgG | 21.2 ± 8.1                               | 0.43 ± 0.03                         | 0.20 ± 0.01 |
| IMC-1H4 IgG | 10.0 ± 1.78                              | 0.61 ± 0.26                         | 0.60 ± 0.14 |
| IMC-1121 IgG| 47.9 ± 2.4                               | 0.25 ± 0.04                         | 0.05 ± 0.01 |

$^a$ All of the numbers are determined by BIAcore analysis and represent the mean ± S.E. from at least three separate determinations.
Further map out the fine binding specificities of IMC-2C6 and IMC-1121. After three rounds of selection, 50% of IMC-2C6-selected phage clones and 81% of IMC-1121-selected phage clones demonstrated positive binding to their respective antibody. Approximately 65% of IMC-2C6-positive phage clones also cross-reacted with IMC-1121, but none of the IMC-1121-positive clones bound to IMC-2C6. A total of 19 IMC-2C6-selected clones and 28 IMC-1121-selected clones were picked and sequenced; 11 different peptides were identified from the IMC-2C6 clones and 15 from the IMC-1121 clones (Table V). Of note, all IMC-2C6-selected clones, including the three clones (clones 2D5, 1A3, and 1A5) identified from the randomized 15-mer linear peptide library, are cysteine-constrained peptides, whereas IMC-1121 binders include both linear and cysteine-constrained peptides. Eight of the 11 IMC-2C6-selected peptides have the same XCX3CX motif, with the consensus motif ECP(H/P/S)X2RC(P/Q). IMC-1121-selected peptides were structurally more diverse; of 15 sequences obtained there were five linear peptides, four peptides with XCX4CX, three with XCX5CX, two with XCX6CX, and one with XCX7CX motif (Table V).

**Table IV**

| First antibody injection | Second antibody injection |
|--------------------------|--------------------------|
| IMC-1C11                 | MAB664                   | IMC-2C6 | IMC-1121 |
| IMC-1C11                 | 112/115                  | 115/334 | 117/190  | 120/234  |
| MAB 664                  | 214/348                  | 189/218 | 218/372  | 227/414  |
| IMC-2C6                  | 174/163                  | 160/337 | 187/160  | 187/200  |
| IMC-1211                 | 210/203                  | 195/382 | 217/213  | 214/217  |

The numbers represent the number of reference units bound to the KDR-coated chip upon the injection of the first antibody followed by the number of reference units bound to the chip after the injection of the second antibody and are the representatives of three separate experiments.
Based on their frequency of occurrence and binding efficiency, four peptide phage clones were selected for each antibody for further analysis. The phage clones were amplified and assayed quantitatively for their efficiency in binding to both IMC-2C6 and IMC-1121 and their ability in blocking the antibodies from binding to their cognate antigen, KDR. As shown in Fig. 7 (top panels), all four IMC-1121-selected phage clones bound specifically to IMC-1121 without any cross-reactivity to IMC-2C6. On the other hand, all the four IMC-2C6-selected phage clones reacted with both IMC-2C6 and IMC-1121. Western blot analysis confirmed the same reactivity pattern of these phage clones (not shown). In another assay, the peptide phages were used to compete with soluble KDR for binding to immobilized antibodies. Consistent with the antibody binding results, all eight peptide phages were able to compete with KDR for binding to IMC-1121, but only IMC-2C6-selected phages blocked KDR/IMC-2C6 interaction (Fig. 7, bottom panels). A control cysteine-constrained phage clone, 9C10 (YCDPRCM-RESKYVIS), did not bind to IMC-2C6 and IMC-1121, nor did it block the antibodies from binding to KDR.

**DISCUSSION**

We previously identified four fully human anti-KDR antibodies from a large human naïve antibody library and demonstrated that these antibodies were able to block KDR/VEGF interaction and neutralize VEGF-induced angiogenic activity (23). Three of these antibodies share, interestingly, an identical VH sequence, suggesting that this single VH domain may pair with different VL domains while preserving KDR binding/blocking activities. In this report, we successfully improved the binding affinity of these anti-KDR antibodies by 30-fold through VL shuffling in association with a tailored selection process. Several observations are noteworthy in our study. First, by following a tailored stringent selection procedure, we were able to identify a consensus VL/VH pair, clone 1121, that showed a picomolar binding affinity because of significant improvement on both the association ($k_{on}$) and dissociation ($k_{off}$) rates. Second, by shuffling against a large VL repertoire, we identified ten different VL sequences (in addition to the original three VL sequences) that could pair with the single VH...
domain while preserving KDR binding specificity. Consistent with our previous study, these different VL/VH pairs bind KDR and block KDR/VEGF interaction with varying efficiency. It is interesting to note that both the V/H9260 and the V/H9261 families are utilized almost equally among the anti-KDR VL/VH pairs, and of the three best KDR/VEGF blocking clones, two (clone 1121 and 4C7) belong to V/H9260, and one (clone 3A1) belongs to V/H9261.

Third, our epitope mapping studies revealed that although clone 1121 competes with 2C6 for binding to certain overlapping epitope(s) on KDR ECD Ig domain 3, the new variant also gained novel binding specificity that is distinct from 2C6. Taken together, these observations suggest that in all of the anti-KDR VL/VH pairs, the single VH domain likely plays a dominate role in determining KDR binding specificity, whereas the VL domains, on the other hand, may contribute to both the binding strength and the fine epitope specificity via influencing the overall folding/presentation of the antigen-binding surface of the antibodies. It is pertinent to note that VL sharing has also been reported in an earlier study among a panel of phage-derived human antibodies directed against several totally unrelated antigens (32), again indicating the dominating role of VH domains in the binding specificities of these antibodies.

A number of approaches have been developed and utilized successfully to improve antibody binding affinity. These approaches usually start with the construction of mutagenized antibody libraries and followed by an array of selections to identify variants with improved affinity. A variety of methods, including randomized mutations (33–35), designed mutations such as “hot spot” and “codon-based” mutations (guided by structural knowledge and/or computer modeling) (36–38) and “CDR walking” (39) have been used to construct the mutagenized libraries. These libraries were then displayed on the surface of filamentous phage (40, 41), bacterial (42–44), yeast (45, 46), or ribosome (47–49) and selected on relevant targets under conditions specifically designed for the enrichment of variants with desired properties (i.e. high affinity) (for review, see Ref. 50). A common practice in these previous studies is that most of them were focused on selection of variant(s) with an improved $k_{\text{off}}$ rate. Selection for improvement on $k_{\text{on}}$ rate, on the other hand, was largely neglected. Because the overall binding affinity ($K_d$) of an antibody equals the ratio of $k_{\text{on}}$ over $k_{\text{off}}$, we reasoned that it should be of significant benefit if we could develop a strategy that would enable us to simultaneously select variants with improvement on not only $k_{\text{off}}$ but also $k_{\text{on}}$.

The three original neutralizing anti-KDR Fab, clones 2C6, 2H2, and 1H4, possess similar overall binding affinity ($K_d$); however, their individual binding kinetics, i.e. $k_{\text{on}}$ and $k_{\text{off}}$, are significantly different. For example, clone 2C6 Fab has the fastest $k_{\text{on}}$ and also the fastest $k_{\text{off}}$ rates, whereas clone 1H4...
Fab has the slowest $k_{on}$ and $k_{off}$ rates (Table III). These observations indicate that VL domains have significant influence on the binding kinetics of these anti-KDR antibodies, which share an identical VH domain. In this report, we therefore constructed a large library by shuffling the single VH domain shared by the three anti-KDR antibodies against the entire VL repertoire from the original naive human library in an attempt to identify the ideal VH/VL pair(s) with improved binding kinetics (i.e., faster $k_{on}$ and slower $k_{off}$). The VL-shuffled library was selected on immobilized KDR protein under conditions of increasing stringency that were designed for both $k_{on}$ and $k_{off}$ selection; the on rate selection was achieved by reducing the numbers of input phages and by shortening the initial binding period, whereas the off rate selection was accomplished by extending the washing process (up to 24 h) in the presence of a high concentration of the soluble receptor protein. This combinatorial selection resulted in the identification of a consensus VL/VH pair (clone 1121) with a $K_d$ of 100 pm, representing >30-fold improvement over the parent Fab fragments. It is pertinent to note that the enhanced affinity of clone 1121 was a result of improvement on both $k_{on}$ and $k_{off}$ rates.

It is generally accepted that to achieve effective tumor binding and antitumor activity, the affinity ($K_d$) of a mAb needs to be $10^{-8}$ M or better (51). Although some investigators presume that high affinity antibody is desirable (52), there is considerable debate whether improving mAb affinity beyond $10^{-9}$ M would result in a further increase in its tumor localization and biological activity (53). Recently, Adams et al. (54) employed several anti-HER2 antibodies of varying affinities derived from the same parent antibody by side-directed mutations and showed a positive correlation between the amount of antibody localized in tumor and antibody affinity up to $10^{-9}$ M. Further increase in affinity beyond $10^{-9}$ M did not, however, result in higher tumor localization of the antibody (55). Histological study on the tumor specimens demonstrated that antibodies with moderate affinity (between $10^{-8}$ and $10^{-9}$ M) penetrated deeper into tumors and percolated more homogeneously into tumor mass than antibodies with high affinity ($<10^{-10}$ M), which were trapped around the perivascular tumor cells with much less localization in the distant tumor tissues (55). The impaired tumor penetrating capacity of antibodies with very high affinity may negatively affect their anti-tumor activity when the antibodies are used as targeting devices to deliver cytotoxic agents to tumor cells within a large tumor mass (53, 55). This argument may not be relevant in the context of anti-KDR therapy, because the target antigen (i.e. KDR) is expressed on the surface of vessel-lining endothelial cells, which are in direct contact with the systemically administered antibody (i.e., no tumor penetration is required for antigen access). Antibodies with higher affinity, such as IMC-1121, should be able to bind more tightly to their target for a prolonged period of time, thus efficiently preventing the growth factors, such as VEGF, from interacting to their receptors. In this setting, high affinity antibodies are therefore likely to be more effective agents than those with low affinity, hence the preferable choice for anti-angiogenesis therapy. Here we demonstrated that the high affinity antibody, IMC-1121, is indeed more potent in inhibiting KDR/VEGF interaction and in neutralizing VEGF-stimulated KDR activation than its parent antibodies. Taken together with our previous observation that IMC-1121 was more potent than IMC-26 in inhibiting KDR-leukemia growth in an animal model (11), these results lend strong support to IMC-1121 for potential clinical evaluation as an anti-angiogenic/antitumor agent.
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