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Production of neutralizing monoclonal antibody against human vascular endothelial growth factor receptor II¹

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ABSTRACT

AIM: To prepare neutralizing monoclonal antibody (mAb) against extracellular immunoglobulin (Ig)-like domain III of vascular endothelial growth factor receptor KDR and study its biological activity. **METHODS:** Soluble KDR Ig domain III (KDR-III) fusion protein was expressed in *E Coli* and purified from the bacterial periplasmic extracts via an affinity chromatography. Monoclonal antibodies against KDR-III were prepared by hybridoma technique. ELISA and FACS analysis were used to identify its specificity. Immunoprecipitation and [³H]-thymidine incorporation assay were also used to detect the activity of anti-KDR mAb blocking the phosphorylation of KDR tyrosine kinase receptor and the influence on vascular endothelial growth factor-induced mitogenesis of human endothelial cells. **RESULTS:** A monoclonal antibody, Ycom1D3 (IgG1), was generated from a mouse immunized with the recombinant KDR-III protein. Ycom1D3 bound specifically to both the soluble KDR-III and the cell-surface expressed KDR. Ycom1D3 effectively blocked VEGF/KDR interaction and inhibited VEGF-stimulated KDR activation in human endothelial cells. Furthermore, the antibody efficiently neutralized VEGF-induced mitogenesis of human endothelial cells. **CONCLUSION:** Our results suggest that the anti-KDR mAb, Ycom1D3, has potential applications in the treatment of cancer and other diseases where pathological angiogenesis is involved.

INTRODUCTION

Angiogenesis, a multiple process that results in the formation of new blood vessels from pre-existing vasculature, is essential for both the growth of solid tumors beyond the size of 2 mm³ and for tumor me-

tastasis^[1]. A number of growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiogenin have been implicated as possible regulators in this process^[2,3]. VEGF is distinct among these factors in that it acts as an endothelial cell specific mitogen during angiogenesis. Furthermore, VEGF is the growth factor most consistently found in a wide variety of conditions associated with angiogenesis^[4].

VEGF binds to and mediates its activity mainly through two tyrosine kinase receptors, the *fms*-like tyrosine kinase (Flt-1, VEGFR-1) and the kinase insert domain-containing receptor (KDR, VEGFR-2), or its

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murine homolog, fetal liver kinase (Flk-1). Numerous studies have shown that overexpression of VEGF and its receptors plays an important role in tumor-associated angiogenesis and hence in both tumor growth and metastasis. Of the two receptors, KDR is generally believed to be the main receptor that mediates VEGF biological activities and thus plays a major role in tumor-associated angiogenesis^[5]. The role of Flt-1 in angiogenesis in adult, on the other hand, remained unclear. Several recent reports have demonstrated that, however, Flt-1 may play an important role in promoting hematopoietic stem cell mobilization and angiogenesis in both ischemic tissues and tumors^[6]. Taken together, these results suggest that both KDR and Flt-1 may represent good targets for developing anti-angiogenic therapeutics.

KDR belongs to the subfamily of class III receptor tyrosine kinases (RTKs) and is characterized by 7 immunoglobulin (Ig)-like domains in the extracellular region and a split kinase intracellular domain. Deletion mutant analysis demonstrated that KDR extracellular Ig-like domain II and III were sufficient for high affinity binding of VEGF. Deletion of domain III alone caused more than 1000-fold reduction in binding affinity, indicating a critical role for this domain in VEGF/KDR interaction^[7,8]. In this study we cloned and expressed the extracellular Ig domain III of KDR (KDR-III) and generated a KDR-III specific monoclonal antibody (mAb), Ycom1D3 (IgG1). The mAb bound specifically to both the soluble KDR-III and KDR expressed on endothelial cells. It blocked VEGF binding to KDR and demonstrated potent inhibition of VEGF-induced receptor phosphorylation and DNA synthesis in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Cell lines and proteins HUVECs were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords and maintained in M199 medium containing 20 % heat-inactivated fetal calf serum (FCS, Hyclone), VEGF, bFGF, glutamine, and antibiotics at 37 °C in a humidified atmosphere containing 5 % CO₂. Cells were used between passage 2 and 4 for all assays. The identity of HUVEC as endothelial cells was confirmed by their polygonal shape and immunoreactivity for factor VIII-related antigens. SP2/0-Ag14 murine myeloma cells were cultured in RPMI-1640 medium supplemented with 10 % FCS. Hybridoma clones were selected in RPMI-1640 medium con-

taining 10 % FCS supplemented with hypoxanthine, aminopterin, and thymidine (HAT) medium, maintained in RPMI-1640 medium after the clonal selection. HI47, a mAb directed against CD20, was produced at Institute of Hematology (Tianjin, China). An anti-CD3 single chain Fv (scFv) was expressed in *E coli* and purified following the procedures described previously^[9].

Expression and purification of the KDR-III protein The cDNA encoding extracellular Ig domain III of KDR (amino acids 225-327) was obtained from HUVECs by reverse transcription-PCR using primers: 5' GCAACTGCGGCCAGCCGGCCGATGTGGTTC TGAGTCCGTC 3' (*Sfi* I restriction site underlined); and 5' GCGCGCTGCGGCCGCTTTTTCATGGACCC TGACAAA 3' (*Not* I restriction site underlined). The resulting product was cloned into the expression vector pCANTAB 5E (Amersham Pharmacia Biotech) in frame with a polypeptide, the E tag, fused to its C-terminal end for purification and assay purposes. Automatic sequencing was done with ABI PRISM 377 sequencer. The nucleic acid sequence and its deduced amino acid sequence were searched against GenBank using Gapped BLAST program. The soluble KDR-III-E tag protein was secreted from *E coli* strain HB2151 containing the expression plasmid grown at 30 °C in a shaker flask and purified by an anti-E-tag affinity chromatography following a procedure described previously^[10]. Briefly, the periplasmic extract of the bacteria 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, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) followed by incubation at 4 °C with gentle shaking for 1 h. After centrifugation at 12×10³ r/min for 15 min, the soluble KDR-III protein was purified from the supernatant by an anti-E tag affinity chromatography using the RPAS Purification Module (Amersham Pharmacia Biotech).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of recombinant KDR III protein The *E coli* periplasmic extract and the purified KDR-III protein preparation were electrophoresed in a 15 % polyacrylamide gel and visualized by staining with a Colloidal Blue Stain kit (Novex). In addition, an identical gel was transferred onto nitrocellulose membrane and subjected to immunoblot analysis using a mouse anti-E tag antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Finally, membrane was developed with diaminobenzidine (Amresco) and H₂O₂. Photoes

were taken to note the results.

Generation of mAbs For production of mAb, BALB/c mice were immunized by purified KDR-III protein as the routine method. Briefly, 1×10^8 lymphocytes from immunized mouse spleen were fused at a ratio of 10:1 with mouse myeloma SP2/0-Ag14 cells in the presence of 50 % polyethylene glycol (PEG1450, Sigma). Indirect ELISA was performed using KDR-III or anti-CD3 scFv for screening assay. The hybrid cells that produce specific antibodies were cloned 3 times by limiting dilution in 96-well microtiter plates. Positive hybridoma cells 2×10^6 were intraperitoneally (ip) injected into pristane sensitized mice for production of ascites antibody. The mAb in the ascites were purified by using protein G affinity chromatography and the purity of the mAb preparation was confirmed by SDS-PAGE analysis.

FACS analysis Early passage HUVECs were grown in growth factor-depleted M199 medium overnight to induce the expression of the KDR. The cells were harvested and washed 3 times with PBS, incubated with Ycom1D3 (50 mg/L) for 1 h at 4 °C, followed by incubation with FITC-labeled goat anti-mouse IgG (Zhongshan, China) for an additional 45 min. The cells were washed and analyzed by a FACS flow cytometry (FACSCalibur, Becton Dickinson, USA)

Quantitative KDR binding assay Various amounts of antibodies were added to KDR-III (4 µg/ml) coated 96-well Maxi-sorp microtiter plates and incubated at room temperature for 1 h, after which the plates were washed three times with PBS containing 0.1 % Tween-20 (PBST). The plates were then incubated at room temperature for 1 h with 100 µL of HRP-labeled goat anti-mouse IgG (Zhongshan, China) for an additional 45 min. The plates were washed 5 times, peroxidase substrate (*o*-phenyldiamine, Invitrogen) was added, and the absorbance at 492 nm was detected by using a microplate reader (SLT Spectra, Austria).

Affinity determination Purified Ycom1D3 (50 µg) was first labeled with 1.85×10^7 Bq [125 I]iodide (Amersham Pharmacia Biotech) following a protocol described previously^[11]. The labeled mAb was separated from free 125 I by gel filtration on a Sephadex G-25 column equilibrated in PBS containing gelatin at 1 g/L. Binding of 125 I-Ycom1D3 to KDR-III was carried out following a protocol as described by Duan *et al*^[12]. Briefly, various amounts of 125 I-Ycom1D3 were added in triplicates to each well of KDR-III-coated (4 mg/L) 96-well Maxi-sorp microtiter plates and incubated for 3 h at room

temperature. The radioactivity in each well was measured using a gamma-counter (1282 Comp Gamma CS, LKB, and Turk Finland) after extensive washes with PBST. Nonspecific binding was determined by incubation of 125 I-Ycom1D3 in the presence of 100-fold excess of unlabeled Ycom1D3. The affinity constant was determined by the Scatchard plot analysis^[13].

Competitive VEGF binding assay Various amounts of antibodies were mixed with a fixed amount of KDR-III protein (100 ng) and incubated at room temperature for 1 h. The mixtures were then transferred to 96-well Maxi-sorp microtiter plates coated with VEGF₁₆₅ (200 ng/well) and incubated at room temperature for an additional 2 h, after which the plates were washed 3 times with PBST. The plates were then incubated at room temperature for 1 h with 100 µL of HRP-labeled mouse anti-E tag antibody. The plates were washed 5 times and the substrate for peroxidase was added to quantify the bound KDR-III molecules. IC₅₀, ie, the antibody concentration required for 50 % inhibition of KDR binding to VEGF, was then calculated.

KDR phosphorylation assay Subconfluent HUVECs were grown in growth factor-depleted M199 medium for 48 h prior to the experiment. After pretreatment with 50 nmol/L sodium orthovanadate for 30 min, the cells were incubated in the presence or absence of antibodies (20 mg/L) for 15 min, followed by stimulation with VEGF₁₆₅ 25 µg/L at room temperature for an additional 15 min. The cells were then lysed in lysis buffer (Tris 50 mmol/L, NaCl 150 mmol/L, 1 % Nonidet P-40, EDTA 2 mmol/L, 0.25 % sodium deoxycholate, PMSF 1 mmol/L, aprotinin 1 mg/L, leupeptin 2 mg/L, pepstatin 1 mg/L, pH 7.5), and the cell lysates were used for KDR phosphorylation assay. The KDR was immunoprecipitated from the cell lysates with protein A agarose beads (Invitrogen) coupled to Ycom1D3. Proteins were resolved with SDS-PAGE and subjected to Western blot analysis. To detect KDR phosphorylation, blots were probed with an anti-phosphotyrosine mAb, PT-66 (Sigma), and the same Western blot analysis was probed with Ycom1D3 to assure that equal amount of protein was loaded in each lane of the gel. All signals were detected using Super Signal West Pico Chemiluminescent Substrate (Pierce)^[14,15].

Anti-mitogenic assay HUVECs (5×10^3 cells/well) were plated onto 96-well tissue culture plates (Nunc) in 200 µL growth factor depleted M199 medium for 72 h prior to the experiment. Various amounts of antibodies were added to duplicate wells and

preincubated at 37 °C for 1 h, after which VEGF₁₆₅ was added to a final concentration of 25 µg/L. After 24 h of incubation, 1.85×10⁴ Bq of [³H]thymidine (Institute of Atomic Energy, Chinese Academy of Science, Beijing, China) was added to each well and incubated for an additional 7 h. The cells were then washed, harvested, and analyzed for their incorporations of [³H]thymidine using a scintillation counter (Beckman, LS 5801)^[14-16].

RESULTS

Expression and purification of recombinant KDR-III protein Total RNA was extracted from HUVECs, and the sequence encoding the extracellular Ig domain III of KDR was amplified and cloned into the expression vector pCANTAB 5E. The nucleotide sequence of the cloned product was verified by automated DNA sequencing. Sequence alignment revealed that it was 100 % homologous to the GenBank accession number AF035121 nucleotide sequence (976-1284) and the reading frame had been maintained. Furthermore, it has little similarity with any other human proteins, so it may serve as immunogen to immunize mice to prepare mAbs against vascular endothelial growth factor receptor KDR. The soluble KDR-III protein was expressed in *E coli* HB2151 and purified from the periplasmic extracts of the bacteria via an anti-E tag affinity chromatography. The yield of the purified KDR-III protein from shake flask culture ranged

from 1-2 mg/L medium. The purified KDR-III protein was analyzed by SDS-PAGE. The polypeptide was resolved under the electrophoretic conditions and gave rise to one major band with mobility close to that anticipated (Fig 1A). Immunoblot analysis using an anti-E tag antibody revealed a single band at ~14 kDa which corresponded to the size of KDR-III fusion protein (Fig 1B).

Production and characterization of anti-KDR mAb The purified KDR-III protein was used to immunize BALB/c mice in an attempt to generate neutralizing mAb. After fusion, the hybridoma cells were screened for binding to KDR-III by indirect ELISA analysis. An E-tag-containing scFv, the anti-CD3 scFv, was also included in the screening in order to distinguish hybridoma cells producing antibodies directed against the E-tag, which was fused to the KDR-III protein for the purpose of purification and detection. In total, four KDR-III-positive clones were identified by initial ELISA screening, and the clone Ycom1D3 was selected after 3 round of further subcloning. mAb Ycom1D3, an IgG1/kappa antibody, was then produced in mouse ascites and purified by Protein G chromatography.

Ycom1D3 bound specifically to both soluble KDR-III and KDR expressed on human endothelial cells The binding specificity and efficiency of the purified Ycom1D3 to KDR were examined by both ELISA and FACS analysis. Ycom1D3 bound efficiently to immobilized KDR-III in a dose-dependent manner

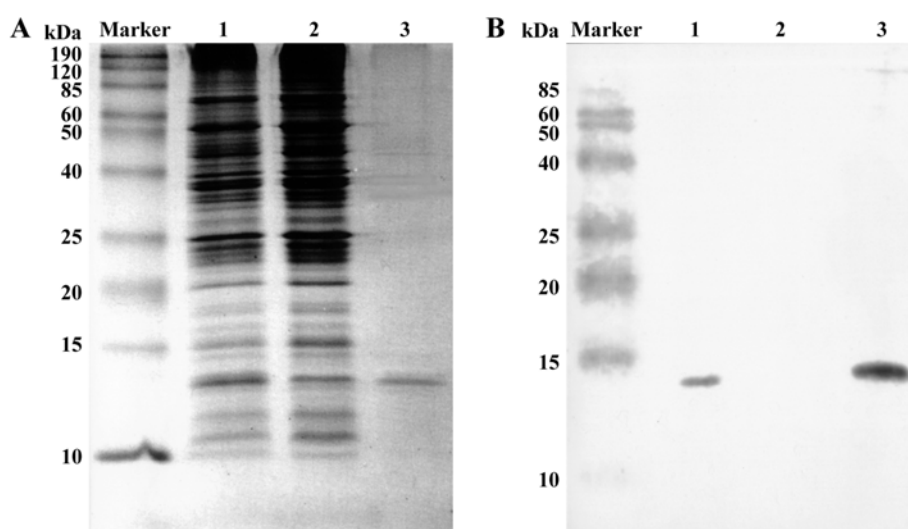


Fig 1. Expression and purification of the recombinant KDR-III protein. **A:** Colloidal staining of a SDS-PAGE gel. **B:** immunoblot analysis of the SDS-PAGE gel with an anti-E tag antibody. Lane 1, periplasmic extract of bacteria cells expressing the KDR-III protein; Lane 2, flow through from the anti-E tag column; Lane 3, KDR-III protein eluted from the anti-E tag column. Also shown on left are the positions of molecular weight markers.

(Fig 2A). In addition, the antibody reacted specifically to KDR expressed on HUVECs as demonstrated by FACS analysis (Fig 2B). The binding affinity (K_d) of Ycom1D3 to KDR-III was about 1.0 nmol/L as determined by Scatchard analysis (Fig 2C), which is similar to that of natural ligand (VEGF) for binding to KDR previously reported^[15].

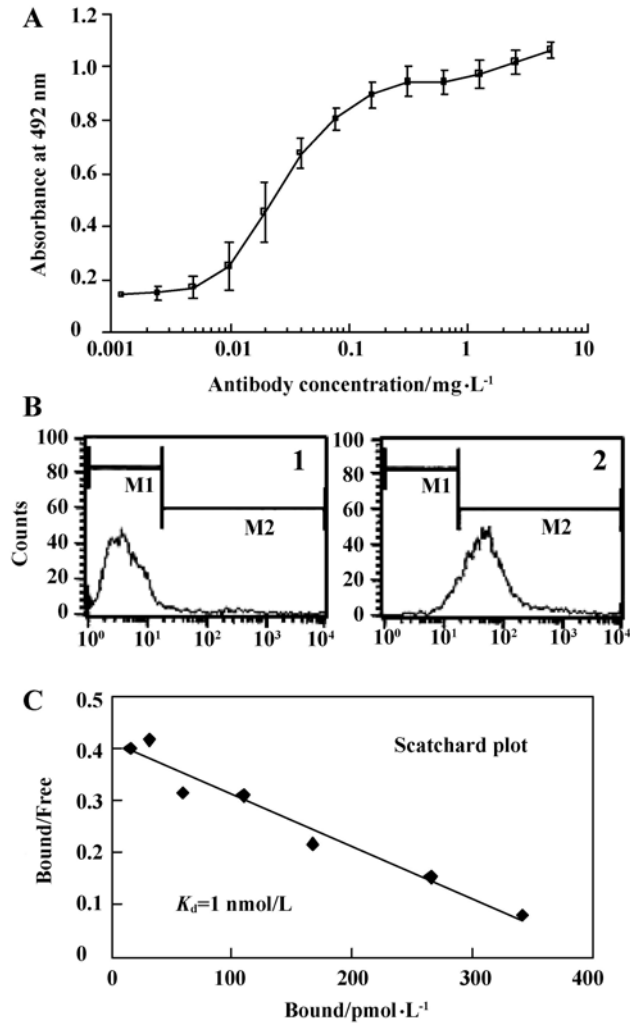


Fig 2. Binding of the anti-KDR-III mAb, Ycom1D3, to KDR. **A:** Binding of Ycom1D3 to immobilized KDR-III as assayed by ELISA. **B:** Binding of Ycom1D3 to KDR expressed on the surface of HUVEC as assayed by FACS analysis. 1. PBS; 2. Ycom1D3 **C:** Scatchard analysis of ¹²⁵I-labeled Ycom1D3 binding to immobilized KDR-III protein. Nonspecific binding was determined by incubation of ¹²⁵I-labeled Ycom1D3 in the presence of 120-fold excess of unlabeled Ycom1D3. Data points represent the means±SD of triplicate determinations.

Ycom1D3 competed effectively with VEGF for binding the KDR Competitive VEGF binding assay was used to determine whether the anti-KDR-III mAb,

Ycom1D3, could compete effectively with VEGF for binding to KDR. Fig 3 showed that Ycom1D3 blocked KDR from binding to immobilized VEGF in a dose-dependent manner, with an IC₅₀, ie, the antibody concentration required to inhibit 50 % of KDR from binding to VEGF, of ~0.3 mg/L. As a control, the anti-CD20 mAb, HI47, did not bind to KDR or blocked VEGF/KDR interaction.

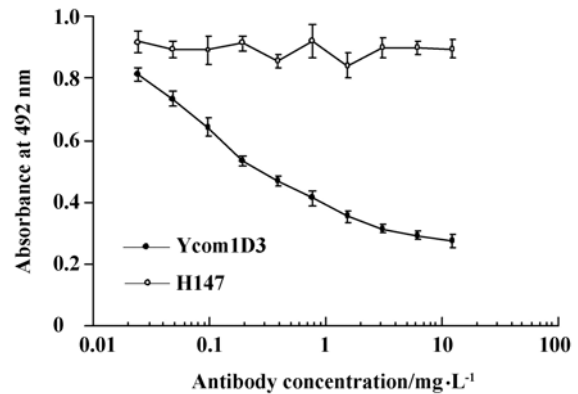


Fig 3. Inhibition of binding of KDR to immobilized VEGF₁₆₅ by Ycom1D3. Data points represent the means±SD of triplicate determinations.

Ycom1D3 inhibited efficiently VEGF-stimulated activation of KDR To investigate the effect of Ycom1D3 on VEGF-stimulated receptor activation, we examined the level of KDR phosphorylation in HUVECs stimulated with VEGF in the presence and absence of the antibody. Ycom1D3 inhibited effectively VEGF-stimulated phosphorylation of KDR (Fig 4). As expected, the control mAb HI47 did not show any inhibition of VEGF-stimulated activation of KDR. In the absence of VEGF, neither Ycom1D3 nor HI47 alone had any effect on KDR activation in HUVECs up to 4 h incubation.

Ycom1D3 neutralized efficiently VEGF-induced mitogenesis of human endothelial cells The effect of Ycom1D3 on VEGF-stimulated mitogenesis of human endothelial cells was determined using the [³H]thymidine incorporation assay in HUVECs. Ycom1D3 inhibited effectively mitogenesis of HUVECs stimulated by VEGF. The antibody concentration required to inhibit 50 % of VEGF-induced mitogenesis of HUVEC is ~0.5 mg/L. The maximum inhibition of HUVECs mitogenesis was achieved at antibody concentration of ~1.5 mg/L (Fig 5). As expected, HI47 did not show any inhibitory effect on VEGF-stimulated endothelial cell proliferation.

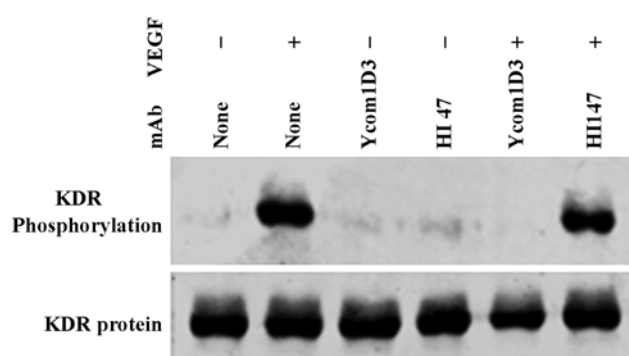


Fig 4. Inhibition of VEGF-induced phosphorylation of KDR by Ycom1D3. Growth factor starved early passage HUVECs were incubated in M199 medium at RT for 15 min in the presence or absence of Ycom1D3 (20 mg/L), followed by stimulation with 25 μ g/L of VEGF₁₆₅ at RT for an additional 15 min. The cells were lysed and the cell lysate were used for KDR kinase phosphorylation assays. Data shown are representative of at least 3 separate experiments.

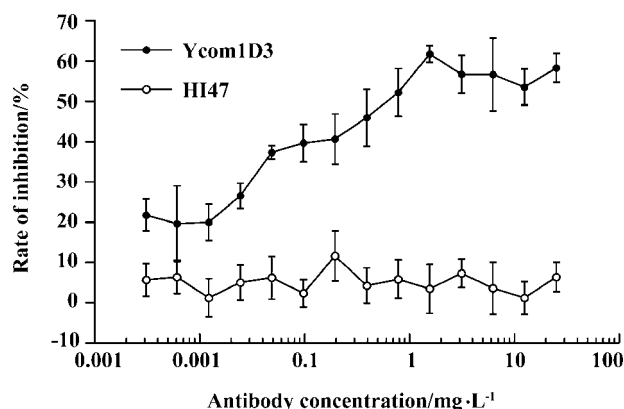


Fig 5. Inhibition of VEGF-stimulated HUVECs proliferation by Ycom1D3. Data shown are representative of at least 3 separate experiments.

DISCUSSION

Compelling evidence to date indicates that VEGF and KDR play an important role in tumor-associated angiogenesis. Inhibition of VEGF/KDR interaction and/or KDR-mediated signal transduction, therefore, represent an excellent approach for anti-angiogenic intervention. Domain-deletion analysis demonstrated that KDR extracellular Ig-like domains I to III were required for high affinity binding of VEGF, and deletion of domain III caused >1000-fold reduction in binding^[7,8,17]. Further, a recent study has revealed that a number of anti-KDR neutralizing antibodies require domain III for efficient binding, whereas all nonblocking antibodies

recognize epitopes that are located outside this domain^[8]. Taken together, these observations suggest that KDR Ig domain III may direct contact VEGF thus playing a critical role in high-affinity VEGF interaction.

Based on the above observations, we hypothesize that the single KDR Ig domain III would serve as a good immunogen for the generation of neutralizing anti-KDR mAb. This approach offers significant benefits in practice because the single Ig domain can be readily produced in *E coli*, whereas the full length KDR, a high molecular weight glycoprotein comprising 7 Ig domains, has to be expressed in mammalian cells. The mAb, Ycom1D3, generated from the KDR-III-immunized mouse reacted to both soluble KDR-III and KDR expressed on cell surface and blocked VEGF/KDR interaction efficiently, confirming the effectiveness of our approach to production of receptor-neutralizing mAb. Since high-affinity interaction between VEGF and KDR requires the first three N-terminal Ig domains of the receptor^[7,8], our results suggest that Ycom1D3 most likely exerts its blocking activity via either directly competing with VEGF for binding site(s) within the Ig domain III of the receptor, or by steric hindrance resulted from interaction between the antibody and the receptor.

Lots of inhibitors of angiogenesis is currently under evaluation as potential cancer therapeutics^[18,19]. Compared to small molecular inhibitors, anti-KDR antibody therapy may provide higher specificity towards tumors, and therefore, better safety profile. In addition, neutralizing antibodies to KDR will not only block the angiogenic activity of VEGF, but also that of other growth factors exerting their angiogenic effects via the receptors, including VEGF-C, VEGF-D, and VEGF-E. In contrast, antibodies to an individual growth factor such as VEGF would only neutralize specifically the angiogenic activity of the single ligand. Whether any of these potential advantages of using anti-KDR antibodies will translate into clinical benefit, however, remains to be seen in human clinical trials.

Furthermore, the VEGF/KDR pathway has been shown to also play a role in other diseases such as diabetic retinopathy, psoriasis, hemangioblastoma, rheumatoid arthritis, and Kaposi sarcomas^[20]. Thus VEGF/KDR antagonists may have broad clinical application in the treatment of a variety of human diseases in which pathological angiogenesis is involved. Here we demonstrated that Ycom1D3 specifically blocked VEGF/KDR interaction and inhibited VEGF-induced KDR activation and proliferation of endothelial cells. Our data lend sup-

port to further evaluation of this antibody as an anti-angiogenic agent in the treatment of cancer and other diseases^[21].

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