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# Potent neutralization of VEGF biological activities with a fully human antibody Fab fragment directed against VEGF receptor 2 ☆

Hua-Quan Miao *, Kun Hu, Xenia Jimenez, Elizabeth Navarro, Haifan Zhang, Dan Lu, Dale L. Ludwig, Paul Balderes, Zhenping Zhu *

*Departments of Antibody Technology, Protein Science, and Cell Engineering and Expression, ImClone Systems Incorporated, New York, NY 10014, USA*

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**Abstract**

Compelling evidence suggest that vascular endothelial growth factor (VEGF) and its receptors, especially receptor 2 (VEGFR2, or kinase insert domain-containing receptor, KDR), play a critical role in angiogenesis under both physiological and pathological conditions, including cancer and angiogenic retinopathies such as age-related macular degeneration (AMD). To this end, inhibition of angiogenesis with antagonists to either VEGF or KDR has yielded significant therapeutic efficacy both in preclinical studies in animal models and in clinical trials in patients with cancer and AMD. We previously reported the identification of a high affinity, fully human anti-KDR antibody fragment, 1121B Fab, through a highly stringent affinity maturation process with a Fab originally isolated from a naïve human antibody phage display library. In this study, we demonstrate that 1121B Fab is able to strongly block KDR/VEGF interaction, resulting in potent inhibition of an array of biological activities of VEGF, including activation of the receptor and its signaling pathway, intracellular calcium mobilization, and migration and proliferation of endothelial cells. Taken together, our data lend strong support to the further development of 1121B Fab fragment as an anti-angiogenesis agent in both cancer and angiogenic retinopathies.


*Keywords:* Angiogenesis; Anti-angiogenic therapy; Endothelial cell; Fab fragment; Human antibody; VEGF; VEGFR2/KDR

Angiogenesis is a tightly regulated multiple step process that results in the formation of new blood vessels from pre-existing vasculature. Accumulating experimental and clinical studies have implicated uncontrolled angiogenesis as a major contributor in tumor growth and metastasis, as well as in a number of other human diseases, such as age-related macular degeneration (AMD), diabetic retinopathy, rheumatoid arthritis, and psoriasis [1–3]. Compelling evidence suggest that vascular endothelial growth factor (VEGF) and its receptors, especially receptor 2 (VEGFR2, or kinase insert domain-containing receptor, KDR), play a critical role in angiogenesis under both physiological and pathological conditions [4–6]. VEGF and KDR are frequently up-regulated in a number of clinically important human diseases, including cancer and AMD [4–6]. Inhibition of angiogenesis with antagonists to either VEGF or KDR, by using antibodies [7–10], aptamers [11], immunotoxins [12], ribozyme [13], soluble receptors [14], and small molecule tyrosine kinase inhibitors [15], has led to significant therapeutic efficacy both in preclinical studies in animal models and in clinical trials in patients with cancer and AMD. Taken together, these findings indicate that both VEGF and KDR represent excellent targets for therapeutic intervention of human diseases where pathological angiogenesis is involved [16–18].

☆ *Abbreviations:* AMD, age-related macular degeneration; AP, alkaline phosphatase; bFGF, basic fibroblast growth factor; GFP, green fluorescence protein; HUVEC, human umbilical vascular endothelial cells; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; KDR, kinase insert domain-containing receptor (also known as VEGFR receptor 2, or VEGFR2); PAE, porcine arotic endothelial cells; PLC-γ, phospholipase C γ; RT, room temperature; VEGF, vascular endothelial growth factor.

* Corresponding authors. Fax: +1 212 645 2054.
*E-mail addresses:* hua-quan.miao@imclone.com (H.-Q. Miao), zhenping.zhu@imclone.com (Z. Zhu).

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Monoclonal antibodies (mAb), owing to their high specificity towards a given target, represent a unique class of novel therapeutics as angiogenesis inhibitors. We previously produced a chimeric anti-KDR antibody, IMC-1C11, and demonstrated that this antibody is capable of blocking KDR/VEGF interaction and inhibiting VEGF-stimulated receptor activation and mitogenesis of human endothelial cells [19,20]. In addition, IMC-1C11, given by intravitreous administration, effectively inhibited ocular, including both retinal and intravitreous, neovascularization in an animal model of high tension oxygen-induced proliferative retinopathy in new-born dogs, but had no effect on the normal retina vascular development in new-born dogs that did not expose to high tension oxygen [21]. We recently produced several fully human anti-KDR Fab fragments from screening of a large naïve human antibody phage display library [22]. These Fab fragments compete efficiently with VEGF for binding to KDR and block VEGF-stimulated mitogenesis of human umbilical vascular endothelial cells (HUVEC). Affinity maturation of one of these Fab clones, 2C6, led to the generation of a new clone, 1121B Fab, that bind to KDR with an affinity of ~0.1 nM [23]. In this report, we further demonstrate the potent neutralizing activity of 1121B Fab on the various biological activities of VEGF, including activation of KDR and downstream signaling molecules, intracellular calcium mobilization, induction of endothelial cell migration, and stimulation of endothelial cell mitogenesis and proliferation.

## Materials and methods

*Reagents and cells.* Porcine aortic endothelial cells stably expressing full-length KDR (PAE/KDR) were generously provided by Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden), and cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Primary-cultured HUVEC were obtained from Dr. S. Rafii at Cornell Medical Center, New York, and maintained in EBM-2 medium (Clonetics, Walkersville, MD) at 37 °C, 5% $CO_2$. 1121B Fab, a high-affinity antibody fragment directed against KDR [23], was produced from a stably transfected mammalian cell line cultured under serum-free conditions, and purified from the cell culture supernatant via several steps of ion-exchange chromatography. The soluble proteins, KDR-alkaline phosphatase (AP) fusion and $VEGF_{165}$, were produced at ImClone Systems (New York, NY) as previously described [24]. Basic fibroblast growth factor (bFGF) was purchased from Invitrogen.

*Generation of PAE/KDR/GFP cell line.* The open reading frame of green fluorescence protein (GFP) was excised from pEGFP-N1 (Clontech, Mountain View, CA) plasmid DNA with restriction enzymes *Eco*RI and *Not*I (New England Biolabs, Beverley, MA). The GFP DNA fragment was then ligated to the linearized pIRESpuro2 vector (Clontech), yielding GFPpuro. The PAE/KDR cells were transfected with the GFPpuro plasmid DNA using FuGENE 6 (Roche, Alameda, CA). Stable clones of PAE/KDR/GFP cells were selected in the presence of 250 μg/ml G418 (Invitrogen) and 2 μg/ml puromycin (Sigma, St. Louis, MO). The uniform expression of GFP in individual clones was confirmed by flow cytometry and fluorescent microscopy. The expression of KDR protein was confirmed both by Western blot and flow cytometry.

*Quantitative KDR binding and blocking assay.* In the direct binding assay, various amounts of 1121B Fab proteins were added to KDR-coated 96-well Maxi-sorp microtiter plates and incubated at room temperature (RT) for 1 h, after which the plates were washed three times with PBS containing 0.1% of Tween 20. The plates were then incubated at RT for 1 h with 100 μl of a rabbit anti-human κ chain antibody-HRP conjugate (Jackson ImmunoResearch Laboratory Inc., West Grove, PA). The plates were washed and developed following a procedure previously described [22,23]. In the competitive KDR/VEGF blocking assay, various amounts of antibodies were mixed with a fixed amount of KDR-AP (100 ng) and incubated at RT for 1 h. The mixtures were then transferred to 96-well microtiter plates precoated with $VEGF_{165}$ (200 ng/well) and incubated at RT for an additional 2 h, after which the plates were washed 5 times 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. $IC_{50}$, i.e., the antibody concentration required for 50% inhibition of KDR binding to VEGF, was then calculated.

*Competitive VEGF binding to PAE/KDR cells.* VEGF (5 μg) was iodinated with 1 mCi $Na^{125}I$ (Perkin-Elmer, Boston, MA) using Iodobeads (Pierce, Rockford, IL) according to manufacturer's instruction. The $^{125}I$-VEGF was purified through a NAP10 column (Amersham Pharmacia Biotech, Piscataway, NJ). The specific activity was $\sim 5 \times 10^4$ cpm/ng protein. Subconfluent PAE/KDR cells cultured in 48-well plate were washed with the binding buffer (F12 medium containing 0.1% BSA, 25 mM Hepes, pH 7.5), and incubated with various concentrations of 1121B Fab for 30 min on ice. The cells were then incubated with 5 ng/ml $^{125}I$-VEGF for 2 h on ice. After washing with binding buffer and PBS, the cells were lysed with 0.5 N NaOH, and the radioactivity was measured using the gamma counter.

*Inhibition of VEGF-stimulated KDR phosphorylations.* Subconfluent PAE/KDR cells cultured in 10-cm dishes were starved overnight in F-12 medium containing 0.1% BSA. The cells were incubated with 1121B Fab for 10 min at 37 °C, followed by stimulation with VEGF (10 ng/ml) or bFGF (10 ng/ml) for 10 min. The cells were lysed with a lysis buffer [0.5% Trition X-100, 150 mM NaCl, 50 mM Hepes, pH7.5, 10 mM NaPPi, 50 mM NaF, 1 mM $Na_3VO_4$, and the protease inhibitors cocktail (Roche)]. In VEGF-treated study, equal amounts of cell lysates were resolved by a 4–20% SDS–PAGE and the proteins were transferred to a PVDF membrane (Millipore, Billerica, MA). The blot was incubated with an anti-phospho-KDR, an anti-phospho-phospholipase C γ (PLC-γ) or an anti-phospho mitogen-activated protein kinase (MAPK) antibody (Cell Signaling, Nanvers, MA). In bFGF-treated group, the cell lysates were precipitated with an anti-FGF receptor antibody, H7 (ImClone Systems). The precipitated proteins were resolved, transferred and blotted with an anti-phospho-tyrosine antibody. Total target proteins loaded on the gels were detected by re-blotting the membrane with an anti-KDR, an anti-FGFR, an anti-PLC-γ, or an anti-MAPK antibody (Cell Signaling). After incubation with the HRP-conjugated protein A (Calbiochem, San Diego, CA), the membrane was incubated with enhanced chemiluminescence reagent (Amersham). The signals were recorded with the Fuji LAS-3000 imaging system (Fuji Medical Systems, Stamford, CT) within linear exposure range.

*Intracellular calcium mobilization assay.* PAE/KDR cells ($1 \times 10^3$ cells/100 μl) were seeded in each well of a black-rim 96-well plate, and starved overnight in F-12 medium containing 0.1% BSA, followed by incubation with 1121B Fab and the FLIPER calcium dye (Molecular Device, Sunnyvale, CA) for 1 h at 37 °C, per the protocol of the manufacturer. The plate was cooled to room temperature for 20 min, and then put onto a FlexStation II fluorometer (Molecular Device). VEGF (10 ng/ml) was then delivered to each well by the robotics. The calcium flux was immediately measured by the fluorometer as reflected by the fluorescence intensity per well. Sensorgrams were obtained at each Fab concentration and IC50, i.e., Fab concentration that yields 50% inhibition of VEGF-stimulated calcium flux, was calculated based on the areas under curve obtained with each sensorgram.

*VEGF-induced chemotaxis assay.* Chemotactic response of PAE/KDR cells to VEGF was studied as previously described [25] using a modified Boyden chamber. VEGF (10 ng/ml) was added to the wells in the lower compartment, whereas the 1121B Fab and the cells were mixed and added to the wells of the upper compartment. After 4 h incubation at 37 °C, the unmigrated cells were scraped, and the migrated cells were fixed with 4% formaldehyde and stained with 2 μg/ml of Hoechst 33342. The number of

nuclei was counted under a Zeiss epifluorescence microscope using an automated protocol with the Image-pro Plus 5.0 software (Media Cybernetic, Silver Spring, MD).

*VEGF-stimulated cell mitogenesis and proliferation.* Two assays were utilized to exam the anti-angiogenic effect of 1121B Fab on endothelial cells. In the first assay, the mitogenesis assay, HUVEC ($5 \times 10^3$ cells/well) were plated onto 96-well tissue culture plates (Wallach, Inc., Gaithersburg, MD) in 200 μl of EBM-2 medium without VEGF, basic fibroblast growth factor or epidermal growth factor and incubated at 37 °C for 72 h. Various amounts of Fab were added to duplicate wells and pre-incubated at 37 °C for 1 h, after which VEGF165 was added to a final concentration of 16 ng/ml. After 18 or 68 h of incubation, 0.25 μCi of [$^3$H]TdR (Amersham) was added to each well and incubated for an additional 4 h. The cells were washed once with PBS, trypsinized, and harvested onto a glass fiber filter (Printed Filtermat A, Wallach) with a cell harvester (Harvester 96, MACH III M, TOMTEC, Orange, CT). The membrane was washed three times with H2O and air-dried. Scintillation fluid was added and DNA incorporated radioactivity was determined on a scintillation counter (Wallach, Model 1450 Microbeta Liquid Scintillation Counter). In the second assay, the endothelial cell proliferation assay, black-rim 96-well plates were coated with Matrigel, diluted 1:10 in PBS (50 μl/well) (BD Bioscience, Franklin Lakes, NJ), for 1 h at 37 °C. PAE/KDR/GFP cells were seeded at a density of $5 \times 10^3$ cells/ml (200 μl/well) in complete F-12 medium. After overnight culture, cells were starved for 3 days in 0.1% FBS/F-12 medium. VEGF and the antibodies were added to the culture for additional 2 days. The cell proliferative response was estimated as GFP fluorescence intensity as measured by a FlexStation II fluorometer (Molecular Device). A linear correlation was established between the fluorescence intensity and the cell numbers per well prior to the experimentation.

## Results and discussion

### *1121B Fab binds efficiently to KDR and blocks effectively KDR/VEGF interaction*

The antigen-binding efficiency of 1121B Fab was determined by ELISA on immobilized receptor. The Fab binds to KDR in a dose-dependent manner, with an ED50, the Fab concentration that yielded 50% of maximum binding, of approximately 0.15 nM (Fig. 1A). The binding affinity of 1121B Fab to KDR, as determined by BIAcore analysis, is approximately 0.1 nM, which is as high as that of several bivalent anti-KDR mAb produced using either phage display library approach or the hybridoma technology from immunized mice [20,26]. We previously reported that the binding affinity between the recombinant KDR and its natural ligand, VEGF, was approximately 0.88 nM when measured by BIAcore analysis under the same conditions [22]. The affinity of VEGF for binding to KDR expressed on the surface of HUVEC was previously determined via Scatchard analysis to be 0.77 nM [27]. Thus 1121B Fab possesses an affinity that is ~8- to 9-fold higher than that of VEGF regarding their binding to KDR.

The Fab fragment also blocks effectively VEGF from binding to KDR, both as a recombinant soluble protein (Fig. 1B) and as surface-expressed receptor in PAE/KDR cells (Fig. 1C). The $IC_{50}$ value, the antibody concentrations required for 50% of inhibition of VEGF from binding to KDR, was approximately 1–2 nM in both assays. As expected, the control Fab, 1E10, an antibody directed

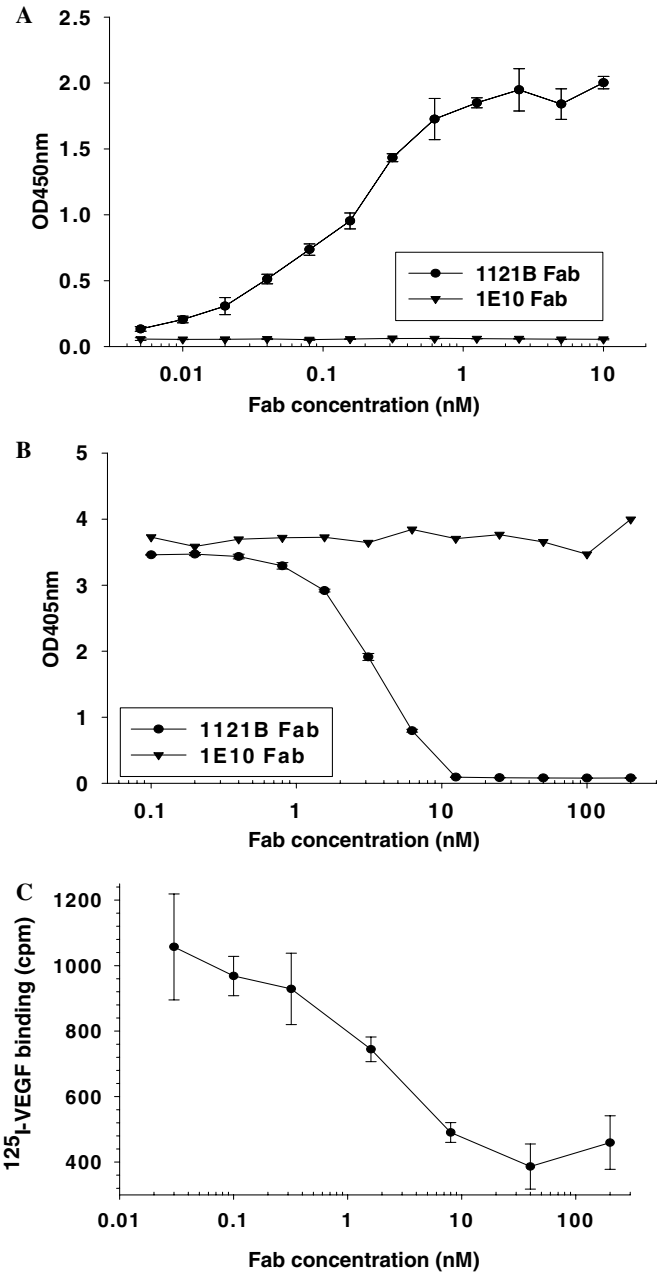


Fig. 1. 1121B Fab binds to KDR and blocks KDR/VEGF interaction. (A) Dose-dependent binding of 1121B Fab to immobilized KDR. Various amounts of Fab were added to microtiter plates coated with KDR and incubated at RT for 1 h, after which the plates were incubated with a mouse anti-human antibody-HRP conjugate. The plates were washed, peroxidase substrate was added, and A450 nm was read. 1E10 Fab, a fragment directed against mouse plate-derived growth factor receptor α, was used as the control. (B) Inhibition of binding of KDR to immobilized VEGF by 1121B Fab. Various amounts of Fab were incubated with a fixed amount of KDR-AP (100 ng) in solution at RT for 1 h, after which the mixtures were transferred to microtiter plate coated with VEGF and incubated for an additional 2 h. The amount of KDR-AP that bound to the immobilized VEGF was quantified by incubation of the plates with AP substrate and reading of A405 nm. (C) 1121B Fab competes with VEGF for binding to KDR expressed on PAE/KDR endothelial cells. Subconfluent PAE/KDR cells cultured in 48-well plate were incubated with various concentrations of 1121B Fab for 30 min on ice, followed by incubation with $^{125}$I-VEGF (5 ng/ml) for 2 h on ice. The cells were lysed with 0.5 N NaOH, and the radioactivity was measured using the γ counter. All data points are the means ± SD of triplicate determinations.

against mouse platelet derived growth factor receptor α, did not bind to KDR nor blocked KDR/VEGF interaction (Figs. 1A and B).

### *1121B Fab neutralizes VEGF-stimulated phosphorylation of KDR, PLC-γ, and MAPK*

VEGF binding induces conformational changes within KDR, followed by receptor dimerization and autophosphorylation of tyrosine residues in the intracellular kinase domain. Tyrosine phosphorylation forms high-affinity binding sites for a variety of Src homology 2 domain- and phosphotyrosine binding domain-containing proteins, including PLCγ, PI3-kinase, Akt, PKC, Raf-1, MAPK, STAT3, and others (28–32). These proteins either possess an intrinsic enzymatic activity, or serve as docking proteins to position other signaling molecules in close proximity with the receptor, to further propagate the VEGF signals that lead to intracellular calcium mobilization, cell migration and proliferation [28–32]. In this experiment, 1121B Fab was examined for its potency in neutralizing VEGF-stimulated activation of KDR and its downstream signaling molecules. VEGF stimulation resulted in significant phosphorylation of KDR, PLC-γ, and MAPK in PAE/KDR cells (Fig. 2A, lane 2). In the presence of 1121B Fab fragment, VEGF-stimulated phosphorylation of KDR, PLC-γ, and MAPK was inhibited in an antibody dose-dependent manner (Fig. 2A, lanes 3–7). The $IC_{50}$, the Fab concentrations that yielded 50% of inhibition of VEGF-stimulated phosphorylation of KDR, PLC-γ, and MAPK, were very similar at about 10 nM. As expected, 1121B Fab did not have any effect on bFGF-stimulated phosphorylation of FGF receptor and downstream MAPK activity (Fig. 2B).

### *1121B Fab inhibits VEGF-induced intracellular calcium mobilization*

One of the first cellular event triggered by VEGF stimulation in endothelial cells is the alteration of cytoplasmic calcium levels [33,34]. Cytosolic levels of calcium play an important role in VEGF-induced vascular permeability [35] and endothelial cell proliferation [36]. Activation of KDR, but not Flt-1, is crucial for VEGF-mediated calcium mobilization in endothelial cells [37], which is regulated through PLC-γ pathway [36]. As shown in Fig. 3, VEGF stimulation of endothelial cells (PAE/KDR) triggered a robust and efficient intracellular calcium mobilization. Treatment of the endothelial cells with 1121B Fab inhibited the effect of VEGF on intracellular calcium mobilization, in a dose-dependent manner (Fig. 3), with an $IC_{50}$, as calculated by the area under the curve obtained at each Fab concentration, of about 7.3 nM. As a positive control, VEGF-induced calcium flux was also efficiently inhibited by a small molecule inhibitor of PLC-γ, U73122 (data not shown).

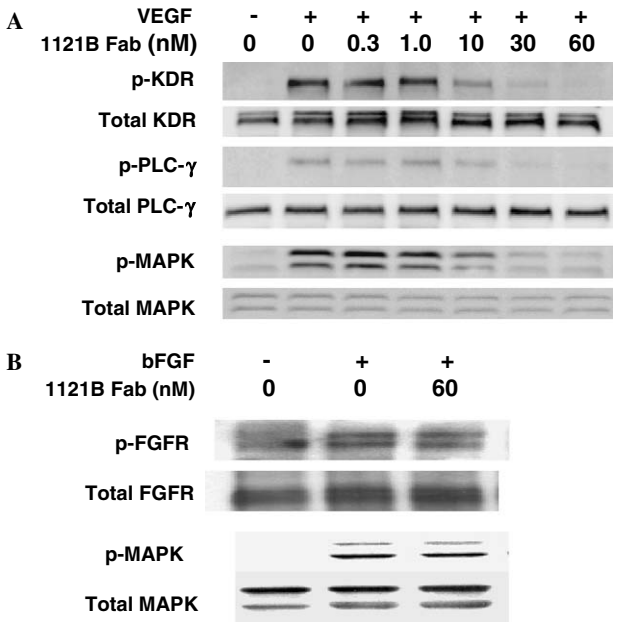


Fig. 2. 1121B Fab inhibits VEGF-stimulated phosphorylation of KDR, PLC-γ, and MAPK in PAE/KDR endothelial cells. Subconfluent PAE/KDR cells cultured in 10-cm dishes were starved overnight in F-12 medium containing 0.1% BSA. The cells were incubated with 1121B Fab for 10 min at 37 °C, followed by stimulation with VEGF (10 ng/ml) (A) or bFGF (10 ng/ml) (B) for 10 min. The cells were lysed, and equal amounts of cell lysates or proteins were resolved by a 4–20% SDS–PAGE and subjected to immunoblotting analysis. The signals were detected using enhanced chemoilluminescence.

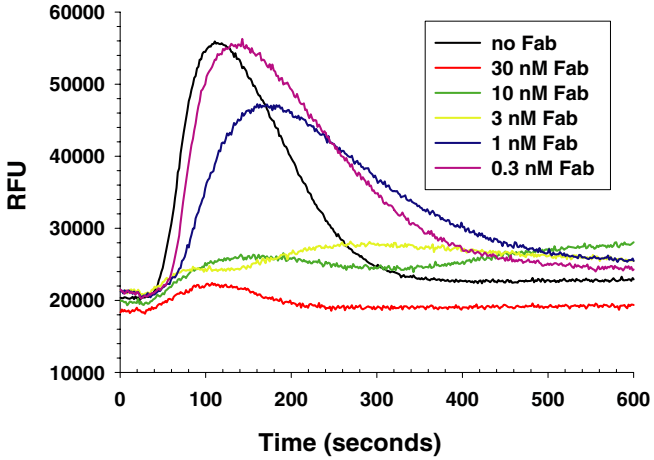


Fig. 3. 1121B Fab inhibits VEGF-induced intracellular calcium mobilization in PAE/KDR endothelial cells. PAE/KDR cells were seeded in a black-rim 96-well plate and starved overnight in F-12 medium containing 0.1% BSA, followed by incubation with 1121B Fab and the FLIPER calcium dye for 1 h at 37 °C, per the protocol of the manufacturer. The plate was cooled to room temperature for 20 min, and then put onto a FlexStation II fluorometer. VEGF (10 ng/ml) was then delivered to each well by the robotics. The calcium flux was immediately measured by the fluorometer as reflected by the fluorescence intensity per well.

### *1121B Fab inhibits VEGF-induced chemotaxis of endothelial cells*

Endothelial migration is an important step during neovascularization. Directional migration of endothelial cells toward the gradient of VEGF is a complex process and distinct from KDR-mediated cell proliferative activity [38]. 1121B Fab effectively blocked VEGF-induced endothelial cell migration with an $IC_{50}$ of ~0.2 nM—at a concentration of 8 nM, the Fab completely blocked VEGF-induced cell migration (Fig. 4). The endothelial cell adhesion to the extracellular matrix protein, fibronectin, was not affected by the antibody fragment (data not shown).

### *1121B Fab inhibits VEGF-stimulated mitogenesis and proliferation of endothelial cells*

Two different methods, a mitogenesis assay and a proliferation assay, were used to examine the antagonistic effect of 1121B Fab on the mitogenic activity of VEGF. In the mitogenesis assay, 1121B Fab was examined for its effect on VEGF-stimulated [$^3$H]TdR incorporation in HUVEC. In this experiment, starved HUVEC were incubated with VEGF, in the presence or absence of 1121B Fab, for 18 or 68 h, followed by incubation with [$^3$H]TdR for additional 4 h. The Fab strongly inhibited [$^3$H]TdR incorporation into VEGF-stimulated HUVEC under both assay conditions, with an $IC_{50}$, the concentration that yielded 50% of inhibition of [$^3$H]TdR incorporation, of approximately 10–15 nM (Fig. 4). C225, an antibody directed against human epidermal growth receptor, did not show any effect on VEGF-stimulated HUVEC mitogenesis in this assay (data not shown).

Next, we investigated if 1121B Fab fragment would inhibit endothelial cell proliferation stimulated by VEGF. A GFP-transfected PAE/KDR cell line was used in this assay, thus the magnitude of cell proliferation could be monitored directly by the intensity of fluorescence at the end of study. Consistent with our previous observations, PAE/KDR/GFP cells did not show significant proliferative response to VEGF stimulation when the cells were seeded on plastic tissue culture plates that were either uncoated or coated with gelatin, collagen, or fibronectin (data not shown). Surprisingly, a rather robust cell proliferation (>3-fold) upon VEGF stimulation was achieved when the endothelial cells were seeded on culture plates coated with diluted (1:10) Matrigel. Under this cell culture condition, 1121B Fab fragment significantly inhibited VEGF-stimulated PAE/KDR/GFP cell proliferation, as evident by the reduction of fluorescence intensity, with an $IC_{50}$ of ~20 nM (Fig. 5). This result is in good agreement with that achieved in the mitogenic assay (Fig. 4).

The biological activity of VEGF is mediated by two structurally related, high affinity tyrosine kinase receptors, the *fms*-like tyrosine kinase (Flt-1, or VEGFR1) and KDR [4–6]. Among these two receptors, KDR appears to be the major transducer of VEGF signals in endothelial cells that lead to cell proliferation, migration, differentiation, tube formation, increase of vascular permeability, and maintenance of vascular integrity (4–6). Inhibition of KDR-mediated signaling pathway, therefore, represents an excellent approach for anti-angiogenic intervention [17,18,26,39]. To this end, a neutralizing antibody to mouse VEGFR2 (flk1), DC101, has demonstrated significant anti-tumor activity in a variety of human tumor xenograft models via anti-angiogenic mechanism [9,39]. Likewise, an anti-KDR antibody, IMC-1C11, has been shown to inhibit high-oxygen-induced retinal neovascularization in newborn dogs [21], and to block gonadotropin-dependent angiogenesis and follicular development in rhesus monkeys [40,41]. Here we showed that an anti-KDR antibody fragment, 1121B Fab, binds to the receptor with high affinity

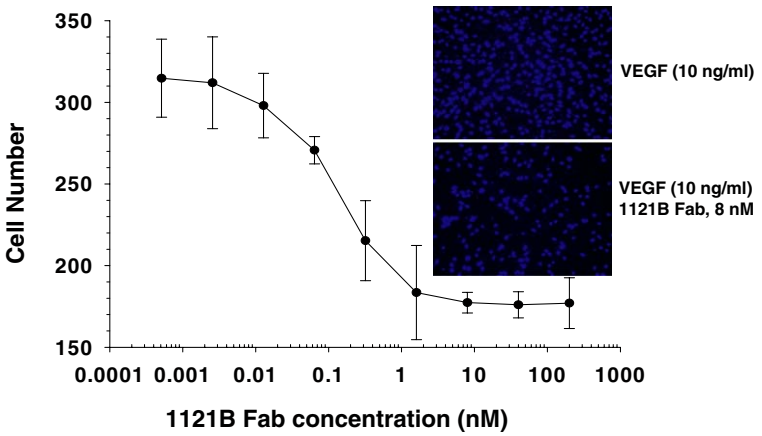


Fig. 4. 1121B Fab inhibits VEGF-induced migration of PAE/KDR endothelial cells. The endothelial cells were mixed with various amounts of 1121B Fab and added to the wells of the upper compartment of a modified Boyden chamber, whereas VEGF (10 ng/ml) was added to the wells in the lower compartment. After 4 h incubation at 37 °C, the unmigrated cells were scraped, and the migrated cells were fixed with 4% formaldehyde and stained with Hoechst 33342. The number of nuclei was counted under a Zeiss epifluorescence microscope using an automated protocol with the Image-pro Plus 5.0 software. Each data point represents the means ± SD of quadruplicate wells. Representative images from the control and the 1121B Fab treated groups were shown in the inset.

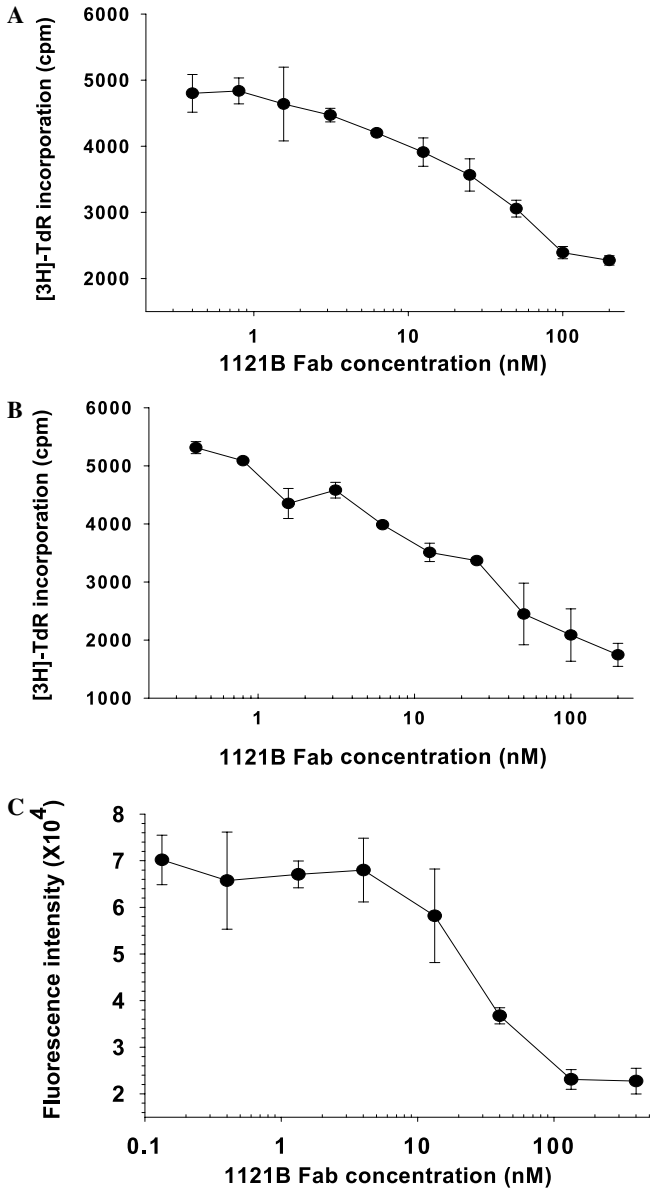


Fig. 5. 1121B Fab inhibits VEGF-stimulated mitogenesis (A,B) and proliferation (C) of endothelial cells. (A,B) HUVEC ($5 \times 10^3$ cells/well) were plated into 96-well plates in 200 μl of EBM-2 medium without VEGF, basic fibroblast growth factor and epidermal growth factor, and incubated at 37 °C for 72 h. Various amounts of 1121B Fab were added to duplicate wells and incubated at 37 °C for 1 h, after which VEGF was added to the wells to a final concentration of 16 ng/ml. After 18 h (A) or 68 h (B) of incubation, 0.25 μCi of [$^3$H]TdR was added to each well and incubated for additional 4 h. The cells were harvested and the DNA incorporated radioactivity was determined with a scintillation counter. Data shown are the means of duplicates and are the representative of at least three separate experiments. (C) PAE/KDR/GFP cells were seeded at a density of $5 \times 10^3$ cells/ml (200 μl/well) in complete F-12 medium in black-rim 96-well plates coated with Matrigel. After overnight culture, cells were starved for 3 days in F-12 medium containing 0.1% BSA. VEGF and 1121B Fab were added to the culture for additional 2 days. The cell proliferative response was estimated as GFP fluorescence intensity as measured by a FlexStation II fluorometer. Each data point represents the means ± SD of quadruplicate wells.

(0.1 nM) and effectively blocks VEGF/KDR interaction at a sub-nanomolar concentration (0.15 nM), leading to complete inhibition of a variety biological events in endothelial cells stimulated by VEGF, including receptor activation and signaling, cell migration and proliferation. Taken together, these results strongly suggest that 1121B Fab fragment may represent an effective antagonist to VEGF/KDR pathway-stimulated angiogenesis.

A growing list of inhibitors of angiogenesis, including small molecule kinase inhibitors and mAb, is currently under evaluation as potential therapeutics for a number of important human diseases, including cancer and proliferative retinopathies such as AMD [8,42–44]. For example, Avastin®, an mAb against VEGF has shown clinical benefits in many cancer patients including those with carcinomas of colorectum, lung, and breast [8,42]. Further, Lucentis® (Ranibizumab, or rhuFabV2), an Fab fragment of Avastin® [45,46], and Macugen® (Pegaptanib), an anti-VEGF aptamer [47,48], have both demonstrated therapeutic efficacy in AMD patients in a number of clinical trials. Anti-KDR antibody approach may provide several therapeutic advantages compared to small molecule kinase inhibitors and VEGF-targeting agents such as Avastin and Macugen. First, unlike most small molecule kinase inhibitors that usually hit several to multiple targets, anti-KDR antibody therapy is very specific, and therefore, may have better safety profile in patient during treatment. Further, neutralizing antibodies to KDR will block the angiogenic activity not only of VEGF, but also that of other growth factors exerting their angiogenic effects via the receptor, 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 associated with the use of anti-KDR antibodies will translate into clinical benefit remains, however, to be seen in human clinical trials.

In summary, here we described a high affinity, fully human neutralizing anti-KDR antibody, 1121B Fab fragment, and demonstrated that the Fab fragment potently blocked VEGF/KDR interaction and inhibited VEGF-induced receptor activation, migration and proliferation of endothelial cells. Taken together with our previous observations with other anti-KDR antibodies [19,20,26,41], these data lend strong support to clinical evaluation of 1121B Fab as an anti-angiogenesis agent in multiple indications where pathological neovascularization is involved, for example, in patients with cancer and proliferative retinopathies such as AMD.

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