Preparation of Extracellular Domain 3 of Human VEGF Receptor-2 and the Monitoring of Its Real-Time Binding to VEGF by Biosensors

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Vascular endothelial growth factor receptor-2 (VEGFR-2) plays an important role in stimulating the proliferation of endothelial cells and improving the permeability of blood vessels, which is involved in tumor angiogenesis, a process that is essential for tumor growth and metastasis. In this study, we describe a method for high yield of recombinant extracellular domain 3 (KDR3) of human VEGFR-2 in an Escherichia coli system with further purification by cation exchange chromatography and immobilized metal affinity chromatography (IMAC). The biological activity of recombinant KDR3 was performed by sequestering VEGF in HUVEC proliferation assay. The real-time binding of human VEGF to immobilized KDR3 was monitored by a label-free biosensor, Optical waveguide lightmode spectroscopy (OWLS). Under the given experimental conditions, the association rate constant k_a *was* $4.2 \times 10^3\ M^{-1}\ s^{-1}$ *and the dissociation rate* k_d *was* $5.1 \times 10^{-3}\ s^{-1}$*. The dissociation constant* K_D *was then calculated to be* 1.2×10^{-6} *M. The obtained values will serve as baseline parameters for the design of improved versions of recombinant soluble VEGF receptors and the evaluation of developed anti-KDR antibodies. In addition, such a scenario established by the use of OWLS will potentiate the kinetic study of ligand/receptor and antigen/antibody. The receptor discussed here, which block VEGF binding to cell membrane KDR, have potential clinical application in the treatment of cancer and other diseases where pathological angiogenesis is involved.* © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.,* 25: 1703–1708, 2009
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Introduction

Angiogenesis is regulated in normal and malignant tissues by the balance of angiogenic stimuli and angiogenic inhibitors that are produced in the target tissue and at distant sites.[1] The most potent direct-acting angiogenic protein known, major mediator of tumor angiogenesis is VEGF-A, usually referred to as VEGF. VEGF and VEGF receptors (VEGFR) have been implicated in angiogenesis that occurs in many human solid tumors including breast cancer, colon cancer, hepatoma, bladder cancer, gastric cancer, and prostate cancer.[2] There are three known VEGF receptors, VEGFR-1—the fms-like tyrosine kinase Flt-1, VEGFR-2—the fetal liver kinase (Flk-1) or kinase insert domain-containing receptor (KDR), and VEGFR-3—the fms-like tyrosine kinase (Flt-4).[3,4] VEGFR-1 binds VEGF with a 10-fold higher affinity than VEGFR-2, but it has a weak signaling ability. The role of VEGFR-1 has been disputed. The most apparent biological function ascribed to it is the induction of monocyte migration.[2] Therefore, the current view is that VEGFR-2 is the major receptor, transducing the effects of VEGF into endothelial cells. These results underscore the pivotal role of VEGFR-2 as a new target in the treatment of tumor and other diseases where pathological angiogenesis is involved.

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The human VEGFR-2 gene, *KDR* encodes 1356 aa, which has 7 immunoglobulin-like (7-Ig) domains in the extracellular region. Amongst the 7-Ig domains, domains 1–3, domain 3 and domains 5–7 have been documented critical in the signal transduction of VEGF.[5,6] Accordingly, the development of anti-KDR monoclonal antibodies (mAbs) generated against the extracellular domain has been reported.[5–9] An alternative approach would be to use a soluble recombinant VEGF receptor to inhibit the VEGF signaling pathway.[10,11] Another approach would be to produce tumor cells transfected with cDNA encoding the native soluble Flt-1 (sFlt-1) truncated VEGF receptor, which functions by sequestering VEGF or by forming inactive heterodimers with membrane-spanning VEGF receptors in a dominant negative fashion.[12] Both of these techniques have demonstrated preclinical efficacy. Although VEGF has been successfully neutralized using these strategies in preclinical models, their potential application may be limited by the significant amount of protein that would be needed to treat humans. Therefore, the development of a high efficacy soluble receptor and target specific mAbs may be a more appropriate means of tumor control in humans. In this study, we present the preparation and characterization of a VEGF specific binding and high yield of soluble extracellular domain 3 of KDR (KDR3), which is indicated to be employed as a unique target for the preparation of anti-KDR Abs.

Optical waveguide lightmode spectroscopy (OWLS) is a particularly sensitive technique for obtaining continuous measurements of the protein mass adsorbed at a waveguide/liquid interface.[13–15] Combined with advanced software, it could measure the binding and dissociation kinetics more precisely and comprehensively than cell based assay. Furthermore, the association constant (K_A) or dissociation constant (K_D) can be determined on the basis of the measured data.

In this report, soluble KDR3 was expressed in a bacterial system and purified by cation exchange chromatography and affinity chromatography. The in vitro specific binding to its ligand was measured by sequestering VEGF in VEGF stimulated proliferation of human umbilical vein endothelial cells (HUVEC). Moreover, OWLS was used to determine the real-time binding activity of VEGF to immobilized KDR3. This was based on the principle of the measurement of changes in the refractive index on the sensor surface.

Materials and Methods

Reagents and cells

VEGF (VEGF165) was expressed from recombinant Pichia Pastoris, which is a generous gift from Dr. Yu Liu (Department of Biochemistry, China Pharmaceutical University, Nanjing) and it was purified with the same method as Dr. Liu reported.[16] Immortal HUVEC cell line was generously provided by Dr. Rui Liu (Nanjing University of Traditional Chinese Medicine).

Plasmid construction

The 314 bp DNA sequence, containing 291 bp extracellular domain 3 of *KDR* gene, was designed according to the nucleotide sequence at Genebank (Genebank accession no.**GI2655411)** and protein sequence at Swiss Prot (EBI accession no. **P35968**). The target sequence was generated by overlapping PCR with four pairs of primers (50 bp each). *Nco I* and *EcoR I* specific sequence was introduced. The annealing temperature in each round of PCR was set according to the melting temperature (T_m) of the primers, respectively.

The product of the last round of PCR was collected, purified and digested with *Nco* I and *EcoR* I, and then ligated with pET-32a (+) vector (Novagene), which was digested by *Nco* I and *EcoR* I, yielding the recombinant vector pET32a-KDR3. The recombinant vector was then transformed to *Escherichia coli* DH5α. Positive colonies were selected from LB plates containing 100 ng mL^{-1} ampicillin, and analyzed by PCR, restriction enzyme digestion and sequencing serially before functional analysis.

Expression of recombinant protein in E. coli

E. coli.Rosetta-gami (DE3) was transformed with recombinant plasmid pET32a-*KDR3*. Bacterial cultures were incubated at 30°C in LB growth medium containing 50 μg mL^{-1} kanamycin until early log phase ($A_{600\ nm}$ = 0.6–0.8). Protein expression was then induced by the supplement of 1 mM isopropyl b-d-thiogalactopyranoside (IPTG) with shaking for 5 h. The bacteria were then harvested by centrifugation at 6,000 rpm for 6 min, washed with cold PBS, resuspended in 0.05 volume of 20 mM Tris-HCl (pH 8.0) and pulse-sonicated for 10·10 s (10 s working and resting for a 10 s pulse, then cooling on ice for 1 min) with a sonicator. The soluble fractions were collected by centrifugation at 12,000 rpm for 10 min at 4°C.

SDS-PAGE and Western blot analysis

The expression of KDR3 protein was characterized with 15% SDS-PAGE. Briefly, Samples were mixed with 20 μL 2 × SDS-PAGE loading buffer and denatured at 100°C for 3 min. SDS-PAGE (15%) electrophoresis was run under reducing conditions. Proteins were transferred to polyvinylidene membrane (Millipore) according to standard Western blot procedure. The membrane was then blocked with 5% (w/v) nonfat milk in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5, TBS) at room temperature for 1 h, blotted with rabbit anti human VEGFR-2 antiserum (1:1,000 dilution) at room temperature for another 1 h, washed with TBS for three times, and then marked with Horseradish peroxidase-conjugated goat anti rabbit IgG antibody (1:10,000 dilution in TBS containing 5% [w/v] milk) for 1 h. Membrane was finally rinsed with TBS containing 0.05% (v/v) Tween-20 and proteins were visualized.

Purification of the recombinant protein

The optimized procedure of combined purification was performed as described below. Briefly, 5 mL of soluble fractions was loaded onto a 20 mL cation exchange column (CM cellulose 32, Waterman), which was pre-equilibrated with 10 volume of binding buffer (8 M urea, 25 mM NaAc, pH 4.0). The column was then washed consecutively with 50 mL binding buffer, a gradient mixture of 50 mL pH 4.0 and 50 mL pH 6.0 binding buffer, 1 volume of pH 6.0 binding buffer and finally eluted with 1 volume of 1 M NaCl at a flow rate of 0.5 mL min^{-1}. At each step, a fraction of the batch volume was retained for characterization with SDS-PAGE. Collections containing target protein were combined for further purification with immobilized metal affinity

chromatography (IMAC). The Ni affinity column (Ni Sepharose 6 Fast Flow, GE Healthcare) with a bed volume of 1 mL was pre-equilibrated with 5 mL binding buffer (25 mM Tris,8 M Urea, pH 8.0). After loading, the column was eluted with 5 mL binding buffer containing 5, 10, 20, 50, 100, 200, and 500 mM imidazole, respectively. The eluted fractions were collected and characterized with SDS-PAGE.

Protein concentrations were determined by the method of Coomassie Brilliant Blue G250 Kit (Nanjing Jian Cheng Bio). The purified protein was balanced in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), dry frozen and stored at −20°C for further use.

Neutralization on VEGF stimulated proliferation of human umbilical vein endothelial cells

HUVEC were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin/streptomycin. The culture was maintained at 37°C in a 5% CO_2 humidified incubator. Cells were fed every 2 days and passaged by 0.25% trypsin-EDTA (Invitrogen) when cells achieved confluence.

HUVEC were plated with 1×10^4 cells/well in a 96-well plate (Corning Incorporated) and incubated for 24 h. Subsequently, HUVEC maintenance media was replaced with RPMI 1640 medium supplemented with 0.5% FBS for another 24-h culture. VEGF (VEGF165, 10 ng mL^{-1}) and different concentrations of KDR3 (0, 0.7, 7, 70, and 700 ng mL^{-1}) were added to each well ($n = 3$). Additionally, wells without VEGF and KDR3 were set as blank control. The 72 h proliferation of the cells was evaluated by the regular methyl thiazolyl tetrazolium (MTT) assay.

Real-time binding studies with OWLS

Real-time binding study was performed using OWLS based measuring system BioSense 2.2 (Microvacuum). Briefly, a sensor chip (OW2400, Microvacuum) was washed with fresh piranha buffer (piranha, H_2SO_4:H_2O_2 = 3:1) for 5 min, hydrated with Millipore-Q water at 90°C for 1 h and silanizated with 10% 3-aminopropyltriethoxysilane (pH 3.5, Sigma) at 75°C for 5 h. The pretreated chip was then washed by ddH_2O, dried by N_2, and incubated at 110°C overnight.[17] Subsequencely, the chip was fixed to OWLS, on-line modified with 2.5% Glutaraldehyde (Sigma) and balanced with 25 mM Tris-HCl (pH 8.0) to stable base line.

KDR3 protein (receptor) diluted with 25 mM Tris-HCl (pH 8.0) was immobilized on the sensor chip by amine coupling reaction. Lysine (5 mM) was injected to block the unoccupied aldehyde group. Transport of the sample to the sensor surface was controlled with a microfluidics cartridge (Zhejiang University Medical Device). NaCl (5 M) was used to regenerate the chip after ligand dissociation. The injection of 25 mM Tris-HCl (pH 8.0) buffer as the background signals were subtracted from the signals in the sample flowcell.

After a base line was established with 25 mM Tris-HCl buffer, freshly diluted VEGF of various concentrations (2, 4, 8, 16, and 32 μg mL^{-1}) flowed through the cuvette at a precisely monitored rate (25 μL min^{-1}) serially. To determine the rate of dissociation, the flow through the cuvette was later switched back to pure 25 mM Tris-HCl buffer at 25 μL min^{-1}. Two effective refractive indices, NTE and NTM were measured by giving rise to continuous Mass density (M, ng cm^{-2}) vs. time, and the change of Mass density dM/dt vs. Mass density. Kinetic constants were calculated in terms of the interaction models that have been described in the literature.[18,19]

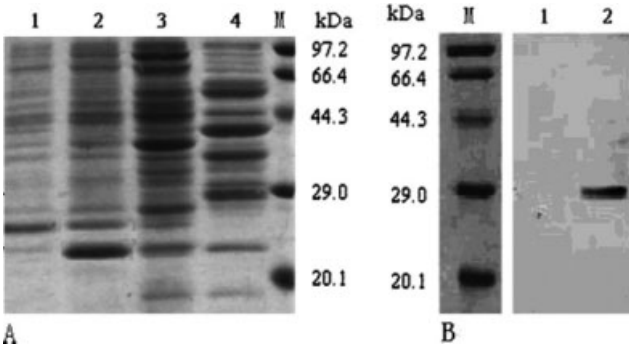

Figure 1. SDS-PAGE and Western blot analysis for the expression of recombinant KDR3.

A: SDS-PAGE assay. Lanes 1 and 2, Supernatant of ultrasonic lysed pET32a *E. coli* uninduced/induced; Lanes 3 and 4, Supernatant of ultrasonic lysed pET32a-KDR3 *E. coli* uninduced/induced. B: Western blot analysis. Lane 1, supernatant of lysed pET32a *E. coli* after induction; Lane 2, supernatant of lysed pET32a-KDR3 *E. coli* after induction. M, protein molecular weight marker.

Results

*Determination of pET32a–*KDR3 *with DNA electrophoresis*

Products from each round of PCR were assessed by 2% agarose gel electrophoresis. The 314 bp DNA was obtained. In the determination of pET32a-*KDR3*, DNA fragment of around 6 kb was obtained after digestion with *EcoR* I, whilst 5865 bp and 310 bp fragments were generated after digestion with *EcoR* I and *Nco* I (data are not shown).The accuracy of the recombinant pET32a-*KDR3* was determined by DNA sequencing and BLAST with Human VEGFR-2 gene.

SDS–PAGE and Western blot analysis

The expression of His 6-tagged KDR3 fusion protein was developed by incubating with 1 mM IPTG at 30°C for 5 h. The bacteria culture was sonicated, and the soluble fractions were collected by centrifugation and then detected by 10% SDS–PAGE under reducing conditions. As shown in Figure 1A, the recombinant *E. coli* carrying pET32a-*KDR3* revealed a protein of Mr = 28 kDa (Figure 1A, lane 4) indicating the expression of soluble fusion protein Trx-KDR3. A typical mass band of 20.4 kDa was visible which indicated the expression of Thioredoxin (Trx) in pET32a *E. coli* (Figure 1A, lane 2).

The immunological characteristics of the target protein was further proved by Dot blot (data are not shown) and Western blot analysis (Figure 1B), indicating that KDR3 with immunity to anti-VEGFR-2 antibody was expressed.

Purification of the recombinant KDR3 protein

The soluble recombinant protein was purified with CM cation exchange chromatography and Ni affinity chromatography in turn. Meanwhile, the purity of the proteins was detected by SDS–PAGE. As shown in Figure 2, the combination of CM cation exchange chromatography and IMAC, yielded nearly homogeneous protein with a purity of 95% (lanes 4 and 5). The production of the purified protein was 26 mg L^{-1} as per the determination of Coomassie Brilliant Blue G250 Kit.

Neutralization on VEGF stimulated HUVEC proliferation

In our pilot studies, under serum-starved culture (0.5% FBS supplemented RPMI 1640 media), the proliferation of HUVEC was remarkably enhanced with the addition of VEGF. An optimal concentration-dependent curve was obtained by incubating HUVEC with serious dilution of VEGF (data are not shown). Cells incubated with the presence of 10 ng mL^{-1} VEGF had remarkably high proliferation rate, however, this stimulation was inhibited by KDR3 (700 ng mL^{-1}) to an extent of 33%, which ascribed to the neutralization of KDR3 to the additional VEGF (Figure 3).

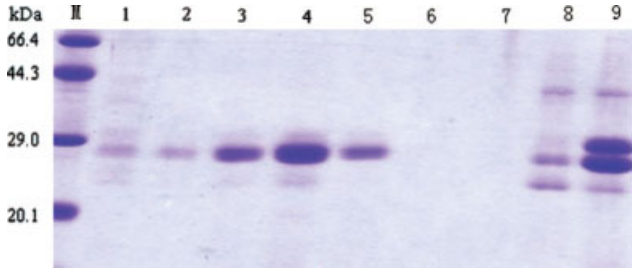

Figure 2. SDS-PAGE assay for the purification of KDR3. Lanes 1–7, eluate from IMAC by 5, 10, 20, 50, 100, 200, and 500 mM imidazole; Lanes 8–9, eluate from CM cation-exchange column by NaCl. M, protein molecular weight marker.

The fraction of Lane 9 was further purified by IMAC. Fractions that eluted by 50 and 100 mM imidazole were combined and quantified with Coomassie Brilliant Blue G250 Kit.

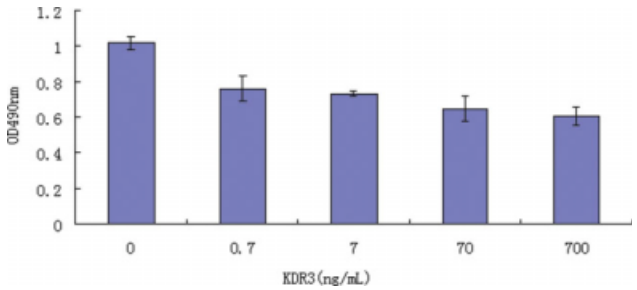

Figure 3. Neutralization of KDR3 to VEGF on the proliferation of HUVEC by MTT assay. (mean ± SD).

Cells were seeded in 96-well plate at a density of 1×10^4 cells/well and treated with 10 ng mL^{-1} VEGF and serial diluted KDR3 at different concentrations for 72 h ($n = 3$, three separate tests). Excessive KDR3 could inhibit the proliferation stimulated by VEGF to an extent of 33%.

Real-time binding study and kinetic constants evaluation

The detection of mass density on sensor chip vs. time and the binding rate vs. mass density, is one of the basic functions of OWLS. The amount of immobilized receptors is calculated from the balanced line via the baseline. A flow of 1 μg mL^{-1} receptor solution to the sensor chip gave rise to an increased mass density of 41.80 ng cm^{-2}, whilst the injection of 7 μg mL^{-1} receptor caused an instant increase to 145 ng cm^{-2}. However, the mass density finally decreased to 52.47 ng cm^{-2} (Figure 4A). It was reported that a high immobilized protein density on the sensor chip surface will promote the mass transport limitation.[19] Therefore, the sensor chip with immobilized KDR3 at mass density of 41.80 ng cm^{-2} was employed in further kinetic study.

Figure 4B demonstrated a representative graph of the kinetics of association and dissociation measured in this study. With the injection of the sample, association of VEGF to immobilized KDR3 was observed as an increase in the mass density. From these data, association rate constant (k_a) can be calculated. At the end of this injection, the sample was replaced by a continuous flow of buffer at rate of 25 μL min^{-1}. The decrease of the mass density reflected the dissociation of VEGF from the immobilized receptor KDR3. Accordingly, dissociation rate constant (k_d) can be calculated in this phase.

The association of the ligand to the immobilized receptor under constant flow was assumed to follow pseudo first-order reaction kinetics as the concentration of the ligand might be considered constant.[19] The association rate constant, k_a, can be determined by carrying out a series of interaction experiments with different concentrations (C) of ligand and by measuring the binding rate, dM/dt, as a function of the OWLS. The equation is:

$$dM/dt = k_a C M_{max} - (k_a C + k_d)M,$$

where M_{max} is the maximum response for VEGF binding. Using linear transformation of the primary data, plots of dM/dt vs. M are made for a range of VEGF concentrations (2, 4, 8, 16, and 32 μg mL^{-1}) and the slopes of these curves,

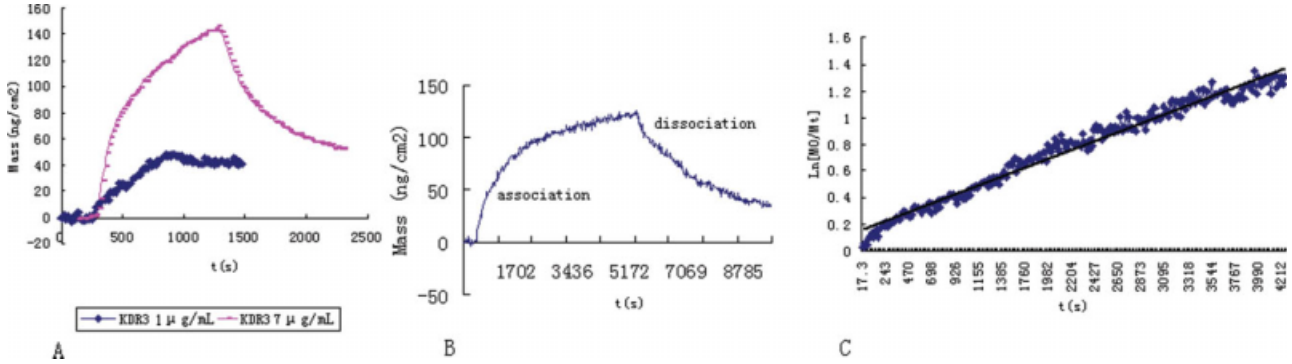

Figure 4. Real-time binding activity of VEGF to immobilized KDR3 as measured using OWLS.

A: The immobilization of various concentrations of KDR3 to sensor chips. A flow of 1 μg mL^{-1} receptor solution to the sensor chip gave rise to an increased mass density of 41.80 ng cm^{-2}, whereas the injection of 7 μg mL^{-1} receptor caused an instant increase to 145 ng cm^{-2} and finally decreased to 52.47 ng cm^{-2}. B: The association and dissociation of VEGF to immobilized KDR3. Mass density increased with the continuous injection of VEGF and decreased with the flow of buffer. Immobilization level in this experiment was 41.80 ng cm^{-2}. C: Dissociation plotted according to ln (M_0/M_t) vs. time. M_t is the response at time t and M_0 corresponds to t_0 (the start of washing). The slope of the curve is given as k_d. The average of k_d at different concentration of VEGF is given in Table 1(k_d).

Table 1. Kinetic Parameters of KDR3-VEGF Interactions

Constant	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_A (M^{-1})	K_D (M)
Value	4.2×10^3	5.1×10^{-3}	8.3×10^5	1.2×10^{-6}

$k_aC + k_d(=k_s)$, are determined. The k_s values are then plotted vs. concentration of VEGF, and the new line obtained will have a slope corresponding to k_a, which was calculated to be 4.2×10^3 M^{-1} s^{-1}.

The dissociation rate constant, k_d, can be calculated from the dissociation phase described by equation:

$$\ln(M_0/M_t) = k_d^{\sim}(t - t_0)$$

where M_t is the response at time t and M_0 is the response at the starting time point (t_0) of dissociation. As shown in Figure 4C, plot of $\ln(M_0/M_t)$ vs. $t-t_0$ gives a straight line with slope k_d. The mean value of the calculated k_d was 5.1×10^{-3} s^{-1}. The apparent affinity (or association) constant K_A, for the receptor-ligand interaction can then be determined as the ratio $K_A = k_a/k_d$. Accordingly, the dissociation constant K_D can be calculated as $K_D = k_d/k_a$. Fitted parameters were given in Table 1.

Statistical analysis

When data are expressed as percentage of inhibition, each value corresponds to the mean of three independent experiments performed with triplicate samples and bars indicate standard error (SD) of the mean.

Discussion

In this study, we have described high yield expression, purification of soluble extracellular domain 3 of VEGF receptor 2 (KDR3), the functional determination by HUVEC proliferation assay and the monitoring of its real-time binding activity to VEGF.

Soluble VEGF-receptor was expressed and Thioredoxin (Trx) and 6 His (Histidine) were tagged to provide a recognition site for affinity purification. In the pET32a system, the addition of a His-tag at the N-terminus of a recombinant protein can inhibit the secretion of the protein into the supernatant despite that the reducing protein Trx was fused to N-terminal. This might be the reason of the poor expression of the His tagged *KDR3*, which we observed when using *E. coli* BL21 (DE3) as expression host. Comparably, the expression of soluble KDR3 increased remarkably when *E. coli* Rosetta-gami (DE3) was employed as expression host. A possible explanation is that *E. coli* Rosetta-gami (DE3) has been modified and it can provide tRNAs which transfer rare code amino acids. Hereby, the expression of soluble proteins was enhanced, which generated a final yield of 26 mg L^{-1} with further purification.

Endothelial cell survival in newly formed vessels is VEGF-dependent. Both autocrine and paracrine VEGF could protect HUVEC from apoptosis. In our preliminary experiment, additional VEGF could enhance the proliferation of HUVEC. However, this effect was neutralized by serial diluted KDR3 in this study (Figure 3). Therefore, the truncated KDR extracellular domain can specifically bind to VEGF and form a relatively stable KDR3-VEGF complex.

To monitor the real-time binding of VEGF to the recombinant KDR3 as mimic of receptor/ligand reaction on cell membrane, a biosensor based system was employed. We used OWLS for three reasons: First, kinetic data can be determined, although they are subject to restrictions due to problems intrinsic for the biosensor systems used here. This is of importance that this method allows to screen improved versions of VEGFR in the future, as obtained by protein engineering, with specific focus on the decrease of dissociation rate. Second, an immobilized sensor chip could be used for more than 10 tests without the loss of the mass density. Finally, OWLS allows binding reactions to be studied in real-time and no secondary labeling reagents are required.

In our system, the calculated association rate constant k_a was 4.2×10^3 M^{-1} s^{-1}, and the dissociation rate constant k_d was 5.1×10^{-3} s^{-1}. The calculated value for the apparent affinity (or association) constant K_A was 8.3×10^5 M^{-1} or dissociation constant K_D was 1.2×10^{-6} M. The reported K_D values of protein–protein interactions were mostly obtained from cell based assays. In terms of Surface Plasmon Resonance (SPR) based analytical systems, variant kinetic data have been reported. The association rate constants for matrix bound monoclonal antibodies interacting with HIV-1 core protein p24 ranged from 3×10^4 to 7.4×10^5 M^{-1} s^{-1} and the dissociation rate constants were in the region from 2×10^{-4} s^{-1} to 2×10^{-2} s^{-1}.[18] Huang obtained the dissociation constant K_D values, with K_D ranging between 1.1×10^{-10} M and 6×10^{-10} M.[20] The numbers range from 1×10^{-8} M (for the proposed low affinity interaction)/3×10^{-10} M (for the high affinity interaction) to as low as 2.5×10^{-12} M, depending on the cell line used.[20] In comparison with previously reported data on VEGF/VEGFR-2, our data indicated a lower affinity interaction between VEGF and KDR3. A presumable reason is that the recombinant KDR3 is one of the 7-Ig extracellular domains, although domain 3 has been documented the most critical role in VEGF binding and signal transduction. However, the affinity is subject to the whole entity or 7-Ig extracellular domains of KDR. Another explanation is the variation from different measurement systems and the variable quality of ligand/receptor prepared in different laboratories. Nevertheless, a scenario of ligand/receptor kinetic study was established by the use of OWLS, which will potentiate the screening anti-KDR3 Abs with high affinity (k_a) and low dissociation rate (k_d).

Conclusion

In summary, we have developed a method to generate functional soluble extracellular domain of VEGFR-2 in high yield. We also described a nonradioactive method for the determination of binding constants, including association and dissociation rates of the receptor/ligand interaction. This method can be easily applied in routine measurements of improved versions of VEGFRs obtained by protein engineering, and such a method could be applied to antibody engineering where antibodies with high affinity are required. In addition, the scenario established by the use of OWLS will potentiate the kinetic study of ligand/receptor an antigen/antibody. We believe the biosensor-based functional assays are becoming essential tools in the molecular biology laboratory.

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