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Expression of Soluble VEGF Receptor 2 and Characterization of Its Binding by Surface Plasmon Resonance

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Vascular endothelial growth factor (VEGF) is an endothelial cell specific mitogen that induces angiogenesis in several pathological conditions. To block angiogenesis, soluble VEGF receptor can be used. In this study, we describe a method for high yield expression of soluble VEGF receptor 2 (sFlk-1) in a baculovirus expression system (30 mg purified sFlk-1 per L of insect cell supernatant). We also determined the binding constants for both human and mouse VEGF to the recombinant receptor by surface plasmon resonance. In this cell-free assay, under the given experimental conditions, the on-rate k_a was 0.5-2.2 x 10^6 $M^{-1}s^{-1}$ and the off-rate k_d was 2-4 x 10^{-4} s^{-1} (K_D = 2-6 x 10^{-10} M). To our knowledge this is the first study to report on- and off-rates for the VEGF:sFlk-1 interaction. Heparin was not required for the binding of VEGF to sFlk-1 in this assay. The obtained values will serve as baseline parameters for the design of improved versions of recombinant soluble VEGF receptor.

Vascular endothelial growth factor (VEGF) and its receptors VEGFR1 (Flt-1, fms-like tyrosine kinase) and VEGFR2 (KDR/Flk-1, kinase insert domain-containing receptor/fetal liver kinase) have recently attracted considerable interest because of their involvement in vasculogenesis and angiogenesis (1–9). Both VEGF and its receptors are overexpressed in a number of angiogenesis dependent diseases such as malignant tumors and diabetic retinopathy. The inhibition of VEGF-mediated signals could therefore offer a valuable approach for the therapy of these diseases. Inhibition can be achieved through neutralizing antibodies and soluble receptor constructs which block the activation of VEGF receptors by preventing either ligand binding or ligand induced dimerization of receptors. Previous reports have demonstrated that both neutralizing VEGF and anti-KDR antibodies as well as soluble VEGF receptor constructs are capable of reducing or inhibiting tumor growth in animal models (10–17).

It is now necessary to develop methods for expressing recombinant soluble VEGF receptor in high yields for extensive preclinical and clinical testing. In this report, we describe the expression of sFlk-1 in a baculovirus system and its purification by affinity chromatography. The yield of highly purified receptor after a single purification step was 30 mg/L supernatant.

Surface plasmon resonance was used to determine the binding of the soluble recombinant receptor in real time to its ligand VEGF. On- and off-rates for the VEGF:sFlk-1 interaction, and the effect of heparin on this interaction are reported.

MATERIALS AND METHODS

1. Construction of plasmids. A Flk-1 cDNA construct was kindly provided by Dr. Ihor Lemischka (Princeton University). This construct contains the full length mouse Flk-1 cDNA. Double stranded DNA encoding for the first 762 amino acids of Flk-1 was obtained by PCR reaction using the primers GCACAGCTGATGGAGAGCAAGGCGCTGCTA and CTGCAGCTGCTATTCCAAGTTGGTCTTTTC with the Flk-1 cDNA construct as a template. The resulting 2.3 kB PCR fragment was cloned into pVL1393 (Pharmingen) and into pBlueBacHis (InVitrogen). The DNA sequence was verified by automated fluorescence sequencing.

2. Expression of soluble Flk-1. Monolayer and suspension cell cultures of Spodoptera frugiperda (Sf9) cells were grown in Sf900II-SFM (Gibco/BRL) supplemented with 50 μg/ml penicillin and 50 μg/ml streptomycin (Gibco/BRL). Recombinant plasmids pVL1393/sFlk-1 and pBlueBacHis/sFlk-1 were cotransformed with baculovirus gold DNA (Pharmingen) into Sf9 cells by using lipofectin (Gibco/BRL) according to the manufacturer's instructions. The recombinant virus was plaque purified as described (18). To confirm the expression of recombinant protein, aliquots of conditioned medium of infected Sf9 cells were analyzed by Western Blot using the monoclonal antibody 1A8 directed against sFlk-1 (Brekken et al.; manuscript in

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Abbreviations used: sFlk-1, soluble fetal liver kinase 1; VEGF121, VEGF 165, vascular endothelial growth factor, 121 or 165 amino acids length (splice variants); VEGFR, vascular endothelial growth factor receptor.

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preparation). Sf9 cells were infected with sFlk-1 recombinant baculovirus at a multiplicity of infection (MOI) of 10. After incubation at room temperature for 1 h the cells were pelleted and resuspended in fresh culture medium. The cells were grown for various time periods at 28 °C. The supernatant was removed and concentrated by membrane filtration using a membrane with a 30 kD cut-off size. The concentrated supernatant was then dialyzed against PBS pH=7.4 for further purification.

3. Optimization of expression conditions. Using the expression vector with the better yields, pVL1393/sFlk, we further optimized the expression conditions. Sf9 cells were infected with sFlk-1-recombinant virus at MOIs of 1, 2, 5 and 10. Samples of the supernatants were taken at 24h, 36h, 48h, 60h, 72h, and 96h post infection and quantified by ELISA. For the ELISA, 96-well plates were coated with 1A8 antibody (see above) and blocked with 5% Casein Acid Hydrolysate (Sigma). Reference standards and samples were added at various concentrations and incubated at room temperature for 1 h. Affinity purified rabbit anti-Flk-1 polyclonal antibody (produced in our laboratory) was used to detect sFlk-1.

4. Affinity purification. An affinity column for liquid chromatography was prepared using the monoclonal antibody 1A8 (see above) and protein G beads (Pharmacia). The antibody was coupled to the beads as described (19).

Supernatant from virus-infected Sf9 cell cultures was concentrated by membrane filtration, dialyzed against PBS and loaded onto the 1A8 affinity column. The column was washed with PBS pH=7.4 and soluble sFlk-1 was eluted with 0.1 M citric acid, pH=3.5. Absorbance at 280 nm was measured and pooled peak fractions were dialyzed against PBS pH=7.4 and then concentrated by membrane filtration.

5. SDS–PAGE analysis. SDS-PAGE was performed on 4-15% gradient gels using the Phastsystem (Pharmacia). To assess the amount of glycosylation, samples were deglycosylated by digestion with peptide N-glycosidase F (PNGase F, Boehringer Mannheim) for 12 h at 37 °C prior to loading on the gel.

6. Real time binding studies. Real time binding analysis was performed using the automated surface plasmon resonance based measuring system Biacore2000 (Biosensor). Recombinant sFlk-1 was immobilized on CM5 sensorchips (Biosensor) by amine coupling using an amine coupling kit (Biosensor) or ligand-thiol coupling using PDEA (2-(2-pyridinyldithio)ethaneamine hydrochloride) as coupling reagent (Biosensor) according to the manufacturer's instructions. For binding studies recombinant human VEGF165 (R&D), recombinant murine VEGF165 (R&D) or recombinant human VEGF121 (R&D) was injected into a flowcell with immobilized sFlk-1 at various concentrations. 4 M $MgCl_2$ was used to regenerate the chip after VEGF injection. A blank flowcell served as negative control and the background signals observed in this flowcell were subtracted from the signals in the sample flowcell. To verify the specificity of the binding, sFlk-1 in 40fold, 200fold, and 1000fold molar excess was coinjected with recombinant human or mouse VEGF.

To estimate the influence of mass transport, kinetic data were obtained at different immobilization densities and at different flowrates (10 μl/min, 30 μl/min, 50 μl/min and 70 μl/min). An immobilization level of 1300 RU and 30 μl/min flow rate were used as standard conditions.

To test for rebinding, the soluble receptor sFlk-1 was injected in the dissociation phase of VEGF-binding, and the off-rate was compared to the off-rate without the competitor sFlk-1.

The influence of heparin on the binding of VEGF to its soluble receptor was tested by preincubation of heparin at various concentrations between 0.1 ng/ml and 10 μg/ml with VEGF. Binding constants were determined using the curve fitting software Biaevaluation 2.1 (Biosensor).

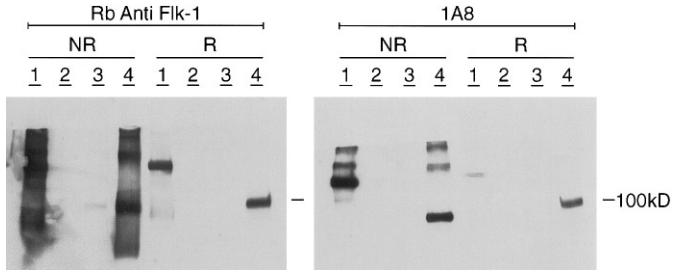

FIG. 1. Western Blot analysis of the conditioned Sf9 cells. Samples were separated by SDS-PAGE and analyzed by western blot with affinity purified rabbit anti-Flk-1 antibody (left) or anti-Flk-1 monoclonal antibody 1A8 (right). Lane 1: purified seap/Flk-1 fusion protein as a positive control; lane 2: conditioned medium from uninfected Sf9 cells; lane 3: medium from pBlueBacHis/sFlk-1 infected Sf9 cells; lane 4: medium from pVL1393/sFlk-1 infected Sf9 cells. SFlk-band at 100 kDa. NR: non reduced; R: reduced.

RESULTS

1. Expression and Purification of Soluble Flk-1

Western Blot analysis of conditioned medium of Sf9 cells infected with recombinant baculovirus carrying either pVL1393/sFlk-1 or pBlueBacHis/sFlk-1 revealed a protein of MW=100 kDa (Figure 1). The signal obtained with pVL1393 as a carrier was much stronger than that with pBlueBacHis, and therefore this plasmid was used in the further experiments.

The results of the expression optimization studies are illustrated in Figure 2. The expression of sFlk-1 was detected as early as 24 h post infection with maximal accumulation and minimal degradation of the protein occurring at 60 h post infection at various infection doses except for MOI=1, which has a maximum production at 72 h post infection. We observed a slightly increased yield at MOI=2, namely 35 mg/L. The best expression conditions therefore are obtained using the infection dose MOI=2 and harvesting 60 hours post infection. After affinity chromatography using immobilized 1A8, 30 mg of purified sFlk-1 could be isolated per L of conditioned medium.

2. SDS–PAGE Analysis

The purified fractions were analyzed by SDS-PAGE. As shown in Figure 3, the affinity purification yielded a nearly homogeneous protein with a mass of 100 kDa. Based on the slower than predicted mobility of sFlk-1 and the 16 potential N-linked glycosylation sites in the cDNA derived amino acid sequence, it was reasoned that sFlk-1 was posttranslationally modified by glycosylation. In Figure 3, an increase in the mobility of sFlk-1 from 100 kDa to 80 kDa can be seen after incubation with PNGase F, demonstrating that sFlk-1 produced by the Sf9 cells is heavily glycosylated.

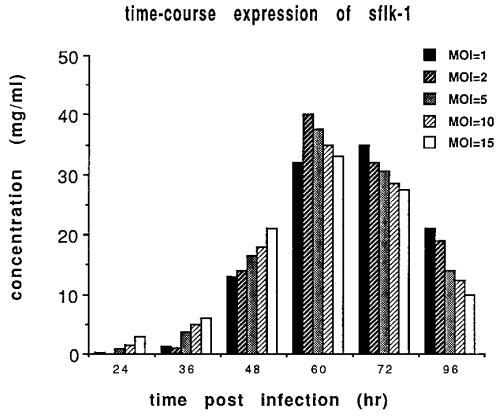

FIG. 2. Time course of sFlk-1 expression in pVL1393/Flk-1 infected Sf9 cells. Insect cells were cultured and infected as described in Materials and Methods. At the indicated times post infection, samples were analyzed for sFlk-1 by quantitative ELISA.

3. Realtime Binding Studies

SFlk-1 was immobilized by either amino- or ligand-thiol-coupling on the CM5 sensorchips at various densities between 520 and 1900 RU (response units). Amino-coupling was the preferred method, since R_{max} values (maximal response) were slightly higher and off-rates slightly lower than with ligand-thiol coupling. We compared the binding abilities of human and mouse VEGF to the murine sFlk-1 and observed almost identical binding curves (Figure 4). The binding could be completely inhibited by addition of a 1000-fold excess of sFlk-1 to the injected VEGF (Figure 5). The variations of calculated binding parameters were within 20% (SD) of the mean value, when different immobilization levels or different concentrations of VEGF were used. Variation of flowrates caused slightly higher variabilities. The values for on- and off-rates under different experimental conditions are shown in Table 1. Addition of heparin to the injected VEGF inhibited the binding of VEGF165 in a dose dependent fashion (Figure 6). At a concentration of 10 μg/ml heparin the inhibition was about 50%. This was due to a decrease in the on-rates, while the off-rates were not significantly changed. No inhibition was seen at concentrations below 0.1 μg/ml. When injections were prolonged to saturation the addition of 0.1 μg/ml or 0.01 μg/ml slightly increased the maximal binding (up to 20%) despite decreased on-rates. VEGF121 binding was not affected by the addition of heparin.

When sFlk-1 was injected in the dissociation phase, off-rates were increased slightly, which indicates, that rebinding occurs to some extent.

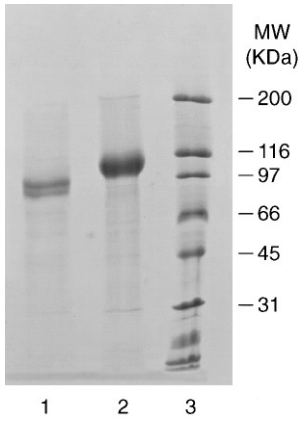

FIG. 3. Affinity purification of sFlk-1. 400 ml culture supernatant from pVL1393/sFlk-1 infected Sf9 cells were purified on a 1A8 affinity column with a bed volume of 10 ml (see Materials and Methods). Samples were analyzed by SDS-PAGE followed by Coomassie Blue staining. The purified product was deglycosylated using PNGase F. Lane 1: sFlk-1 after deglycosylation; lane 2: sFlk-1 before deglycosylation; lane 3: molecular weight marker. Numbers on the right indicate molecular mass in kDa.

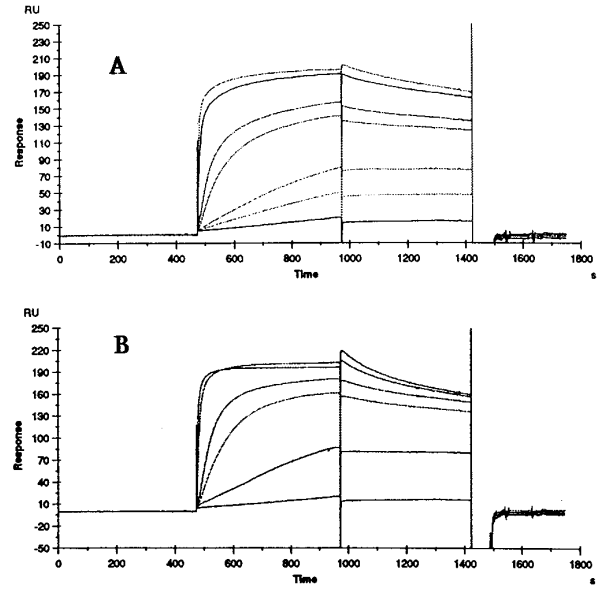

FIG. 4. Binding of recombinant VEGF165 to immobilized sFlk-1. A: human VEGF165; concentrations are 100nM, 50nM, 10nM, 5nM, 1nM, 0.5nM, 0.1nM; B: mouse VEGF165; concentrations are 100nM, 50nM, 10nM, 5nM, 1nM, 0.1nM. Saturation of binding sites is achieved between 10nM and 50nM in both cases.

DISCUSSION

In this study we describe the high yield expression and affinity purification of soluble VEGF-receptor 2 and the characterization of its binding to VEGF. Flk/KDR is one of two known VEGF receptors. It has been shown to be responsible for the transmission of a mitogenic signal to endothelial cells (20). Therefore Flk/KDR is a potential target for angiogenesis inhibition. We cloned and expressed the murine receptor Flk-1. Cloning, expression and purification of the human homolog KDR, can be performed in the same manner,

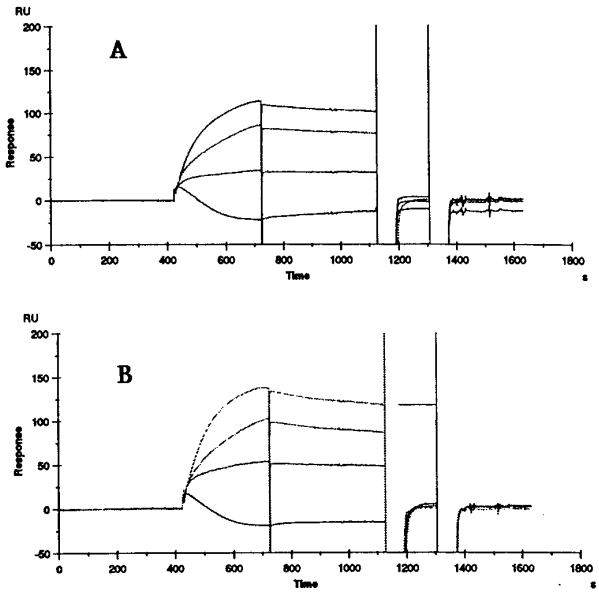

FIG. 5. Inhibition of VEGF binding to immobilized sFlk-1 by excess of soluble sFlk-1. Concentrations of soluble sFlk-1 in solution are 0, 40fold, 200fold and 1000fold molar excess over VEGF. Concentration of VEGF is 5nM. A: human VEGF165; B: mouse VEGF165.

since the antibody used for affinity purification, 1A8, also reacts with KDR (Brekken et al., manuscript in preparation). In previous studies in which soluble VEGF-receptor was expressed, tags had to be added to the receptor to provide a recognition site for affinity purification. These tags include SEAP (secreted alkaline phosphatase) and IgG fragments (21–24). Where yields were reported, they ranged between 150 μg/L and 5 mg/L. Another recently reported tag is 6His (Histidine), (17) for Ni-NTA affinity purification. In the baculovirus 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. This might be a reason for the poor expression of His-tagged sFlk-1, which we observed when using the pBlueBacHis transfer vector. Using the pVL1393 transfer plasmid, which contains no tags, we obtained at least 100-fold higher yields of recombinant protein as determined by Western Blot (Figure 1). With 1A8, a monoclonal antibody against Flk-1, we were able to affinity purify sFlk-1 without using any tags. After a single purification step we obtained nearly homogenous protein with a yield of 30 mg/L crude supernatant.

To analyze the binding of the recombinant sFlk-1 to its ligand VEGF, we used surface plasmon resonance for two major reasons: First, surface plasmon resonance is a cell free assay. Cell based assays are important to understand the biology of VEGF binding and signalling in its natural environment. The soluble receptor, however, is designed to capture VEGF in solution, where no membrane environment is involved. For our studies, a binding assay that lacks any cell membrane components was warranted. Other cell free assays, where KDR-Fc fusion proteins were captured on microtiter plates, have been successfully used (25), but the sFlk-1 without an Fc-component cannot be immobilized on microtiter-plates without impairing its binding capabilities (data not shown). Second, kinetic data (on- and off-rates) can be determined as well, although they are subject to restrictions due to problems intrinsic for the Biosensor systems used here (26). This is of importance, since this method allows to screen improved versions of VEGFR, as obtained by protein engineering, with specific focus on the decrease of off-rates. In addition, surface plasmon resonance allows binding reactions to be studied in real time and no secondary labeling reagents are required.

In a first set of experiments we showed that sFlk-1 binds specifically to human or mouse VEGF 165 (Figures 5 and 6). A curve fitting software (BIAevaluation, Biosensor) was used to determine on- and off-rates from the obtained binding curves. Dissociation constants (K_D) were calculated from the on- and off-rates.

TABLE 1

Binding of VEGF to Immobilized sFlk-1 under Various Conditions Using Surface Plasmon Resonance

VEGF species and additions	response [RU]	k_a (on-rate) [$M^{-1}s^{-1}$]	k_d (off-rate) [s^{-1}]	calculated K_D [M]
hVEGF165 50nM	173	1.2×10^6	4.1×10^{-4}	3.4×10^{-10}
hVEGF165 50nM + Heparin (1 μg/ml)	165	3.3×10^5	3.3×10^{-4}	1.0×10^{-9}
hVEGF165 5nM	113	2.2×10^6	2.4×10^{-4}	1.1×10^{-10}
hVEGF165 5nM + 1000 × sFlk-1	no binding	n/a	n/a	n/a
mVEGF165 20nM	175	1.9×10^6	6.2×10^{-4}	3.3×10^{-10}
mVEGF165 20nM + 1 μg/ml Heparin	135	4.0×10^5	2.7×10^{-4}	6.8×10^{-10}
mVEGF165 5nM	140	2.2×10^6	3.0×10^{-4}	1.4×10^{-10}
mVEGF165 5nM + 1000 × sFlk-1	no binding	n/a	n/a	n/a

Note. Immobilization level in this experiment was 1700 response units. Response levels for the given concentration as well as binding constants are shown.

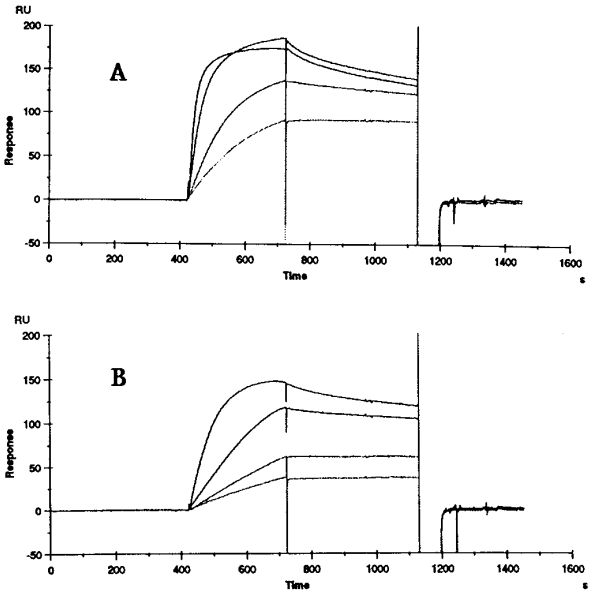

FIG. 6. Inhibition of VEGF165 binding to immobilized sFlk-1 by heparin. Human and mouse VEGF165 showed identical inhibition curves. Shown here are the sensorgrams for mouse VEGF165 at 20nM (A) and 5 nM (B). Concentrations of heparin are 0, 0.1 μg/ml, 1 μg/ml, 10 μg/ml (from top to bottom). Heparin concentrations lower than 0.01 μg/ml had no effect.

In our system, the on-rates were 0.5-2.2 x 10^6 $M^{-1}s^{-1}$, and the off-rates were 2.0-6.2 x 10^{-4} s^{-1} with no significant differences between human and mouse VEGF165. The calculated values for the dissociation constant K_D ranged between 1.1 x 10^{-10}M and 6 x 10^{-10}M. VEGF121 showed a lower affinity for sFlk-1, due to a lower on-rate (data not shown). Previously reported K_D values for Flk-1/KDR binding to VEGF were mostly obtained from cell based assays. The numbers range from 1 x 10^{-8}M (for the proposed low affinity interaction)/3 x 10^{-10}M (for the high affinity interaction) to as low as 2.5 x 10^{-12}M, depending on the cell line used. In two studies, the binding of KDR-Fc to VEGF was assessed in cell free assays (24, 25) with K_D values of 3-4 x 10^{-11}M and 1.5 x 10^{-10}M, respectively. In a crosslinking assay Kendall (27) reported a K_D of approximately 1 nM for the VEGF:KDR interaction. Previous data are not available for on- and off-rates of the VEGF:VEGFR2 interaction.

We were also interested in assessing the influence of heparin on the VEGF:VEGFR binding in our system, because in a therapeutic setting heparin-like molecules are not necessarily available at the site of VEGF:sVEGFR binding. Heparin at concentrations above 0.1 μg/ml dose dependently inhibited the binding by reducing the on-rate. This could be because heparin either bound to the injected VEGF or to sFlk-1 and impeded the rate of association of VEGF with its receptor. SFlk-1, when added to the injected VEGF inhibited its binding to immobilized sFlk-1 without addition of heparin. These findings strongly suggest that heparin is not essential for the binding of VEGF to its receptor, nor does it cement the sFlk-1:VEGF complex once formed. Kaplan (24), who studied the KDR-Fc binding to VEGF in a radioligand binding assay on agarose beads, also found no requirement for heparin in VEGF:VEGFR binding. The dissociation constant of the VEGF:KDR-Fc interaction was slightly higher in the presence of heparin (1.7 x 10^{-10}M versus 1.0 x 10^{-10}M). Similarly, Keyt (25) found that heparin was not needed for VEGF:VEGFR binding in another cell free assay.

These findings are in contrast with the observations made in cell based assays (21, 28–30), where heparinase treatment of VEGFR-bearing cells abolishes their ability to bind VEGF. Also, cells deficient in heparan sulfate biosynthesis cannot bind VEGF (30). Addition of exogenous heparin can increase VEGF binding when applied at an optimal concentration which varies from cell line to cell line. High concentrations of heparin (10 μg/ml or above) generally inhibit the VEGF-binding. Gitay-Goren (28) reported that an increase in binding of VEGF to its receptor in the presence of heparin correlates with an increase in the number of receptors, while decrease of binding correlates with a decreased receptor number. A possible explanation for these findings is that the negatively charged heparin molecules on the surface of cells are required to present the receptor in a form that can bind VEGF. If an excess of heparin is added, receptor binding sites might become covered and unavailable for VEGF binding.

In summary, we have developed a method to generate functional soluble VEGFR2 in high yields. We also describe a nonradioactive method for determination of binding constants, including on- and off-rates, for the VEGF receptor:ligand interaction. This method can be easily applied in routine measurements of improved versions of VEGFR obtained by protein engineering.

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REFERENCES

1. Fong, G-H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) *Nature* **376,** 66–70.
2. Shalaby, F., Rossant, J., Yamaguchi, T. P., *et al.* (1995) *Nature* **376,** 62–66.
3. Frank, S., Hubner, G., Breier, G., Longaker, M. T., Greenhalgh, D. G., and Werner, S. (1995) *J. Biol. Chem.* **270,** 12607–12613.
4. Burke, P. A., Lehmann-Bruinsma, K., and Powell, J. S. (1995) *Biochem. Biophys. Res. Comm.* **207,** 348–354.
5. Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J., and Keshet, E. (1995) *Nature Med.* **1,** 1024–1028.

6. Malecaze, F., Clamens, S., Simorre-Pinatel, V., *et al.* (1994) *Arch. Ophthalmol.* **112,** 1476–1482.
7. Detmar, M., Brown, L. F., Claffey, K. P., *et al.* (1994) *J. Exp. Med.* **180,** 1141–1146.
8. Plate, K. H., Breier, G., Weich, H. A., Mennel, H. D., and Risau, W. (1994) *Int. J. Cancer* **59,** 520–529.
9. Claffey, K. P., Brown, L. F., del Aguila, L. F., *et al.* (1996) *Cancer Res.* **56,** 172–181.
10. Presta, L. G., Chen, H., O=Connor, S. J., *et al.* (1997) *Cancer Res.* **57,** 4593–4599.
11. Sioussat, T. M., Dvorak, H. F., Brock, T. A., and Senger, D. R. (1993) *Arch. Biochem. Biophys.* **301,** 15–20.
12. Kondo, S., Asano, M., and Suzuki, H. (1993) *Biochem. Biophys. Res. Commun.* **194,** 1234–1241.
13. Asano, M., Yukita, A., Matsumoto, T., Kondo, S., and Suzuki, H. (1995) *Cancer Res.* **55,** 5296–5301.
14. Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S., and Fusenig, N. E. (1997) *Nature Med.* **3,** 1222.
15. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., and Phillips, H. S. (1993) *Nature* **362,** 841–844.
16. Borgstrom, P., Hillan, K. J., Sriramarao, P., and Ferrara, N. (1996) *Cancer Res.* **56,** 4032–4039.
17. Lin, P., Sankar, S., Shan, S-Q., *et al.* (1998) *Cell Growth Differ.* **9,** 49–58.
18. Summers, M. D., and Smith, G. R. (1987) *Texas Agricultural Experiment Station Bulletin,* 1555.
19. Simanis, V., and Lane, D. P. (1985) *Virology* **144,** 88–100.
20. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C-H. (1994) *J. Biol. Chem.* **43,** 26988–26995.
21. Tessler, S., Rockwell, P., Hicklin, D., *et al.* (1994) *J. Biol. Chem.* **269,** 12456–12461.
22. Aiello, L. P., Pierce, E. A., Foley, E. D., *et al.* (1995) *Proc. Natl. Acad. Sci.(USA)* **92,** 10457–10461.
23. Chiang, M-K., and Flanagan, J. G. (1995) *Growth Factors* **12,** 1–10.
24. Kaplan, J. B., Shridharan, L., Zaccardi, J. A., Dougher-Vermazen, M., and Terman, B. I. (1997) *Growth Factors* **14,** 243–256.
25. Keyt, B. A., Berleau, L. T., Nguyen, H. V., Chen, H., Heinsohn, H., Vandlen, R., and Ferrara, N. (1996) *J. Biol. Chem.* **271,** 7788–7795.
26. Schuck, P., and Minton, A. P. (1996) *Anal. Biochem.* **240,** 262–272.
27. Kendall, R. L., Wang, G., DiSalvo, J., and Thomas, K. A. (1994) *Biochem. Biophys. Res. Commun.* **201,** 326–330.
28. Gitay-Goren, H., Soker, S., Vlodavsky, I., and Neufeld, G. (1992) *J. Biol. Chem.* **267,** 6093–6098.
29. Terman, B. I., Khandke, L., Dougher-Vermazan, M., *et al.* (1994) *Growth Factors* **11,** 187–195.
30. Dougher, A. M., Wasserstrom, H., Torley, L., Shridaran, L., Westdock, P., Hileman, R. E., Fromm, J. R., Anderberg, R., Lyman, S., Linhardt, R. J., Kaplan, J., and Terman, B. I. (1997) *Growth Factors* **14,** 257–268.