Ligand-Receptor Assay for Evaluation of Functional Activity of Human Recombinant VEGF and VEGFR-1 Extracellular Fragment

A. V. Leopol'd[1], V. P. Baklaushev[1,2], A. A. Korchagina[1], S. A. Shein[1,2], N. F. Grinenko[2], K. A. Pavlov[1,2], I. A. Ryabukhin[2], and V. P. Chekhonin[1,2]

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cDNA encoding VEGF and Ig-like extracellular domains 2-4 of VEGFR-1 (sFlt-1_{2-4}) were cloned into prokaryotic expression vectors pET32a and pQE60. Recombinant proteins were purified (metal affinity chromatography) and renatured. Chemiluminescent study for the interaction of recombinant VEGF and sFlt-1_{2-4} showed that biotinylated VEGF specifically binds to the polystyrene-immobilized receptor extracellular fragment. Biotinylated recombinant sFlt-1 interacts with immobilized VEGF. Analysis of the interaction of immobilized recombinant VEGFR-1 and VEGF with C6 glioma cells labeled with CFDA-SE (vital fluorescent dye) showed that recombinant VEGFR-1 also binds to native membrane-associated VEGF. Recombinant VEGF was shown to bind to specific receptors expressed on the surface of C6 glioma cells. Functional activity of these proteins was confirmed by ligand-receptor assay for VEGF and VEGFR-1 (sFlt-1) and quantitative chemiluminescent detection.

Key Words: *ligand-receptor assay; vascular endothelial growth factor; vascular endothelial growth factor receptor*

Vascular endothelial growth factor (VEGF) serves as the major proangiogenic effector under normal and pathological conditions [2]. There are three tyrosine kinase receptors that bind to VEGF. The affinity of interaction between VEGF and VEGFR-1 (Flt-1) is highest [2,8]. Similarly to other tyrosine kinases, VEGFR-1 includes a conservative domain with GXGXXG repeat, ATP-binding site, HTRLA motif for catalysis, and one of the two sites for tyrosine autophosphorylation [7]. The extracellular part of VEGFR-1 consists of seven Ig-like domains. The 2nd and 3rd domains play the main role in the interaction with VEGF [1,7]. Since binding of VEGF to VEGFR-1 does not activate endotheliocyte proliferation, VEGFR-1 can serve as a natural trap of VEGF. VEGFR-1 firmly binds BEGF and prevents its interaction with VEGFR-2, which is responsible for mitogenic signal transduction in endotheliocytes [2,3,7,8]. Alternative splicing is followed by the formation of a shortened VEGFR-1 isoform (sFlt-1), which includes the first six Ig-like domains. It probably plays a role of a natural angiogenesis inhibitor [7,8].

Measurement of sFlt-1 concentration in blood plasma holds much promise for the diagnostics of various diseases. For example, blood sFlt-1 content increases during preeclampsia, liver cirrhosis, atherosclerotic diabetes, progressive retinopathy, and other diseases that are accompanied by the impairment of angiogenesis [8]. Moreover, the sFlt-1/VEGF ratio is inversely proportional to tumor malignancy (*e.g.*, astrocytic glioma, breast cancer, cancer of the pancreas, and acute

[1]Department and Division of Medical Nanobiotechnologies, Medico-Biological Faculty, N. I. Pirogov Russian State Medical University; [2]Department of Basic and Applied Neurobiology, V. P. Serbskii Sate Research Center of Social and Forensic Psychiatry, Ministry of Health Care and Social Development ofd the Russian Federation, Moscow, Russia. ***Address for correspondence:*** ahnavleopold@yandex.ru. A. V. Leopol'd

myeloid leukemia). The lower is the sFlt-1/VEGF ratio, the lower is survival rate of cancer patients [3].

This work was designed to obtain functionally active recombinant proteins in a prokaryotic expression system. Biological activity of these proteins was evaluated from the ligand-receptor interaction with chemiluminescence and fluorescence detection.

MATERIALS AND METHODS

cDNA encoding a part of the VEGFR-1 receptor extracellular fragment (95-520 b.p.) that corresponds to 2-4 Ig-like domains (sFlt-1_{2-4}) was subjected to PCR amplification from a human brain cDNA library (Invitrogen) with 5'-GCATCCATGGTCAGCTACTGGGACAC-3' and 3'-GCATAGATCTTAGAGTG-GCAGT-GAG-GTTTT-5' primers (Sintol). A purified product of PRC was cloned into the pQE60 vector by recognition sites for Ncol and Bglll restriction endonucleases (Sibenzim).

Human VEGF-encoding cDNA was PCR-amplified from a human brain cDNA library (Invitrogen) with 5'-GCAT-GAATTCATGAACTTTCTGCTGTC TTGGG-3' and 3'-GCATCTCGAGCCGCCTCGGC TTGTCACATC-5' primers (Sintol). A purified PRC product was cloned into the pET32a vector encoding the N-terminal fusion protein thioredoxin (trx) by recognition sites for EcoRI and Xhol restriction endonucleases (Sibenzim). Cloning of VEGF in fusion protein trx was performed to increase the yield of a soluble recombinant protein. Sequencing of the constructs showed that the nucleotide sequence of cloned DNA is identical to the corresponding structural genes in the database.

E. coli BL21 (DE3) was transformed with expression constructs pQE60-sFlt-1 and pET32-VEGF/trx. *E. coli* cultures were grown in 250 ml medium LB with 100 μg/ml ampicillin in a temperature-controlled shaker at 37°C and 200 rpm. Isopropyl-β-thiogalactoside (final concentration 0.4 mM) was added when the optical density of c *E. coli*-containing culture fluids reached 0.8 (A_{600}). Incubation was continued for 4 h. *E. coli* soluble fraction proteins were analyzed. The culture pellet (1 ml) was resuspended in cell lysing buffer under native conditions (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, and 10 mM imidazole). The cells were lysed by 3-fold freezing in liquid nitrogen and defrosting at 37°C. After centrifugation at 10,000*g*, the supernatants were tested for recombinant proteins by electrophoresis in 12% PAAG with sodium dodecyl sulfate (SDS). sFlt-1 was isolated by metal chelate chromatography under hybrid conditions according to Invitrogen protocol. VEGF/trx was isolated by metal chelate chromatography under denaturing conditions and renatured as described previously [1].

VEGF/trx and sFlt-1_{2-4} were biotinylated in 0.1 M carbonate buffer using ProtOn kit according to manufacturer's recommendations (VectorLaboratories). Biotinylation reagent (2 μl) was added to 1 ml solution of recombinant protein in carbonate buffer (pH 8.0, 1 mg/ml). The mixture was agitated and incubated at room temperature. The reaction was stopped by adding 2 μl 1 M ethanolamine to the reaction mixture. The excess of biotinylation reagent was removed by dialysis against 0.1 M carbonate buffer for 3 h.

sFlt-1_{2-4} (0.5 μg/well) and VEGF/trx (0.5 μg/well) were immobilized in wells of a 96-well plate (Corning) at 4°C for 10 h. The well was washed with potassium-phosphate containing 0.1% Tween-20 (working buffer). Biotinylated recombinant VEGF/trx (diluted from 500 to 0.5 ng/ml) was added to wells with immobilized sFlt-1_{2-4}. Biotinylated recombinant sFlt-1_{2-4} (same dilutions) was added to wells with immobilized VEGF/trx. Incubation was performed at 37°C for 1 h. The well was washed with a working buffer. To study competitive inhibition, the wells were simultaneously treated with biotinylated and non-biotinylated sFlt-1_{2-4}. The concentration of non-biotinylated sFlt-1_{2-4} was 5-fold higher than the concentration of biotinylated sFlt-1_{2-4}. Quantitative analysis of biotinylated VEGF/trx and sFlt-1_{2-4} that bound to plastic-immobilized sFlt-1_{2-4} and VEGF/trx, respectively, involved biotin-conjugated peroxidase, streptavidin (ABCkit, VectorLab) and ECL reagent (GEHealthcare) as the substrate. Chemiluminescence was recorded using a VICTORX3 plate analyzer (PerkinElmer). Electrophoresis of recombinant VEGF/trx in 12% PAAG with sodium dodecyl sulfate was performed for ligand blotting. Protein bands were transferred from polyacrylamide gel to a PVDFc membrane using a wet transfer blotter (Bio-Rad). The membrane was incubated in a working buffer with 5% dry milk for a night to inactivate the protein-binding chemical groups. The membrane with immobilized proteins was incubated overnight in the presence of recombinant sFlt-1_{2-4} at a concentration of 100 ng/ml. The membrane was washed with the same buffer, incubated with commercial monoclonal antibodies to Flt0-1 (Sigma) for 1 h, repeatedly washed, and incubated with a solution of peroxidase-labeled secondary antibodies to mouse IgG (2 μg/ml, Sigma). Binding of sFlt-1_{2-4} to VEGF/trx was detected with ECL reagent (GEHealthcare) and roentgen film (Kodak).

To study cell adhesion, recombinant proteins and monoclonal anti-VEGF antibodies were immobilized on polystyrene in a 96-well plate (Corning) and incubated overnight at 4°C. The plate was washed with a working buffer. C6 glioma cells were labeled with a vital fluorescent dye CFDASE (10 μM, Invitrogen) according to the manufacturer's instructions and added to the wells (2×10^5 cells/well). The plate with labeled

cells was incubated in a temperature-controlled orbital shaker at 37°C and 160 rpm for 1 h and washed to remove unbound cell proteins. Fluorescence was detected using a VICTORX3 plate analyzer (PerkinElmer) and DMI 6000B fluorescence microscope (LeicalMicrosystems).

RESULTS

The first purpose of the study was to obtain soluble recombinant proteins. The recombinant receptor of VEGF was mainly present in an insoluble fraction (up to 98%). Therefore, it was isolated under hybrid conditions according to the Invitrogen protocol. Recombinant protein was put on a column with NiNTA under denaturing conditions. The column was balanced with a buffer to isolate the proteins under native conditions (50 mM NaH_2PO_4 and 0.5 M NaCl, pH 8). The protein was eluted with 100 mM imidazole. The renaturation efficiency of sFlt-1 from *E. coli* inclusion bodies was 10% of the total amount of recombinant protein.

Despite the presence of N-terminal fusion protein thioredoxin that improves solubility of recombinant proteins in the *E. coli* cytoplasm [4], the recombinant protein VEGF/trx synthesized by bacteria was detected in the insoluble fraction. Therefore, the isolation under hybrid conditions was ineffective. Hence, VEGF/trx was denatured according the protocol described previously [1]. This procedure yielded 30% soluble recombinant protein.

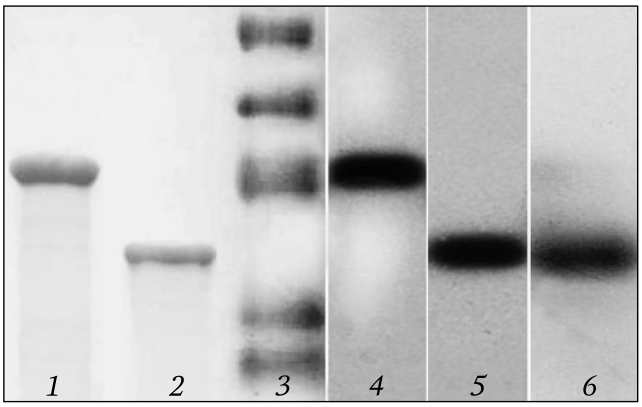

Fig. 1. Disc electrophoresis in 12% PAAG with SDS, immunoblotting, and ligand blotting of sFlt-1_{2-4} and VEGF/trx. *1*) purified renatured sFlt-1_{2-4} (50 kDa); *2*) purified renatured VEGF/trx (37 kDa); *3*) molecular weight markers (Chemichrome, Sigma; bottom-up: 20, 30, 45, 60, and 100 kDa); *4*) immunoblotting of sFlt-1_{2-4} with monoclonal antibodies against VEGFR-1 (Sigma); *5*) immunoblotting of VEGF/trx with monoclonal antibodies against VEGF (Abcam); *6*) ligand blotting of PVDF-immobilzied VEGF/trx and soluble sFlt-1_{2-4}.

Purity of isolated VEGF/trx and sFlt-1_{2-4} was at least 80-90% (Fig. 1, bands *1* and *2*). Minor admixtures of a lower molecular weight were probably related to the partially hydrolyzed or incompletely synthesized protein.

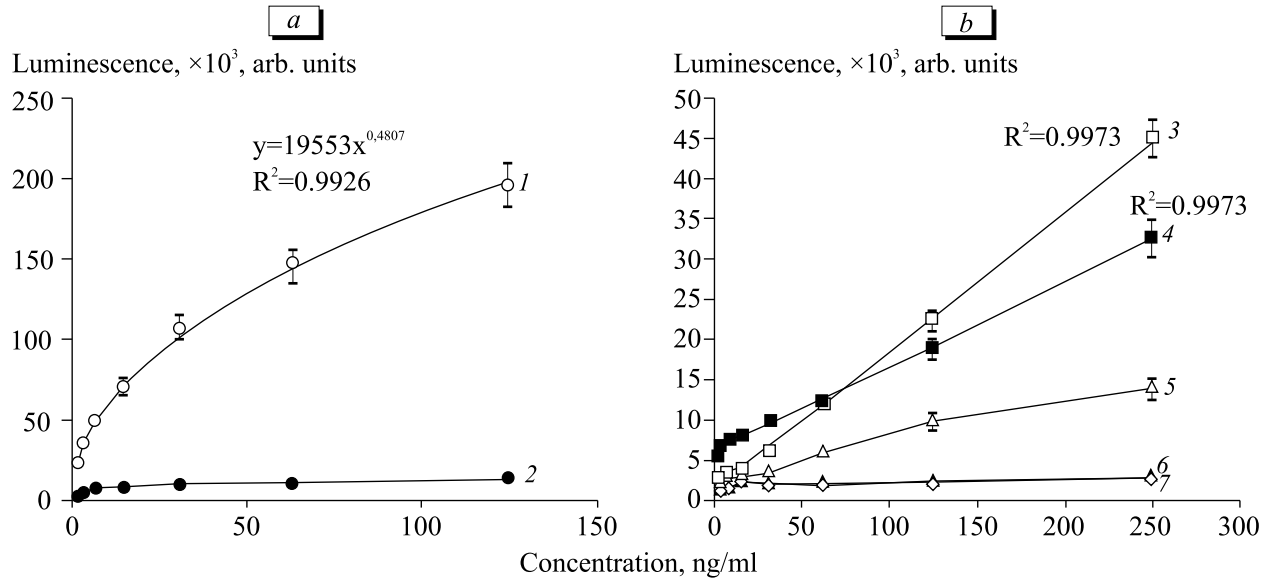

Fig. 2. Quantitative chemiluminescence detection of ligand-receptor interaction of sFlt-1 and VEGF/trx. *a*) study of VEGF/trx with polystyrene-immobilized sFlt-1 (working segment of the curve, 1-125 ng/ml); *b*) study of sFlt-1_{2-4} with immobilized VEGF/trx. *1*) detection curve for consecutive dilutions of streptavidin/biotin peroxidase-developed biotinylated VEGF/trx (ABC kit, VectorLab); *2*) negative control (immobilized sFlt1_{2-4} after incubation with ABC in the absence of biotinylated VEGF/trx); *3*) curve for binding of biotinylated recombinant sFlt1_{2-4}, development with ABC; *4*) curve for the interaction of sFlt1_{2-4} with immobilized VEGF, development with monoclonal antibodies to Flt1 and conjugate of anti-mouse IgG antibodies with peroxidase (Sigma); *5*) competitive inhibition of interaction between sFlt1_{2-4} and VEGF/trx in the presence of non-biotinylated sFlt1 (5-fold excess), development with ABC; *6*) negative control without biotinylated sFlt (immobilized VEGF/trx+ABC); *7*) negative control for antibodies (immobilized VEGF/trx+Flt1+conjugate of anti-mouse IgG antibodies with peroxidase).

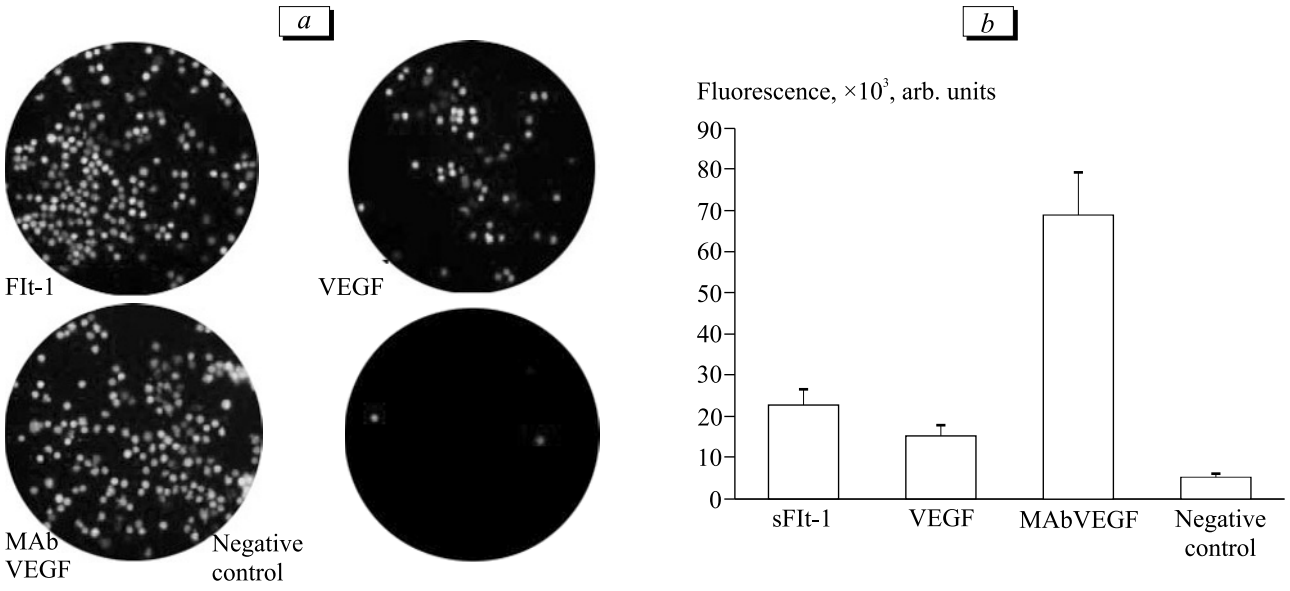

Fig. 3. Adhesion of CFDA SE-labeled C6 glioma cells to polystyrene with immobilized sFlt-1_{2-4}, VEHF/trx, and monoclonal antibodies against VEGF (Abcam). Fluorescent microscopy (*a*); intensity of fluorescence (data obtained on a plate analyzer, *b*).

Immunoblotting with commercial antibodies allowed us to perform the immunochemical identification of sFlt-1_{2-4} and VEGF/trx. In immunoblotting with chemiluminescence detection, both purified preparations were visualized as discrete single bands of the corresponding molecular weight (Fig. 1, bands *4* and *5*).

Functional activity of recombinant proteins was tested from ligand blotting on the PVDF membrane, quantitative ligand-receptor assay, and adhesion of CFDA-labeled cells.

Ligand blotting assay revealed specific luminescence of VEGF/trx on the PVDF membrane after successive incubation with sFlt-1_{2-4}, specific antibodies, peroxidase-labeled secondary antibodies to mouse immunoglobulins (Fig. 1, band *6*). No luminescence was detected under control conditions, when membrane-transferred VEGF/trx was incubated with the same antibodies in the absence of sFlt-1_{2-4}.

Ligand-receptor assay with immobilization of one of the components on polystyrene and addition of another component in consecutive dilutions allowed us to perform a quantitative analysis of interaction between sFlt-1_{2-4} and VEGF/trx. Renatured and biotinylated recombinant VEGF specifically bound to polystyrene-immobilized sFlt-1_{2-4} (similarly to ligand blotting). Soluble sFlt-1_{2-4} specifically bound to immobilized VEGF. Working segment of the calibration curve for VEGF/trx corresponded to 1-125 ng/ml (Fig. 2, *a*). In sFlt-1_{2-4} assay using biotinylated receptor or monoclonal antibodies to the receptor extracellular fragment, the concentration range of this agent that corresponded to a linear segment of the calibration curve was 2-250 ng/ml (Fig. 2, *b*). Taking into account the sensitivity limit of chemiluminescence detection, it can be suggested that optimization of this analysis with high-affinity monoclonal antibodies to sFlt-1 holds much promise for the diagnostics (detection of 0.1 ng/ml or lower).

Competitive inhibition analysis of the ligand-receptor interaction confirmed its specific type. After addition of non-biotinylated sFlt-1, the chemiluminescence signal in experiments with the biotinylated receptor was reduced by more than 50%. Therefore, non-biotinylated sFlt-1_{2-4} competes with the biotinylated receptor for binding to a VEGF active site in the reaction with immobilized VEGF/trx.

Analysis of cell adhesion showed that labeled C6 glioma cells strongly bind to polystyrene-immobilized recombinant proteins (sFlt-1_{2-4} and VEGF/trx). Monoclonal anti-VEGF antibodies served as positive control (Fig. 3).

Adhesion of VEGF-positive C6 glioma cells to plastic-immobilized sFlt-1_{2-4} is related to binding of immobilized recombinant sFlt-1_{2-4} to membrane-associated VEGF, which is expressed by C6 glioma cells. Another ligand of VEGFR-1, placental growth factor P1GF, is absent in glioma [5]. Neither VEGFR-1 nor VEGFR-2 is overexpressed in glioma cells [6], which contributes to low-efficiency adhesion of C6 glioma cells to recombinant VEGF/trx. High-efficiency adhesion of glioma cells to plastic-immobilized antibodies was not surprising, because C6 glioma cells express various isoforms of VEGF [6].

We showed that recombinant renatured VEGF can bind not only to recombinant and plastic-immobilized

$sFlt_{2-4}$, but also to specific native receptors expressed on the surface of C6 glioma cells. The constant of VEGF binding to VEGFR-1 is much higher than that for other receptors, including VEGFR-2. These data explain the fact that antiangiogenic therapy with a genetic construct containing three first Ig-like domains of VEGFR-1 is 500-fold more effective than that with VEGFR-2 construct [3].

We conclude that functionally active recombinant $sFlt_{2-4}$ obtained in a prokaryotic system holds much promise for the measurement of VEGF in biological fluids and antiangiogenic therapy of VEGF-dependent tumors.

REFERENCES

1. B. Barleon, F. Totzke, C. Herzog, *et al*., *J. Boil. Biochem*., **272**, No. 16, 10,382-10,388 (1997).
2. N. Ferrana, *Endocr. Rev*., **25**, No. 4, 581-611 (2004).
3. J. Holash, S. Davis, N. Papadopoulos, *et al*., *Proc. Natl. Acad. Sci. USA*, **99**, No. 17, 11,393-11,398 (2002).
4. E. R. LaVallie, E. A. DiBlasio, S. Kovacic, *et al*., *Biotechnology*, **11**, No. 2, 187-193 (1993).
5. A. Luttun, M. Tjwa, and P. Carmeliet, *Ann. N. Y. Acad. Sci*., **979**, 80-93 (2002).
6. R. Mentlein, F. Forstreuer, H. M. Mehdorn, and J. Held-Feindt, *J. Neurooncol*., **67**, Nos. 1-2, 9-18 (2004).
7. N. Rahimi, *Front. Biosci*., **11**, 818-828 (2006).
8. F. T. Wu, M. O. Stefanini, F. MacGabhann, *et al*., *J. Cell. Mol. Med*., **14**, No. 3, 528-552 (2010).