Expression of a Recombinant Extracellular Fragment of Human Vascular Endothelial Growth Factor Receptor VEGFR1 in *E. coli*

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Human vascular endothelium growth factor receptor VEGFR1 is a type III fms-like tyrosine kinase with weakly pronounced tyrosine kinase function. The second and third IgG-like domains of the extracellular part of VEGFR1 act as "traps" for VEGF and are prospective candidates for antiangiogenic therapy of VEGF-dependent tumors. cDNA encoding extracellular Ig-like domains 2, 3, 4 of VEGFR1 was cloned in expressing vectors pET28a, pET32a, and pQE60. The recombinant protein was expressed in *E. coli* cells and purified by metal affinity chromatography. An expressing construction and a superproducer strain were created, allowing the production of high amounts of recombinant VEGFR1 extracellular fragment, needed for experimental *in vivo* antiangiogenic therapy.

Key Words: *vascular endothelial growth factor; vascular endothelial growth factor receptor; VEGFR1; expression in E. coli*

Vascular endothelial growth factor (VEGF) is the main regulator of angiogenesis in health and disease, for example, of neoangiogenesis in poorly differentiated tumors [13]. VEGF expressed by the tumor is an attractive target for antitumor therapy, as inhibition of proangiogenic action of VEGF suppresses tumor vascularization in many poorly differentiated carcinomas and gliomas characterized by VEGF-dependent angiogenesis [3]. Two type III tyrosine kinases serve as VEGF receptors: VEGFR1 and VEGFR2 [9]. They have a cytoplasmatic C-terminal domain, a transmembrane domain, and a large extracellular site consisting of seven Ig-like domains [7]. The VEGFR1 and VEGFR2 are characterized by high homology [9], but in contrast to VEGFR2, VEGFR1 much stronger binds to VEGF, while its tyrosine kinase activity is rather low [9,13]. The gene encoding full-length VEGFR1 mRNA also encodes the short mRNA from which the sflt-1 amino acid sequence is translated. This sequence corresponds to the first six Ig-like domains of the full-size receptor [7] and acts as the natural inhibitor of VEGF-dependent angiogenesis [3,9].

The second and third Ig-like loops of flt-1 extracellular domain are important for VEGF binding, while loop 4 is needed for receptor dimerization [1,8]. Glycosylation is not obligatory for VEGFR1 binding to its ligand [1], and hence, functionally active recombinant receptor binding VEGF can be obtained in eukaryotes (*Pichia pastoris* [8], insect cells [1]) and in *E. coli* cells [1]. Recombinant protein consisting of VEGFR1 extracellular domains 2 and 3 also inhibits the VEGF-dependent angiogenesis [8]. Interestingly that the growth of some human tumors transplanted to mice was significantly inhibited (but not completely suppressed) after injection of monoclonal antibodies to VEGF [3], while injections of chimerical receptor

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molecules containing the first three VEGFR1 domains [4] or its second Ig-like VEGFR1 domain connected to the third VEGFR2 domain [5] led to virtually complete cessation of tumor growth and tumor cell necrosis in the mouse model of human rhabdomyosarcoma [4,5].

Hence, recombinant flt-1 can be regarded as a candidate for suppression of VEGF-dependent angiogenesis. Realization of these experiments *in vivo* requires high amounts of functionally active protein, and hence, creation of extracellular VEGFR1 domain superproducer strain is an important task. In a relevant study, the recombinant Ig-like loop 3 of extracellular VEGFR1 domain was expressed in *E. coli*, but the output of recombinant protein was no more than 1.1 mg/liter [14].

We attempted to create an expressing construct and a superproducer strain for the production of high amounts of extracellular VEGFR1 fragment in *E. coli* cells.

MATERIALS AND METHODS

Cloning of the gene encoding amino acid sequences of flt-1 Ig-like loops 2, 3, 4. The cDNA encoding part of the extracellular domain receptor (from 95 to 520 aa) was amplified by PCR from human brain cDNA library (Invitrogen) using primers (Sintol) flunking the coding part of the respective nucleotide sequence from 5' and 3' terminals. The primers' nucleotide sequences included restriction sites needed for recognition by respective restriction endonucleases and subsequent cloning in expressing vectors (Table 1).

The amplicon containing the coding VEGFR1 sequence was treated with the corresponding restriction endonucleases and ligated in expressing vector treated by the same restriction endonucleases. Expressing vectors pET28a (Novagen), pET32a (Novagen), and pQE60 (Quiagen) were used. As a result, plasmids pET28a/flt-1(95-520), pET32a/flt-1(95-520), and pQE60/flt-1(95-520) were constructed (Fig. 1).

Sequencing (Sintol) of all resultant constructs showed identity of nucleotide sequences of the cloned gene and gene known by published data [10].

Evaluation of the efficiency of recombinant flt-1(95-520) biosynthesis. Cultures carrying different plasmids were reproduced in LB Miller, TB (Terrific broth), 2YB, and SB (Superbroth) media in order to evaluate the efficiency of recombinant flt-1(95-520) biosynthesis. *E. coli Rosetta(DE3)* cultures transformed with pET28a/flt-1 and pET32a/flt-1 plasmids were incubated overnight in a thermoshaker (Heidolph) at 200 rpm and 37°C in 5 ml LB Miller medium with 150 mg/liter kanamycin and 15 mg/liter chloramphenicol or 150 mg/liter carbenicillin and 15 mg/liter chloramphenicol, respectively. *E. coli* M15 [pREP4] culture carrying pQE60/flt-1 plasmid was incubated overnight in 5 ml LB Miller with 150 mg/liter carbenicillin and 15 mg/liter kanamycin. Bacteria from overnight cultures were then inoculated in LB, 2LB (double LB medium), TB, 2YB, and SB with antibiotics in 1:50 proportion. The cultures were incubated till reaching OD_{550}=0.6 in a thermoshaker at 200 rpm and 37°C, after which isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures to the final concentration of 1 mM and incubation at 37°C and intense shaking was carried out for 4 h more. The cells were then precipitated by centrifugation and frozen. The cell precipitate was resuspended in a single SDS-PAGE buffer with β-mercaptoenthanol and incubated at 100°C for 5 min. The cell lysates were compared by

TABLE 1. Oligonucleotide Sequences Used in the Study

Name	Oligonucleotide structure
flt-1 for 1	5'-GCATCATATGATGGTCAGCTACTGGGACAC-3 FauNDI
flt-1 rev 1	3'-GCATGAATTCTAGAGTGGCAGTGAGGTTTT-5' EcoRI
flt-1 for 2	5'-GCATGAATTCATGGTCAGCTACTGGGACAC-3' EcoRI
flt-1 rev 2	3'-GCATCTCGAGTAGAGTGGCAGTGAGGTTTT-5' Sfr274I
flt-1 for 3	5'-GCATCCATGGTCAGCTACTGGGACAC-3' NcoI
flt-1 rev 3	3'-GCATCTCGAGTAGAGTGGCAGTGAGGTTTT-5' BglII

12% PAGE-SDS electrophoresis. The gel was stained and all lysate proteins were visualized by Coomassie staining (0.4 g Coomassie R250, CH_3OOH 10%, CH_3CH_2OH 40%); hybrid protein with the histidine tag in bacterial lysate was detected using Invision His-tag In-gel stain (Invitrogen).

Biosynthesis and purification of recombinant flt-1(2-3). Recombinant flt-1(95-550) in preparative amounts was reproduced in SB medium. Overnight culture was incubated as described above, after which the medium with cells from overnight culture was inoculated in 200 ml SB with appropriate antibiotics and reproduced to OD_{550}=0.6, and IPTG was then added to final concentration of 1 mM and the cultures were incubated overnight in a thermoshaker (Heidolph) at 200 rpm and 37°C. The cells were precipitated by centrifugation at 4000*g* and resuspended in lysing buffer (6 M guanidine chloride, 20 mM Na_3PO_4, 500 mM NaCl, pH 8.0) with protease inhibitor (PMSF, 1 mM). The cells were lysed for 1 h at 4°C. The lysate was centrifuged at 8000*g*, after which it was applied onto a column with 2 ml Ni-NTA (Invitrogen). The column was washed with buffer (8 M urea, 20 mM Na_3PO_4, 500 NaCl, pH 8.0) with imidasole concentrations increasing from 10 to 40 mM and then to 50 mM. Recombinant protein was eluted with 8 M urea with 200 mM imidasole. The fractions were analyzed by 12.5% SDS PAGE electrophoresis.

RESULTS

Step 1 of the work was to reproduce flt-1(95-520) recombinant protein. Its gene was therefore cloned into pET28a plasmid with a "strong" T7 promotor providing high intensity of recombinant protein biosynthesis in *E. coli* cells [11,12]. However, the biosynthesis of recombinant protein with this construct was ineffective, with the output of just 300 μg/liter of culture. In addition, because of low intensity of the recombinant protein biosynthesis, a traditional contaminant,

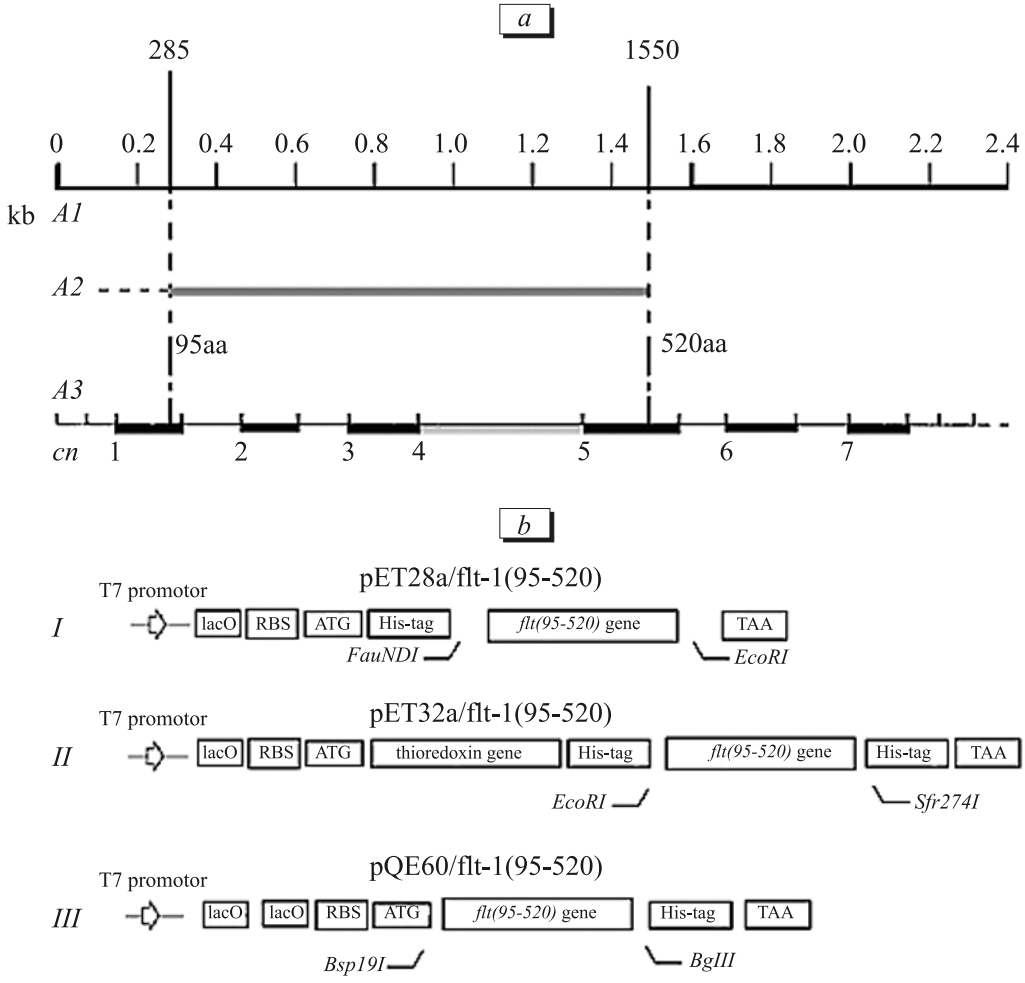

Fig. 1. Scheme of semidark expressing constructions. *a*) *A1*: VEGFR1 extracellular domain cDNA (thousands of base pairs are numbered); *A2*: cDNA fragment encoding VEGFR1 extracellular domain part; *A3*: VEGFR1 extracellular domain amino acid sequence (Ig-like domains are numbered). *b*) scheme of expressing vectors.

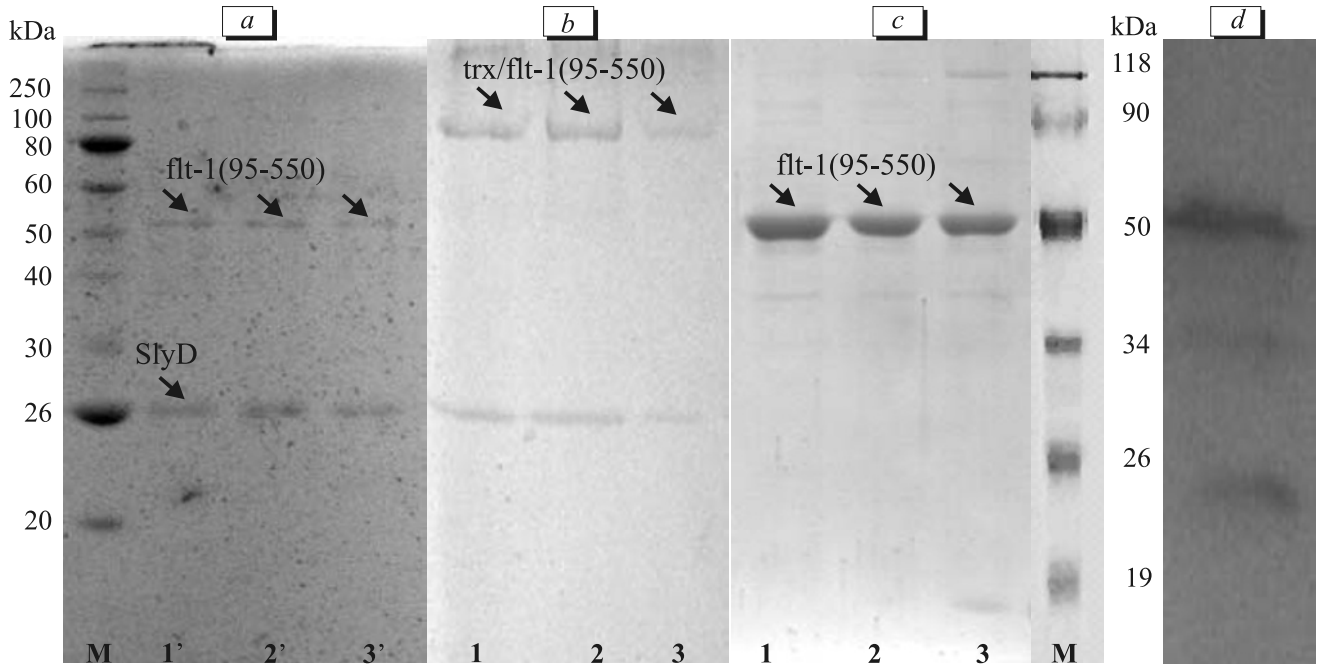

Fig. 2. Analysis of purified fractions of recombinant flt-1 in 12% PAGE. *a*) biosynthesis was controlled by T7 promotor in pET28a plasmid (Sibenzyme markers). 1', 2', 3': elution No.; M: markers. Band corresponding by electrophoretic mobility to SlyD (contaminant released together with recombinant proteins at low intensity of their biosynthesis). *b*) biosynthesis in pET32a plasmid; 1, 2, 3: elution fractions. *c*) biosynthesis in pQE60 plasmid. 1, 2, 3: elution fractions; M: molecular weight markers (Fermentas). *d*) result of immunoblotting with commercial monoclonal antibodies (anti-flt1; Sigma), antispecies antibodies (goat antimouse HRP-conjugated Ab; A9917; Sigma) and ECL-advance chemiluminescent reagent (GE Health care).

SlyD bacterial protein, was released together with the recombinant protein during its purification by metal affinity chromatography (SlyD contains several histidines and competes with the recombinant protein for binding to nickel ions if the intensity of its biosynthesis is low) (Fig. 2) [2].

No contamination with SlyD protein takes place if the recombinant protein is highly effectively synthesized in *E. coli* cells, as in that case SlyD cannot compete with the recombinant product excess with the hexahistidine motif for Ni ion binding.

In order to increase the recombinant protein output and improve its purification, the VEGFR1 coding sequence was re-cloned into pET32a plasmid allowing co-expression of recombinant proteins with N-terminal thioredoxin. Co-expression of recombinant proteins in the system in which thioredoxin was used as an N-terminal partner resulted in sufficintly high expression of eleven mammalian growth factors and cytokines in *E. coli.* cells. In addition, we significantly increased VEGF expression to 10 mg/ml culture by co-expressing it with thioredoxin in *E. coli Rosetta(DE3)* cells (our unpublished data). However, after cloning of flt-1 sequence in pET32a the protein output in that construct did not exeed than 3 mg/liter (Table 2). In addition, it was impossible to get rid of all admixtures during protein purification (Fig. 2, *b*). The biosynthesis efficiency was virtually the same in different media (LB Miller, TB, SB, 2YB), including those with proteolysis inhibitors. Poor intensity of recombinant protein biosynthesis in the system with T7 promotor could be caused by disagreement between the rates of transcription and translation because of bacterial promotor replacement with T7 promotor. The resultant transcript not protected by ribosomes became a target for nucleases [6].

Because of the negative result with the T7 system of expression, the relevant coding sequence was cloned

TABLE 2. Recombinant Protein Output (mg/liter culture)

Protein	Plasmid and strain	Output, mg/liter
Flt-1(95-520) with histidine tag	pET28a/flt-1(95-520)	0.3
Flt-1(95-520) with histidine tag and thioredoxin	pET32a/flt-1(95-520)	1-3
Flt-1(95-520) with histidine tag	pQE60/flt-1(95-520)	10

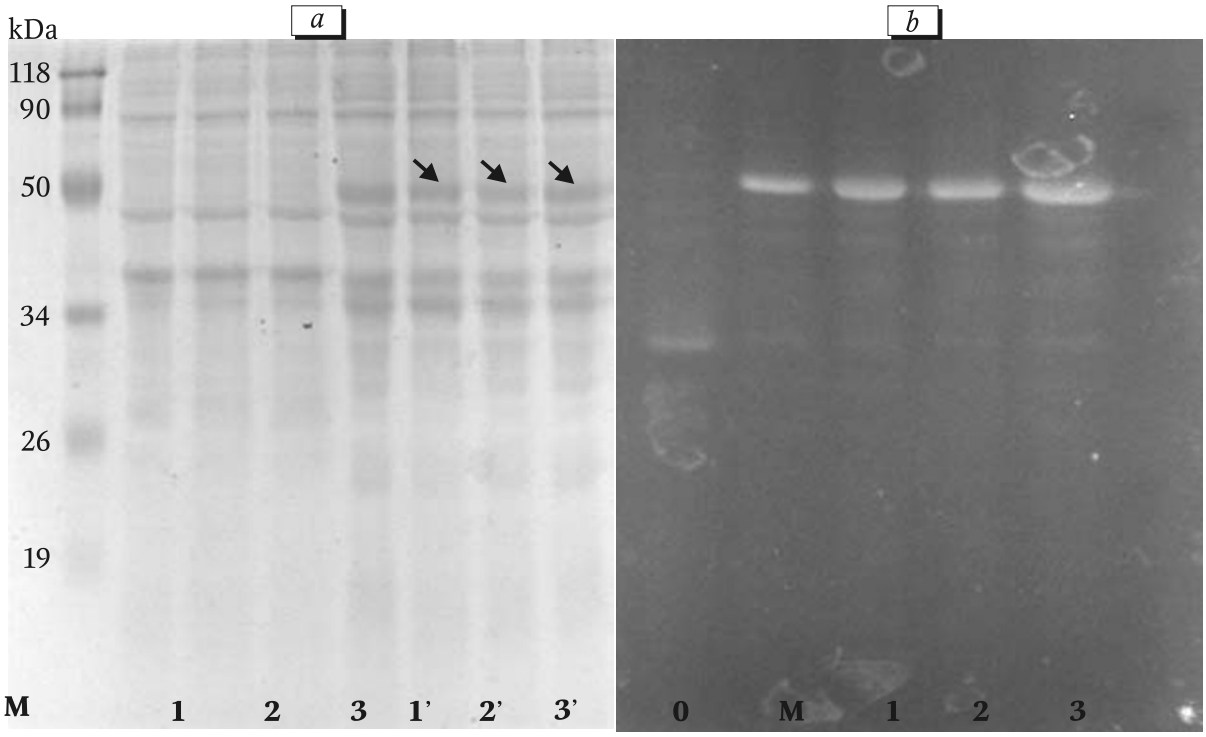

Fig. 3. Comparison of *E. coli* proteomes after IPTG induction in 12% PAGE. *a*) 1, 2, 3: proteomes of M15[pREP4] cells transformed with pQE60/flt-1 in 2LB, 2YB, SB, respectively, before IPTG induction; 1', 2', 3': proteomes of M15[pREP4] cells, transformed with pQE60/flt-1 after IPTG induction in 2LB, 2YB, SB, respectively. *b*) analysis of cell lysates for expression of hybrid protein with histidine tag; 0: cell lysate before IPTG induction of protein synthesis; M: flt-a(95-520) purified by metal affinity chromatography; 1, 2, 3: proteomes of M15[pREP4] cells transformed with pQE60/flt-1 after IPTG induction in 2LB, 2YB, SM, respectively.

in pQE60 expressing factor (Quiagen), in which the recombinant protein expression was controlled by *E. coli* T5 promotor.

Transformation of *E. coli* strain M15[pREP] with pQE60/flt-a(95-520) plasmid DNA resulted in creation of a superproducer strain. The level of recombinant protein expression in LB Miller medium did not differ from that in previous strains with T7 promotor. However, intense expression of recombinant protein was seen in enriched media SB and 2YB and in 2LB medium, constituting 20% of all bacterial proteins (Fig. 3). Staining of gels with Invision His-tag In-gel stain visualized the histidine tag in hyperexpressed protein (Fig. 3). Using metal chelate chromatography, highly purified recombinant extracellular VEGFR1 fragment without any admixture proteins was obtained.

Hyperexpression of extracellular VEGFR1 domain in plasmid DNA with T5 promotor can be presumably explained by synchronization of transcription and translation in the producer strain. The immunochemical identity of the resultant recombinant Flt-1 protein was proven by immunoblotting with commercial antibodies to extracellular VEGFR1 fragment (Flt-1) (Fig. 2, *d*).

Hence, cloning of cDNA of IgG-like domains 2-4 in hybrid proteins with hexahistidine motif in different expressing vectors resulted in creation of strains producing the desired recombinant proteins. The synthesis of VEGFR1 recombinant extracellular fragment was the most effective in M15[pREP4] strain transformed with pQE/flt-1(95-550) plasmid. The output of recombinant protein after synthesis induction in that case was 10 mg/liter medium, *i.e.* almost 10-fold higher than in available reports.

The recombinant protein including human extracellular IgG-like VEGFR1 domains 2-4 working as VEGF "traps" is regarded as a prospective candidate antiangiogenic drug for therapy of VEGF-dependent tumor neoangiogenesis, similar by activity to humanized antibodies to VEGF (Bevacizumab, Avastin) and by some data, even superior to them.

The resultant superproducer strain can be used for production of recombinant flt-1(95-550) protein for preclinical trials of its antiangiogenic activity towards poorly differentiated tumors *in vivo*.

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