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Production of polyclonal anti VEGF antibodies and establishment of sELISA system for detection of serum VEGF level in tumor patients

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ABSTRACT

Vascular endothelial growth factor (VEGF), the most potent endothelial cell mitogen and also a regulator of vascular permeability, is emerging as a powerful prognostic tool. The aim of the present study is to produce specific rabbit polyclonal antibodies against bacterially expressed human VEGF165 using a recombinant DNA approach. A plasmid has been constructed to direct the synthesis of recombinant human VEGF165 (rhVEGF165) in Escherichia coli as a fusion protein containing a His6-tag at the N-terminus. The rhVEGF165 was purified based on its 6xHis-tag by affinity chromatography using Nickel-Agarose Column and refolded by dialysis. Two rabbits were immunized with the purified recombinant protein to produce polyclonal anti-human VEGF antibody. The titer was checked with indirect ELISA and the final titer was 1:25600.We used the purified recombinant protein and polyclonal antibody to detect VEGF expression levels in the serum of 33 tumor patients and 25 healthy controls by an established sandwich ELISA. The mean antigen concentration in serum of patients was approximately 407 pg/ml and significantly higher than those in the healthy controls in which the circulating VEGF antigens concentration was not detectable (P <0.05). Thus, this newly ELISA could be a rapid, effective and cheap method for tumor diagnosis using serum VEGF as a biomarker.

Key words: VEGF; prokaryotic expression; polyclonal antibodies; ELISA; tumor detection

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INTRODUCTION

VEGF, also known as VEGF-A or vascular permeability factor, is an endothelial mitogen and stimulator of angiogenesis and is a member of a family of growth factors that includes VEGF-B, -C, -D, -E and placental growth factor [1]. The human VEGF gene, which is localized in chromosome 6p12, is organized in eight exons separated by seven introns. The coding region spans approximately 14 kb. The initial discoveries of VEGF were followed by the identification of several splicing variants of VEGF gene transcripts, each encoding an active protein product. At least five VEGF protein products have been identified and designated as VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 [2], [3] with VEGF165 is the most potent and the most abundant in the cell.

Because of the potency and specificity of its angiogenic effect, VEGF is the most widely studied angiogenic factor for its clinical significance**.** Numerous studies have demonstrated that tumor over expression of VEGF correlates with high tumor microvessel density (MVD) and is associated with advanced tumor stage or tumor invasiveness

(metastasis) and decreased patient survival in various common human malignancies such as gastric cancer [4]; esophageal cancer [5]; liver cancer [6]; breast cancer [7]; Chronic Myeloid Leukemia [8] and so on.

Antibodies are important tools used by many investigators in their research and have led to many medical advances. Mammalian sera represent a remarkable and economical source of immunoglobulins widely used in diagnostic and therapeutic applications [9], [10]. In this study we report a constructed prokaryotic expression system and preparation of a polyclonal antibody against the bacterially expressed His-tag VEGF165 fusion protein. This low-coast antibody can be used in the detection of VEGF antigen in serum samples using ELISA method in an attempt to reduce the coast of such detection and to provide scientific basis for screening metastasis and recurrence of tumor using serum VEGF level as biomarker.

EXPERIMENTAL SECTION

1. ***$VEGF_{165}$ gene, Bacterial strains, plasmid and growth conditions***

The coding sequence of VEGF165 gene has been already cloned into the pCI-neo vector (Promega) in our laboratory. The *E.coli* strain DH5α (life technologies, Inc., Rockville, MD, USA) was used for all routine cloning experiments whereas, the *E.coli* strain Rosetta (DE3) was used for recombinant protein expression.

The pET-28a (+) Vector (Novagen)
Lauria-Bertani medium (LB) was used for culture with supplement of 25 mg Kanamycin per ml and 50mg/ml Chloramphenicol.

Serum samples were collected from cancer patients, Jilin Hospital (China)

2. ***Construction of the recombinant plasmid***

The coding region of the mature human VEGF165 (without the signal peptide) was amplified using PCR method from a pCIneo-VEGFplasmid that carried the VEGF gene. Primers were designed according to the sequence submitted in GenBank database under Accession No. NM_003376.3.The upstream primer contained an *Eco*RI restriction site (5'-TCT GAATTC GCA CCC ATG GCA GAA GG- 3'). The downstream primer included the stop codon and a *Xho*I restriction site (underlined) (5' – GCGC CTCGAG TCA CCG CCT CGG CTT GT- 3'). The PCR temperature was programmed as follows:denaturation step: 94^o C/ 2 min-one cycle; annealing step: 94^o C/15 s, 60^o C/15 s, 72^o C/2min-35 cycles; extension step:72^o C /5 min –one cycle.The PCR mixture(25µL) was composed of: 12.5 µL premix Taq, 5µL pCIneo-VEGF plasmid (template), 1.5µLwater, and 3 µL of the primers (2.5 µmol/ml each).The resultant PCR product was purified by routine procedures. The purified DNA fragment and the plasmid pET-28a (+) were both digested with *Eco*RI and *Xho*I at 37 °C overnight. Then, the VEGF gene was ligated into the pET-28a (+) plasmid by reaction with T4 DNA ligase in 10XT4 DNA ligase buffer at 16°C/overnight. The constructed recombinant plasmid was transferred into *E. coli* DH5 α. Positive transformants were selected after performing a bacterial PCR and double enzyme digestion. The DNA sequence was analyzed by Dingan biotechnology company (Shanghai, China).

3. Expression and analysis of recombinant VEGF165 protein

The expression plasmid harboring the VEGF gene was transformed into *E. coli* Rosetta (DE3) strain. Preculture in 200 ml of Luria-Bertani medium containing 25 mg/ml kanamycin and 50mg/ml Chloramphenicol was inoculated directly from glycerol stock at −20 °C and grown overnight at 37°C. This culture was used to inoculate 1000 ml of LB/kan/Cm and allowed to grow at 37 °C with vigorous shaking (230 rpm).When the OD_{600} of the medium reached 0.6, IPTG was added to a final concentration of 0.1mM. The cultures were incubated for an additional 4.5 h. Cells (typically 2–3 g wet weight per 500 ml culture) were collected from the IPTG induced1-liter culture by centrifugation at 8000 rpm/3 min/4°C. The cells were then resuspended in 25 ml lysis buffer containing 50 Mm Tris-Cl, 100 mMNaCl pH 8.0 and disrupted by sonication on ice. Sonication of the cells was conducted using a digital Sonifier at 5s / cycle for 75 cycles with 5s cooling after each sonication cycle. The inclusion bodies were collected by centrifugation at 4°C (14.000 rpm/ 15 min).The supernatant was discarded, and the pellet was resuspended in 20 ml washing buffer (100 mM NaH_2PO_4, 100 MmTris-Cl, 2M Urea, pH 8), centrifuged again at 14.000 rpm / 15 min and resuspended in 25 ml solubilizing buffer (10 mM NaH_2PO_4, 10 MmTris-Cl, 8M Urea, pH 8.0) and then incubated at 4°C overnight. Following centrifugationat 14.000 rpm for 20 min, protein fractions in both supernatant and pallet were analyzed to determine protein solubility by SDS-PAGE analysis.

4. Purification of His-tagged VEGF Protein by Ni-NTA affinity Chromatography

The purification of the recombinant VEGF protein was accomplished using Ni-NTA affinity chromatography. Prior to sample loading, the resin was washed twice with 3 volumes of the binding buffer (200mM NaH_2PO_4, 20mM Tris-ClpH7.8), the cleared lysate was then loaded into the Ni^{+2}-NTA column (Qiagen), washed twice with 4 ml wash buffer (100mM NaH_2PO_4, 10mM Tris-Cl，8M urea, 20 Mm imidazole pH 6.8).The target protein was eluted 4 times with 0.5 ml elution buffer (100mM NaH_2PO_4, 10mM Tris-Cl, 8M Urea, 20 Mm imidazole pH 5.9 and 4.5 respectively).20 Mm imidazole was added to all buffers to minimize non specific binding of untagged protein contaminants . The fractions were collected and analyzed by SDS-PAGE gel and stained with Coomassie blue. The gel was destained with 10% acetic acid and 10% methanol (v/v).

5. Refolding and dialysis

VEGF inclusion body protein was refolded by dialysis at 4 °Cwith gradually decreasing the concentration of the denaturant (8M Urea).Step dialysis was performed with buffer changes occurring every 4 h to facilitate complete removal of urea from the protein solution. Briefly, the protein fractions eluted from the Ni^{2}-NTA column were transferred to dialysis tubing with a molecular weight cut off of 12-14 kD and dialyzed against 1L of buffer I [100 mMTris-HCl, 10 mM EDTA, 0.5 % SDS, 3M Urea (pH 8.0)] at 4°C for 4 h. The recombinant protein was then further dialyzed twice against 1L ml of buffer II [100 mMTris-HCl, 10 mM EDTA, 0.1 % SDS, 1M Urea (pH 8.0)] and 1L of buffer III [100 mMTris-HCl, 10 mM EDTA, 0.1 % SDS, (pH 8.0)] at 4°C for 4 h/dialysis cycle, respectively. A final dialysis (4°C, 4h) was performed twice against buffer IV containing 50 mMTris-HCl, 5 mM EDTA (pH 8.0) and buffer V containing 20 mMTris-HCl, 2 mM EDTA (pH 8.0).To confirm the dialyzed protein remained intact, SDS–PAGE analysis was performed.The refolded protein was then concentrated by incubating the dialysis tubing containing the protein solution with Silica Gel at 4°C / overnight and subsequently stored in 1 ml aliquots containing 0.5 mg/ml purified VEGF protein.

The protein concentration was measured as described by Bradford [13], with bovine serum albumin as reference.

6. Animal immunization and Production of polyclonal anti-h*VEGF *Antibody

Two New Zealand white rabbits, about 4-month-old and 2-kilogram-weight,

were chosen for preparing antibody against rVEGF protein. Rabbits were first immunized using 500μg of recombinant protein in Freund's complete adjuvant (Sigma). After a 15-day growth, three enhancing immunization was further carried using incomplete Freund's adjuvant, with a 15-day interval between each implantation, and serum was obtained after the last bleeding. Needle injections and serum collection were performed according to institutional guidelines for animal care and handling. Antibody titer was determined by indirect ELISA.

7. Purification of Polyclonal Anti-h*VEGF*Antibody

The immunoglobulin G (IgG) fraction of 1 ml of the immune serum against rVEGF was purified in a HiTrap Protein GHp column (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The purity of the various IgG preparations was checked by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Antigenic specificity of the antiserum raised after immunization with rVEGF was examined using the Western blot procedure.

8. Establishment of ELISA procedure for detection of serum VEGF

Duplicate rows in multiwell ELISA plates were coated with 0.5μg/ml monoclonal anti VEGF antibody in 50 mM sodium bicarbonate buffer, pH 9.4–9.6. The following day, the plates were washed five times, (each time for 3 min) with PBS containing 0.3% Tween 20 (TPBS) and one time with PBS and blocked with 200 μl 0.25% non-fat dry milk (BSA) in TPBS (blocker) for 1 h at 37°C. To prepare an ELISA standard curve, a serial dilution of recombinant human VEGF was prepared in the range 0.045–200 ng/ml. Appropriate control (without antigen) was also included. One hundred microliters of recombinant VEGF proteins (standard) or serum samples (1:200), diluted in wash/ dilution buffer, were added to the wells and incubated for 1h at 37 °C. After washing in wash/dilution buffer, 100 μl of polyclonal anti VEGF antibody was added at the dilution 1:50 and incubated at 37 °C for 1h. Extensive washing was carried out after this stage to remove unbound reagents .Then, 100 μl of a 1:5000 solution in the blocker of goat anti-rabbit IgG specific secondary antibody conjugated to HRP was added to each ELISA well and the plates incubated at 37°C for 45 min. After washing the wells 5 times with TPBS and 1 time with PBS, 100 μl of TMB solution was added to each ELISA well. The color was allowed to develop for 12 min at room temperature and the reaction stopped by the addition of 50 μl of 2N solution of sulfuric acid H_2SO_4. The plates were read at 450 nm

using ELISA reader. A total of 25 cases of healthy human serum and 33 cases of patients with various types of tumor were detected with the above-mentioned method.

9. Statistical analysis

Student's t-test was used to evaluate differences between serum VEGF level in tumor patients and healthy individuals group. Significance was presumed at $P \leq 0.05$.

RESULTS AND DISCUSSION

1. VEGF gene cloning

In this study, the *VEGF* gene was PCR amplified from plasmid pCI-neo-$VEGF_{165}$ and cloned into the prokaryotic expression vector pET-28a(+). PCR products were confirmed to contain 498 bp as expected by 1% agarose electrophoresis (Figure 1). The recombinant plasmid was transferred into *E. coli* DH5 α. Positive transformants were selected after performing double enzyme digestion (Figure 2) followed by sequencing.

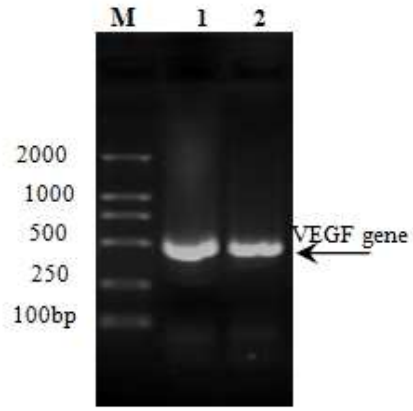

Figure 1: VEGF gene amplification. M: DL 2000 DNA Marker; lane 1, 2: VEGF gene.

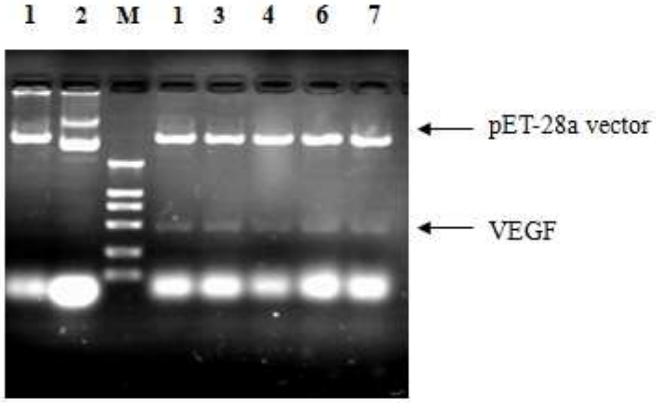

Figure 2: Double enzyme digestion of pET-28a-VEGF recombinant plasmid.
1. pET-28a digested plasmid, 2.pET-28a plasmid,
M: DL200 marker, 3.4.5.6.7 recombinant plasmids.

Several investigators have encountered difficulties in expressing a sufficient amount of protein product due to the presence of the signal peptide at the N terminus, which causes rapid transportation of the product out of the bacterial cell [11], [12]. Removal of the signal peptide leads to cytoplasmic retention of the protein, resulting in higher yields of expressed products. In order to obtain a sufficient amount of fusion, we have excluded the signal peptide at the N terminus of the VEGF gene during the preparation of the construct.

2. Recombinant VEGF Proteinexpression and purification

As 6 rare codons are present in the VEGF gene, a modified *E. coli* strain (Rosetta (DE3) was employed for transformation of the expression plasmid in order to increase protein yield. This strain is derived from BL21. It contains extra t-RNA to prevent codon usage bias and to enhance mammalian protein expression in *E.coli* expression system.

The recombinant plasmid was transformed to expression host *E.coli* Rosetta (DE3) strain, and induced with IPTG after the OD_{600} showed a value of 0.6. After induction, a predominant band corresponding to the expected size of the His-Tag VEGF fusion protein (24kD) appeared only in the total cell extract of induced *E.coli* cells. The identity of the expressed protein was investigated by Western blot using commercial available anti-VEGF antibody (R&D). The un-induced and induced *E. coli* protein extracts (figure 3, Lane 1 and 2) were separated by SDS–PAGE and transferred onto nitrocellulose membrane. Western blot with anti-VEGF antibody was performed, one band on the membrane corresponding to the size of rVEGF was detected only in the bacteria extract after IPTG induction (figure3, Lane 4), and not in the un-induced bacteria extract (figure 3, Lane 1). The Western blot result supported that the observed induced protein is rVEGF indicating an efficient expression of recombinant VEGF in the form of

insoluble and inactive inclusion bodies.

3. Inclusion bodies isolation

Inclusion bodies were isolated from soluble protein of *E.coli* cells by different solubilizing and washing steps. The initial cell pallet was first suspended in Tris-buffer to isolate the easily soluble *E.coli* proteins. The inclusion bodies were collected by cells disruption followed by centrifugation, and washed with 2 M urea and then the pellet was fully solubilized by denaturing with 8 M Urea overnight to permit Ni–NTA affinity chromatography.

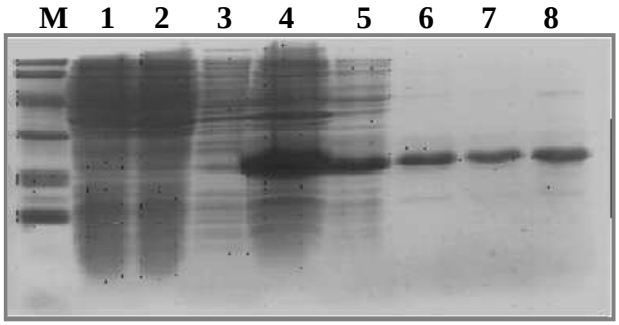

Figure 3: SDS-PAGE analysis showing the different steps of VEGF protein purification M: molecular mass markers, 1: non induced total fractions, 2: culture with empty Plasmid, 3: soluble Fractions, 4: insoluble fractions (inclusion bodies), 5: solubilized inclusion body using 8M Urea, 6, 7: purified VEGF protein, 8: refolded VEGF protein after dialysis and concentration.

His-tagged-VEGF fusion protein was efficiently purified by Ni–NTA affinity chromatography where the fractions of the VEGF protein obtained by purification, corresponding to a molecular weight of 24 kDa, could be observed. By carefully adjusting the imidazole concentration (10 mM) during the binding reaction to the Ni^{2+}–NTA affinity column, as well as in the washing buffer and elution buffer, it was possible to suppress the nonspecific binding and to wash contaminating proteins before eluting the histidine-tagged target protein. (figure 3 lane 6,7)

The purified protein was then subjected to a refolding step using dialysis method at 4 °Cwith gradually decreasing the concentration of the denaturant (8M Urea).Step dialysis was performed with buffer changes occurring every 4 h to facilitate complete removal of urea from the protein solution. The refolded protein was then concentrated by incubating the dialysis tubing containing the protein solution with Silica Gel at 4°C / overnight. This component is a high activated desiccant (drying agent). It has a very strong affinity for water and will absorb it in presence to most other substances. The protein was then subsequently stored in 1 ml aliquots containing 0.5 mg/ml purified VEGF protein. The protein concentration was measured as described by Bradford[13]**,** with bovine serum albumin as reference.We could get the high purity of fusion protein as shown in figure3 (lane 8)

4. Titer and specificity of polyclonal antibody

Rabbits are usually used for antibody preparation due to some traits, such as good immune characteristics, high affinity of antibodies and enough amounts of sera [14]. Meanwhile, the amount of immunogen and the approach of injection might have influences on antibody production. In order to survey production of antibody in rabbits and evaluating effectiveness of immunization, indirect ELISA test was performed after each immunization. In this work, a final 30-ml volume of antiserum against *r*VEGF was obtained from 2 rabbits. The titer of the antiserum in ELISA test was 1:25,600 (Figure 4).

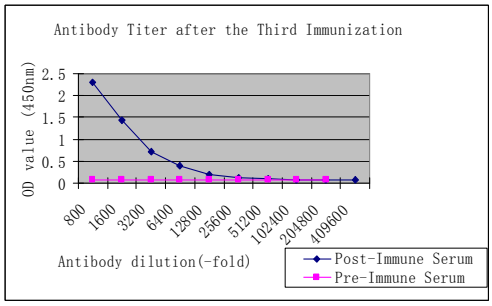

Figure 4: Analysis of rabbit anti-VEGF serum titer using indirect ELISA

On the other hand, Western blot analysis was used to ensure that the serum generated detected a band of the expected size. Preimmune serum was used as a control. A band of 24 kD was detected using the immune serum but not with the pre-immune serum control (Figure 5).This is consistent with the known size of VEGF. This result confirms that the antiserum obtained is specific to its immunogen, *r*VEGF protein. The remaining weak bands are probably due to impurities.

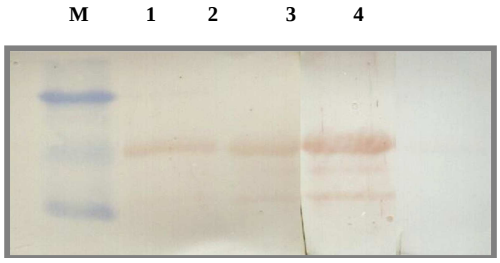

Figure 5: Validation of the anti-VEGF antibody by Western blot
M: molecular mass markers, 1,2,3: immune serum, 4: Preimmune serum(control).

5. Purification of polyclonal anti-VEGF Antibody

Purification of IgG fraction from immunized rabbit sera by HiTrap Protein G HP column resulted in a highly pure fraction which was appeared as pure bands in SDS-PAGE analysis. The protein content of this fraction was 1.67 mg/ml as determined by Bradford assay. The results of SDS-PAGE for determining the purity of IgG, which was purified onHiTrap Protein G HP column,has been shown in Figure 6. Distinct polypeptide band with molecular weight about 50 kDa corresponds to rabbit IgG heavy chains and the diffused bands between molecular weights of 20-30 kDa correspond to rabbit IgG light chain (figure 6).It is likely that diffused band of light chain could be related to different level of deglycosilation of protein during manipulation process.

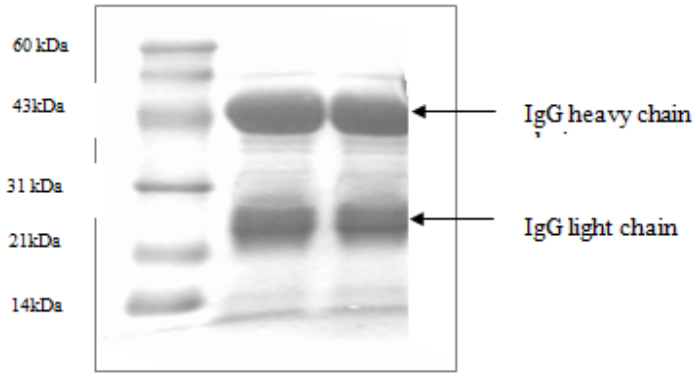

Figure 6: SDS-PAGE analysis of rabbit anti VEGF Antibody purified by HiTrap Protein GHp column. M: Molecular mass markers, 1, 2: purified antibody

6. Establishment of ELISA procedure for detection of serum VEGF

6.1 Checkerboard Titration

Chessboard reagent titration experiments were carried out to define optimal assay parameters. Based on this analysis (Table 4) using excess antigen, we found that absorbance plateaued from 8 µg/ml to 0.5 µg/ml of capture antibody, with the drop occurring using 0.25 µg/ml. Thus, we determined the optimal capture antibody concentration as 0.5 µg/ml. In the second checkerboard assay (Table 1), 0.5 µg/ml was used as the coating concentration and 200 ng VEGF protein as an antigen with a serial dilution of detecting antibody (polyclonal anti-VEGF antibody) from 1:25 to 1:800. Two wells without antigen were considered as a negative control. Using 200ng/ml VEGF protein, the absorbance plateaued between the dilution 1:25 and 1:50 of the capture antibody, with a drop occurring at 1:100. Thus, we determined the optimal dilution of detection antibody as 1:50.

6.2 Standard curve and measurement of VEGF levels in samples

A standard curve was generated by spiking negative control serum with known quantities of VEGF antigens (from 0.045 to 200 ng/ml). The optical density at 450 nm (OD450) of spiked negative control serum was plotted against its known antigen protein concentration. By use of the standard curve, ODs of sera from patients were transformed into antigen concentrations. Negative/positive discrimination levels for test results were determined by using 25 negative serum samples derived from healthy subjects and 33 serum samples derived from patients with different type of tumor. Mean OD of the negative group was 0.063 + 0.03 (SD).

Sera from tumor patients showed a range of ODs between 0.184 and 0.379, with a mean value of 0.28+ 0.06 (SD).To determine approximate amounts of circulating VEGF antigens in sera of patients, OD_{450} values were compared with the standard curve. As shown in Table 1, sera from all patients had detectable circulating VEGF antigens (between 167 and 655 pg/ml) and significantly higher than those in controls (P <0.05). The mean antigen concentration in serum of patients was approximately 407 pg/ml whereas in the healthy individuals (n=25) the circulating VEGF antigens concentration was not detectable.

Table 1: Sensitivity of MAb-sandwich ELISA for the detection of VEGF antigens in 33 tumor patients

Standard curve and controls		**33 Patients with different type of tumor**		
VEGF antigen concn (ng/ml)	OD ± SD*	No.	OD ± SD	Concn (pg/ml)
200.0	2.484 ± 0.03	1	0.303 ± 0.01	465
50.0	1.289 ± 0.006	2	0.306 ± 0.001	472
12.5	0.768 ± 0.01	3	0.359 ± 0.02	605
3.125	0.559 ± 0.03	4	0.346 ± 0.02	572
0.780	0.351 ± 0.01	5	0.320 ± 0.05	507
0.390	0.249 ± 0.04	**6**	**0.379 ± 0.02**	**655**
0.195	0.214± 0.02	7	0.297 ± 0.02	450
0.097	0.202 ± 0.01	8	0.368 ± 0.03	628
0.045	0.190 ± 0.02	9	0.370 ± 0.03	633
0.00	0.00	10	0.311 ± 0.03	485
		11	0.268 ± 0.02	377
Healthy controls		12	0.253 ± 0.004	340
Controls (n= 25)	0.063 ± 0.03	13	0.275 ± 0.01	395
Not detectable		14	0.323 ± 0.02	515
		15	0.317 ± 0.002	500
		16	0.360 ± 0.055	608
		17	0.338 ± 0.06	553
		18	0.269 ± 0.02	380
		19	0.322 ± 0.02	513
		20	0.367 ± 0.04	625
		21	0.197 ± 0.01	200
		22	0.297 ± 0.01	450
		23	0.222 ± 0.02	263
		24	0.236 ± 0.007	297
		25	0.211 ± 0.006	235
		26	0.223 ± 0.006	265
		27	0.211 ± 0.01	235
		28	0.194 ± 0.006	193
		29	0.234 ± 0.009	293
		30	0.193 ± 0.03	190
		31	0.195 ± 0.03	195
		32	0.188 ± 0.02	178
		33	**0.184 ± 0.01**	**167**

***** OD ±SD: Mean ±standard deviation. (P <0.05)***

CONCLUSION

In the present paper we describe the expression and purification of an N-terminally His-tagged VEGF protein and polyclonal antibody raised against it. In summary, a modified *E. coli* strain was used, which allows rare codons to be expressed more efficiently, and the conditions for cell growth and cell lysis were modified. The recombinant VEGF protein was produced in the form of intracellular insoluble, biologically inactive inclusion bodies. The insoluble aggregates in the inclusion bodies turned out to be advantageous as they prevented the degradation of protein as has been described for other recombinant proteins and it also facilitates its purification.

As the VEGF is accumulated in the form of insoluble inclusion bodies, other soluble proteins of *E. coli* can be separated from the inclusion bodies by using different solubilizing steps. In this protocol *E. coli* cells expressing recombinant protein as inclusion bodies were solubilized in a suitable buffer in the presence of 8 *M* urea concentration. The protein solution was loaded onto a Ni –NTA column and the bound proteins were eluted using a *VEGF* gradient decreasing pH and refolded by dialysis.

Then, rabbits were immunized with recombinant VEGF protein expressed in *E. coli*. After multiple immunizations, blood was collected and affinity chromatography was used for antibody purification. To evaluate polyclonal antibody titer, designed ELISA test was applied. Because of directed evaluation of antigen-antibody complex, the test has a high degree of precision [15]. Determining a titer of 25,600 in this test indicates the high quality of the product. Thus, this antibody is highly economical and regarding the volume of 30 mL of serum taken from two rabbits at the final sampling, considerable amount of anti-VEGF IgGs can be obtained.

ELISA and Western blot analysis confirmed that the polyclonal antibodies have high titers and specific bonds to the VEGF protein. Therefore, the antibodies generated can serve as an effective tool to facilitate our future researches on VEGF.

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