Generation of Monoclonal Antibodies to Recombinant Vascular Endothelial Growth Factor

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Female BALB/c mice were subcutaneously immunized with recombinant VEGF-164. After 3 immunization cycles, splenic B cells from immunized mouse were fused with immortalized myeloma culture SP2/0-Ag14 cells. Screening of hybrid cells producing anti-VEGF antibodies was performed by ELISA and immunocytochemical analysis on cultured C6 glioma cells. Subsequent cloning yielded hybridoma stably expressing monoclonal anti-VEGF antibodies recognizing recombinant and native VEGF.

Key Words: *monoclonal antibodies, anti-VEGF antibodies; vascular endothelial growth factor*

The formation of new vessels and maintenance of their functional and structural integrity are going on throughout ontogeny. Angiogenesis is regulated by a number of proangiogenic (VEGF, angiopoietin 1 and 2, platelet growth factor, TGF-α, and bFGF) and antiangiogenic factors (angiostatin, endostatin, thrombospondin-1) [3] acting in dynamic equilibrium. This equilibrium is disturbed in some pathological states, *e.g.* psoriasis, diabetic retonopathy, benign and malignant neoplasms, which leads to uncontrolled growth of the vascular network.

Vascular endothelial growth factor (VEGF-A) is an important inductor of physiological and pathological angiogenesis [4]. Products of *VEGF-A* gene are presented by 6 proteins, results of alternative splicing. VEGF-165, the predominant isoform is a glycoprotein; two subunit of this glycoprotein form a bioactive homodimer [11]. Some isoforms carry a heparin-binding site and through heparan sulfate are anchored to the membrane and extracellular matrix. Some enzymes, *e.g.* plasmin, heparinase, and matrix metalloproteinase can release bound isoforms thus increasing the concentration of soluble VEGF [3].

VEGF-A binds tyrosine-kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR), which leads to activation of second messenger cascade IP_3, DAG, Ca^{2+}, PKC, Raf, MEK, Erk, PI3K, Akt *etc*. [7]. VEGF is a endotheliocyte-specific factor exhibiting antiapoptotic and mitogenic activity. VEGF triggers the expression of antiapoptotic proteins Bcl-2, XIAP, Bcl-A1, and BIRC5 [2,6], thus improving cell survival. Proliferation is regulated via activation of the Erk pathway (MAPK) inducing endotheliocyte entry into the cell cycle. This is paralleled by activation of integrins stimulating cell adhesion and migration of endothelial cells [8]. It should be noted that VEGF expression depends on oxygen concentration in tissues. Under hypoxic conditions, factor HIF1α (hypoxia-inducible factor 1α) is stabilized and dimerized with HIF2α, which leads to activation of the corresponding promoter and expression of VEGF [9,12].

It is now proven that the development of solid tumors largely depends on the density microvessels and blood supply to the malignant tissue [5]. The tumor cells can remain dormant until angiogenic switch,

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which is believed to result from imbalance of inhibitory and stimulatory factors [3]. Numerous genetic aberrations, *e.g.* activation of *RAS*, *SRC,* and *ERBB2* oncogenes, and dysfunction of tumor-suppressing genes *TP53* and *VHL* (von Hippel-Lindau tumor suppressor) leading to malignant transformation of cells also affect regulation of VEGF expression [10].

Enhanced expression of VEGF is detected in various malignant neoplasms (cancer of the colon, liver, lungs, thyroid gland, breast, kidney, urinary bladder, carcinoma of the ovaries and cervix, angiosarcoma, multiform glioblastoma). VEGF plays an important role in the survival of neoplastic vessels, because reduced content of VEGF leads to apoptosis of endothelial cells and regression of pathological vessels [1].

Here we performed immunoenzyme and immunofluorescent analysis of obtained anti-VEGF antibodies.

MATERIALS AND METHODS

Nucleotide sequence encoding rat VEGF-164 was amplified using primers GCA TCATATGAT GAA CTT TCT GCT GTC TTG GG and ATA TGAATCCCC GCC TCG GCT TGT CAC ATC from rat brain cDNA library (Invitrogen) carrying recognizion sites for NdeI and EcoRI restriction endonucleases. The sequence was cloned by NdeI and EcoRI restriction sites into pET28a plasmid (Quiagen). The resultant plasmid pET28a/VEGF-164 was used for transformation of BL21(DE3) strain. The strain producing recombinant VEGF-164 was cultured in LB medium at 37°C in Erlenmeyer flasks in a thermo-shaker. After attaining OD=0.6 in *E. coli* culture, expression of recombinant protein was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a concentration of 0.1 M. Recombinant protein VEGF-164/His was isolated by metal affinity chromatography on Ni-NTA agarose.

Female BALB/c mice were immunized with purified recombinant preparation of rat VEGF-164. Three immunization cycles were performed with 4-week intervals. Each cycle included three subcutaneous injections of 25 μg VEGF-164 with complete Freund adjuvant (Difco) and final booster injection of the preparation. In 5-7 days after booster immunization, the immune response was verified by testing the serum of immunized animals by ELISA. The spleen from the immunized animal was homogenized, splenocytes were mixed with SP2/0-Ag14 myeloma cells lacking hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Fusion was performed routinely by the method of Kohler and Milstein in our modification. The cells were grown for 2 weeks in a medium containing hypoxanthine, aminopterin, and thymidine, a selective medium for hybridoma cells that can synthesize nucleotides in the presence of aminopterin.

Hybridoma screening was performed by two methods in paralllel. For supernatant testing by ELISA, chimeric protein thioredoxin/VEGF-164 was immobilized on high-adhesion polystyrene in 96-well plates (Sarstedt) in a concentration of 5 μg/ml. Goat antibodies to $F(ab)_2$ fragments of mouse Ig conjugated with horseradish peroxidase and pre-absorbed with rat serum (A3682, Sigma) diluted 1:20,000 were used as second antibodies. The reaction was visualized with tetramethylbenzidine (ready-to-use solution, Zymed). For selection of antibodies interacting with native VEGF, supernatants were tested on C6 glioma cells. The cells grown on highly adherent polystyrene to 80-100% confluence were fixed with 4% paraformaldehyde, washed with PBS, incubated with anti-VEGF for 12 h, and again washed with PBS. For immunoperoxidase staining, the specified second antibodies labeled with peroxidase (A 3682, Sigma; dilution 1:1000) were visualized with diaminobenzidine with hydrogen peroxide (Vector Labs). Immunocytochemical analysis was performed with goat antimouse antibodies conjugated with Alexa

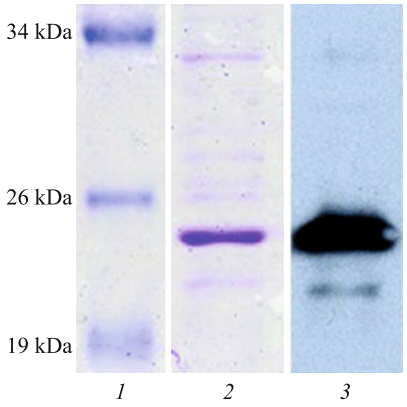

Fig. 1. Immunoblotting of recombinant VEGF with commercial antibodies. *1*) molecular weight markers (Fermentas); *2*) disc-electrophoresis of cell lysate *E. coli* producer strain after induction of VEGF synthesis; *3*) immunoblotting of VEGF using ab1316 antibodies (Abcam).

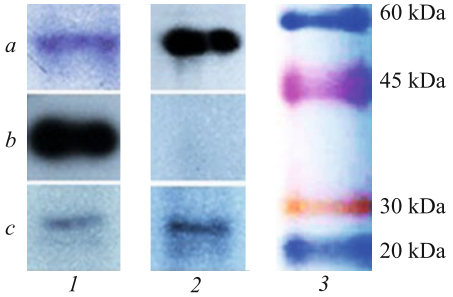

Fig. 2. Immunovisualization of native VEGF in lysate of glioma C6 cells (*a*, *c*) and recombinant fusion protein thioredoxin/VEGF-164 (*b*). *1*) immunoblotting with commercial ab1316 antibodies (Abcam); 2) immunoblotting with antibodies obtained by us; 3) molecular weight markers Chemichrome™ Western Control (Sigma).

Fluor 488 (Invitrogen; dilution 1:1000); nuclei were poststained with TOTO 633 (Invitrogen). PBS (pH 7.4) with 0.2% Tween-20 and 0.2% Triton X-100 served as working buffer. Analysis of cell preparations was performed using a Leica DMI 6000 inverted microscope.

Monoclonal antibodies were purified from ascitic fluid by affinity chromatography on protein A-Sepharose (Invitrogen). Sodium phosphate buffer (0.02 M, pH 7.4) and citrate buffer (0.1 M, pH 3.0) served as working and eluting buffers, respectively.

Recombinant thioredoxin/VEGF-164, lysates of VEGF-positive glioma C6 strains, and primary astrocyte culture were used in immunoblotting for antibody testing. Visualization was performed using ECL-advance kits (GE Healthcare) according to manufacturer's instructions.

For analysis of cell adhesion, monoclonal anti-VEGF antibodies were immobilized on polystyrene in a 96-well plate (Corning) and incubated overnight (12 h) at 4°C. The plate was washed with the working buffer and glioma C6 cells labeled with vital fluorescent CFDA dye (Invitrogen) in a concentration 10 μM (according to manufacturer's instruction) were added to wells (2×10^5 cells per well). The plate with labeled cells was incubated in an orbital thermo-shaker (37°C, 160 rpm) for 1 h and then washed to remove unbound cells. Fluorescence was detected on a VICTORX3 plate reader (PerkinElmer).

RESULTS

The obtained recombinant VEGF-164 was analyzed by Western-blotting using commercial antibodies to VEGF (Abcam). Recombinant VEGF-164 isolated under denaturating conditions was presented by a monomer with a molecular weight about 24 kDa and was immunochemically identical to native protein (Fig. 1).

Four successive cloning procedures with evaluation of anti-VEGF antibody production at each stage yielded several clones stably producing specific monoclonal antibodies. For production of antibodies, the hybridoma cells were intraperitoneally injected to fe-

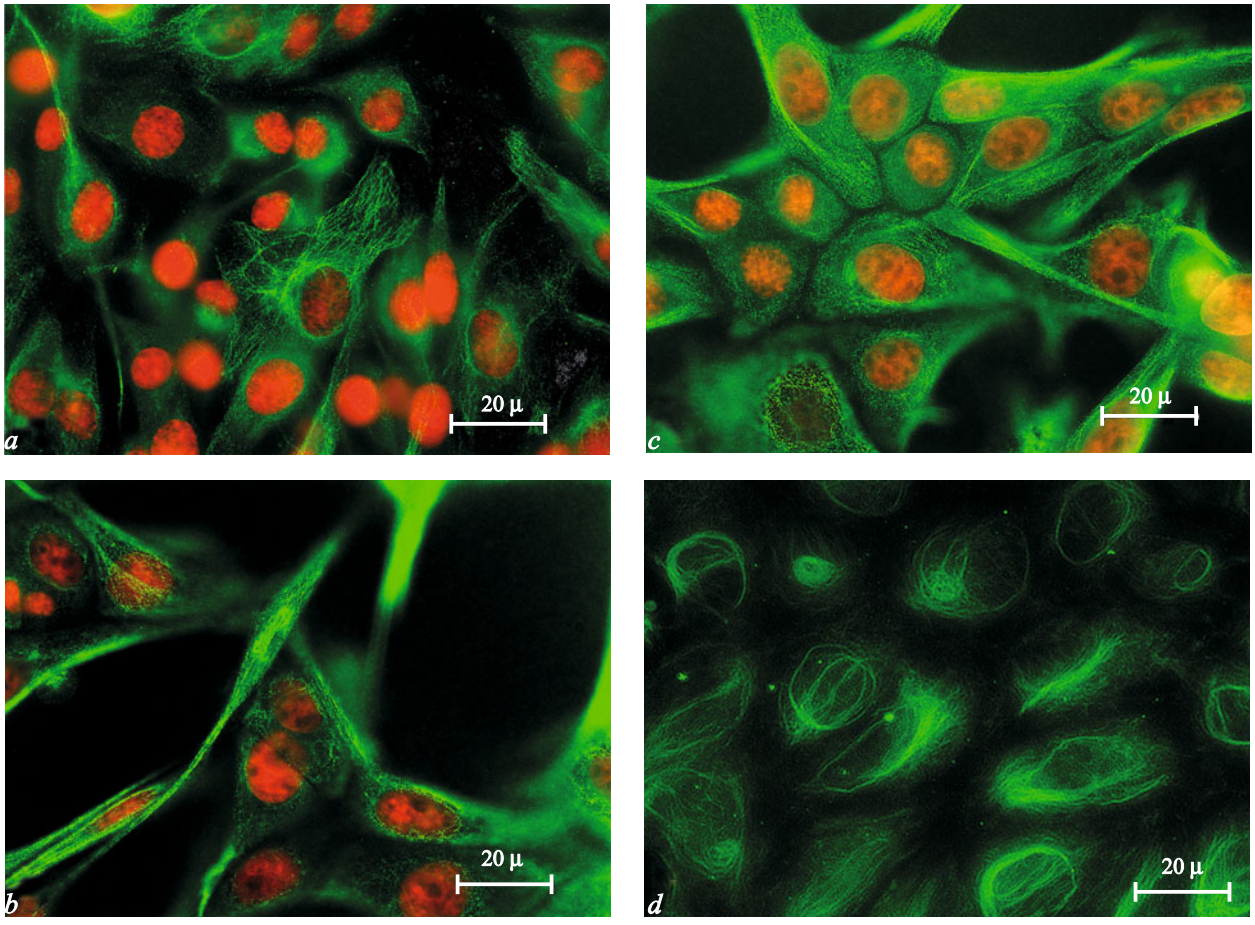

Fig. 3. Immunocytochemical analysis of VEGF with monoclonal antibodies obtained by us. *a*) glioma C6 cells; *b*) human glioblastoma U251 cells; *c*) HEK293 cells; *d*) positive control: monoclonal antibodies to VEGF (ab1316, Abcam) on HUVEC cells. Secondary antibodies are Goat antimouse Alexa Fluor 488 (Invitrogen). *a-c*) cell nuclei are post-stained with TOTO 633 (Invitrogen).

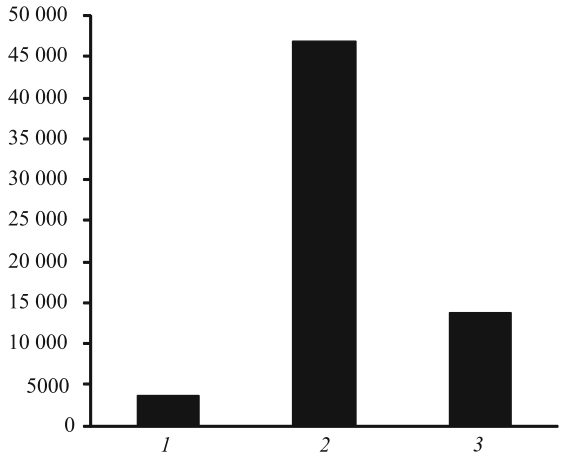

Fig. 4. Adhesion of glioma C6 cells labeled with CFDA on polystyrene with immobilized monoclonal antibodies to VEGF. 1) control (cell adhesion on polystyrene); 2) cell adhesion on polystyrene with immobilized antibodies to VEGF; 3) cell adhesion on polystyrene with immobilized antibodies pre-incubated with recombinant VEGF.

male BALB/c mice and ascitic fluid was collected after 10 days. Monoclonal antibodies purified on protein A-Sepharose and concentrated to 2.5 mg/ml were analyzed by ELISA, immunoblotting, and immunocytichemical analysis of VEGF on fixed preparations.

Immunoblotting with recombinant fusion protein thioredoxin/VEGF-164 (37 kDa) and lysates of VEGF-positive glioma C6 cells allowed immunohistochemical identification of monoclonal antibodies to VEGF. The obtained antibodies (clone 14) intensively visualized native VEGF dimer and slightly weaker VEGF monomer (Fig. 2: *2a*, and *2c*, respectively) and almost did not interact with recombinant VEGF (Fig. 2: 2*b*). On the contrary, commercial antibodies recognize with high affinity recombinant thioredoxin/VEGF-164 (Fig. 2, *1b*) and with lower affinity native VEGF in cell culture lysates (Fig. 2, *1a* and *1c*). These results suggest that the obtained antibodies to VEGF primarily recognize with native VEGF dimer, the most abundant functionally active isoform of this protein.

In immunocytochemical analysis, anti-VEGF antibodies visualized cytoplasmic pool of VEGF; characteristic staining was observed in preparations of all analyzed VEGF-positive cultures: rat C6 glioma, human U251 glioblastoma, HUVEC, and HEK293 (Fig. 3). Staining of reticular compartment in endoplasma observed in all cell preparations corresponded to localization of endoplasmic reticulum, the site of VEGF translation. In the control, no staining was observed in the absence of primary antibodies. In the ectoplasm, weak fluorescence visualizing the vacuolar system was observed. Similar granular pattern is typical of export proteins synthesized in the granular endoplasmic reticulum and accumulating in secretory vacuoles.

Analysis of cell adhesion demonstrated significant binding of glioma C6 cells labeled with fluorescent label (CFDA) with monoclonal antibodies to VEGF immobilized on polystyrene (Fig. 4). Selective adhesion of C6 cells on polystyrene with immobilized antibodies to VEGF is possible due to interaction of antibodies to membrane-bound isoforms of VEGF. Specificity of this adhesion was demonstrated in the experiment with competitive binding of antibodies. To this end, recombinant VEGF was added to wells with immobilized antibodies to VEGF, incubated for 30 min, and labeled cells were added. Due to competitive binding of antibodies with VEGF, the level of cell adhesion on polystyrene with immobilized antibodies decreased by 3.4 times. This suggests that cell adhesion is determined by immunoaffinity interaction of antibodies and membrane-associated VEGF.

Thus, we obtained hybridomas stably expressing mouse monoclonal antibodies to native VEGF. The obtained antibodies can be used for immunochemical visualization of VEGF-positive cells by both immunoperoxidase and immunofluorescent methods. Taking into account predominant interaction of antibodies with functionally active isoform of VEGF we can hypothesize that the obtained antibodies can be successfully used for antiangiogenic therapy of pathologies associated with uncontrolled growth of the vascular network, in particular, in malignant neoplasms, and for targeted delivery of diagnostic and therapeutic agents into neoangiogenesis foci. Moreover, monoclonal antibodies can provide the basis for creation of highly sensitive test-system for immunoenzyme measurements of VEGF concentrations in biological fluids.

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