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Nanoantibodies for Detection and Blocking of Bioactivity of Human Vascular Endothelial Growth Factor A_{165}

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Abstract—Nanoantibodies (single-domain antibodies, nanobodies) derived from noncanonical single-chain immunoglobulins provide an attractive tool for *in vitro* and *in vivo* diagnostics as well as for development of targeted drugs for clinical use. Nanoantibodies against several clinically important targets have been developed and are actively investigated. However, no development of nanoantibodies against vascular endothelial growth factor VEGF-A_{165} has been reported. We describe here the generation of nanoantibodies derived from single-chain Bactrian camel immunoglobulins directed against VEGF-A_{165}. We demonstrate that these nanoantibodies are suitable for enzyme-linked immunoassay to quantify human VEGF-A_{165} as well as for blocking its activity. Our results provide a basis for diagnostic kit development for quantification of VEGF-A_{165}, which emerges as a biomarker useful in various pathological conditions. In addition, the nanoantibodies might be used for development of therapeutic molecules targeting VEGF-A_{165}-dependent pathological neoangiogenesis.

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Key words: nanoantibodies, human vascular endothelial growth factor VEGF-A_{165}, diagnostics, ELISA, VEGF-A_{165} inhibitor

Discovery of noncanonical immunoglobulins in some species, in particular, of the Camelidae family, has had a great influence on research and developments in biotechnology by providing the availability of the unique antibody property of recognizing specific antigenic determinants. This property of antibodies provides the basis for enzyme-linked assay broadly used for diagnostics, and antigenic reagents are now successfully used as drugs based on binding of target antigens, which play a significant role in pathogenesis or specificity of target cells. The specific characteristic of single-domain antibodies distinguishing them from classical antibodies is that the antigen-recognizing region is formed of one polypeptide chain rather than specific folding of light and heavy chains [1]. This unique feature of single-domain antibodies has a great practical value. First, it allows using just a small antibody fragment rather than a full-size protein. Second, it significantly simplifies gene engineering manipulations for deriving recombinant proteins. Moreover, the single-domain nature of these antibodies provides for easy combination of an antigen-recognizing determinant with other functional modules, for instance, with epitopes for purification or detection of recombinant antibody, providing interaction with another protein, etc. by creating chimeric molecules or incorporating sequences responsible for the formation of protein–protein complexes. Finally, antigen-recognizing fragments of noncanonical antibodies called nanoantibodies (single-domain antibodies, nanobodies), because of their small size, are characterized by high stability and solubility. Prokaryotic expression systems can be used for the production of recombinant nanoantibodies, which significantly reduces the price of their production compared with the production of classical antibodies for which eukaryotic expression systems are commonly used. All this provides wide perspectives for using nanoantibodies as an alternative to classical antibodies in different practical applications [2-5].

Neoangiogenesis is a therapeutic target in broad spectrum of oncological diseases as well as during neovas-

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt; ELISA, enzyme-linked immunoassay; PBS, phosphate buffered saline; PCR, polymerase chain reaction; TMB, 3,3′,5,5′-tetramethylbenzidine; VEGF-A_{165}, vascular endothelial growth factor A_{165}.

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cular (wet) form of age-related macular degeneration [6, 7]. One of the ways used for its suppression is blocking the activity of vascular endothelial growth factor A_{165}, VEGF-A_{165}, implemented using humanized monoclonal mouse antibodies (bevacizumab, brand name Avastin) or its Fab-fragment (ranibizumab, brand name Lucentis). Furthermore, the level of VEGF-A_{165} in blood was shown to have diagnostic and prognostic significance.

Considering the advantages of nanoantibodies, the derivation of VEGF-A_{165}-recognizing nanoantibody molecules is a problem of high interest. Its solution will provide not only for developing an instrument for VEGF-A_{165} monitoring in biological solutions but also for better ways of antiangiogenic therapy, for example, by providing tumor-specific localization of nanoantibody blocking VEGF-A_{165} activity by combining it with specific peptide motifs or another nanoantibody with corresponding specificity [8].

In this work, nanoantibodies against human VEGF-A_{165} were produced for the first time, and their possible application for VEGF-A_{165} detection in biological samples using enzyme-linked immunoassay (ELISA) was demonstrated. Moreover, we have shown the ability of nanoantibodies to block biological activity of VEGF-A_{165}. This provides a basis for the development of a new class of antiangiogenic agents based on the nanoantibody platform with improved or absolutely new properties compared to existing drugs.

MATERIALS AND METHODS

Immunization with VEGF-A_{165} protein. Cells of CHO line were transfected using Unifectin-56 agent (Rusbiolink, Russia) with a transfection plasmid containing VEGF-A_{165} cDNA under control of the CMV promotor. To obtain an expression plasmid, the fragment of human VEGF-A_{165} cDNA was amplified in polymerase chain reaction (PCR) associated with reverse transcription on total RNA matrix from human placenta. The amplified fragment of human VEGF-A_{165} cDNA containing the whole reading frame, Kozak sequence, and termination codon was cloned into plasmid pEGFP-N2 (Clontech, USA) instead of cDNA of green fluorescent protein (the details of cloning are available on request). In 24 h after transfection, the cells were washed with phosphate buffered saline (PBS) and incubated in serum-free medium for 3 days to obtain conditioned medium containing human VEGF-A_{165} recombinant protein. The conditioned medium was collected, filtered through a PES-filter with 0.2 μm pores (Corning-Costar, USA), and concentrated using an Immersible CX-10 cartridge with nominal pore size 10 kDa (Millipore, USA). The amount of VEGF-A_{165} recombinant protein was measured by color intensity of the corresponding protein band after separation of an aliquot of the medium in SDS-PAGE followed by Coomassie staining.

Conditioned medium containing about 300 μg of VEGF-A_{165} protein in final volume 7.5 ml diluted in PBS was used for each immunization of a Bactrian camel. The first immunization was performed with complete Freund's adjuvant in 1 : 1 ratio. The second immunization was performed with incomplete Freund's adjuvant (1 : 1) 1 month later. The three additional immunizations were performed each 2 weeks after the previous one. Blood (150 ml) was taken 5 days after the last immunization. Heparin (35 units/ml) and EDTA (2 mM) were added to the blood to prevent coagulation.

Separation of mononuclear cells. The collected blood was diluted 2-fold with PBS containing 1 mM EDTA. To separate mononuclear cells (lymphocytes and monocytes), 35 ml of diluted blood was layered on 15 ml Histopaque-1077 (Sigma, USA) and centrifuged for 20 min at 800*g*. Mononuclear cells were obtained from the interphase zone and then washed in PBS with 1 mM EDTA.

Amplification of cDNA fragments encoding variable antibody domains of noncanonical immunoglobulins. Total RNA separated from isolated mononuclear cells with TRIzol (Invitrogen, USA) was used for separating poly(A+) RNA using affinity chromatography on oligo(dT)-cellulose. One microgram of poly(A+) RNA was used as template in the reverse transcription reaction with reverse transcriptase M-MuLV (Fermentas, Lithuania) and oligo$(dT)_{15}$ as a primer for synthesis of the first cDNA chains according to the protocol of the enzyme producer. The products of reverse transcription were used as template in two-stage PCR, and then the products of amplification were cloned by NcoI (or PstI) and NotI sites into phagemid vector pHEN4 as described in [9, 10] with only slight modifications. For that purpose, 1 μl of reverse transcription products was used as the template for PCR, containing 50 : 1 DNA-polymerases Taq and Pfu (Silex, Russia), 20 pmol of primers 5′-gtcctggctgctcttctacaagg-3′ and 5′-ggtacgtgctgttgaactgttcc-3′ under following conditions: 95°C for 90 sec – 1 cycle; 95°C for 30 sec, 59°C for 120 sec, 72°C for 90 sec – 30 cycles; 72°C for 5 min. Amplification products 600-800-bp long corresponding to noncanonical antibodies separated from gel using QIAEX II kit (Qiagen, USA) were used as template in the analogous reaction with primers 5′-ccggccatggccctgcaggtgcagctggtggagtctgg-3′ and 5′-ggactagtgcggccgcttgaggagacggtgacctgggt-3′, which were internal for previously used ones and contained recognizing sequences for restriction endonucleases NcoI and PstI, and NotI, correspondently.

Preparation of library of variable domains of single-chain antibodies. The amplification products were cleaved by restriction endonucleases NcoI (or PstI) and NotI and cloned into phagemid vector pHEN4, with bacteriophage M13KO7 (New England Biolabs, USA) being used as helper phage for obtaining a phage library with surface

expression of variable domains of single-chain antibodies [9, 10].

Selection of nanoantibodies specifically recognizing VEGF-A_{165} protein. The antibodies were selected using the phage display method [9, 10] with recombinant protein VEGF-A_{165} produced in insect cells (R&D Systems, USA) used as antigen immobilized in wells of a MICROLON 600 96-well plate for enzyme-linked immunoassay (Greiner, USA). The selection and following amplification of selected phage particles was subsequently performed three times. The sequences of cDNA fragments were grouped according to their fingerprint similarity determined using electrophoretic separation of hydrolysis products of amplified sequences of single-domain antibodies using three frequent-cleaving endonucleases (HinfI, MspI, RsaI) (HMR analysis) [11]. One representative from each group was used for subsequent analysis.

Production of recombinant nanoantibodies. The selected cDNA fragments from pHEN4 vector were subcloned into plasmid vector pHEN6 [12] thus linking HA- and 6xHis-epitopes to the C-terminus of the produced protein. Due to the presence of signal peptide (pelB) at the N-terminus of the expressed sequence, the produced recombinant protein (nanoantibody) was accumulated in periplasm of bacteria allowing for its effective separation using osmotic shock without damaging the bacterial cells. Nanoantibodies were expressed in *E. coli* cells strain BL21. The expression was induced by the addition of 1 mM indolyl-β-D-galactopyranoside, and the cells were incubated at intense mixing for 7 h at 37°C or overnight at 29°C. The recombinant proteins were separated from periplasm extract by affinity chromatography on Ni-NTA-agarose using the QIAExpressionist purification system (Qiagen) according to the producer's recommendations. The purified nanoantibodies were dialyzed against PBS and stored at 4°C.

Biotinylation of recombinant proteins. Biotinamidocaproate N-hydroxysuccinimide ester (Sigma) was chosen for protein biotinylation. Labeling was performed according to the producer's protocol. After this reaction, the proteins were dialyzed against PBS. The introduction of biotin into the protein was monitored by changes in protein mobility in SDS-polyacrylamide gel and Western blotting using conjugate of streptavidin and horseradish peroxidase (Calbiochem, USA) for detection of biotinylated proteins.

Enzyme-linked immunoassay. The enzyme-linked immunoassay was performed according to a standard protocol. For the detection of occluded recombinant protein VEGF-A_{165} with nanoantibodies, we used anti-HA mouse antibodies (Sigma) and secondary antibodies against mouse immunoglobulins conjugated with horseradish peroxidase (Sigma) for nanoantibody recognition. Colorimetric detection was performed with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ammonium salt (ABTS) (Sigma). For sandwich-ELISA, solutions of recombinant protein VEGF-A_{165} of different concentrations were prepared in PBS containing 1% albumin from bovine serum. The conjugate of streptavidin with horseradish peroxidase (Calbiochem) and chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) were used for detection of biotinylated nanoantibodies.

Analysis of VEGF-A_{165}-dependent HUVEC cell proliferation. Human umbilical vein endothelial cells (HUVEC) were cultivated in EGM-2 medium (Lonza, Switzerland). Cells for the analysis were seeded in 96-well plate (2000 cells per well) in 50 μl of basal medium for endothelial cells EBM-2 with 2% fetal bovine serum (Lonza), and after 4 h we added 50 μl of same medium containing VEGF-A_{165} and nanoantibody (1.25 μg/well) or bevacizumab (0.6 μg/well) (Roche, Switzerland). The number of cells was determined after 96 h using CellTiter-Glo Luminescent Cell Viability Assay (Promega, USA). The number of cells proportional to luminescent signal was evaluated in relative luminescence units as mean ± S.D. for three independent wells.

RESULTS

Preparation of VEGF-A_{165}-recognizing nanoantibodies. For induction of immune response to VEGF-A_{165}, a Bactrian camel was immunized with recombinant protein VEGF-A_{165}, obtained in a eukaryotic expression system that provides for preservation of protein glycosylation, which is important when obtaining antibodies that recognize and block the activity of endogenous VEGF-A_{165}. Total RNA from peripheral blood B-lymphocytes of the immunized camel was used as the template in RT-PCR for selective amplification of the whole repertoire of variable regions of the camel antibodies consisting of the dimer of only one heavy chain. The amplification products were dissociated in agarose gel, and 600-800 bp PCR products corresponding to noncanonical antibodies were separated from the gel and cloned into phagemid vector pHEN4 to obtain the library of variable domains of noncanonical immunoglobulins that are expressed on the phage surface. From the resulting library using phage display, we selected phages expressing variable antibody domains able to recognize VEGF-A_{165} protein. After three selection rounds, individual clones of phage pools enriched by VEGF-A_{165}-recognizing variable antibody domains were analyzed for the variety of variable domain sequences. Clone sequences of selected nanoantibodies incorporated in pHEN4 plasmid were grouped according to fingerprint similarity, determined using electrophoretic separation of hydrolysis products of amplified sequences of single-domain nanoantibodies simultaneously by three frequent cleaving restriction endonucleases (HinfI, MspI, RsaI) (HMR analysis). As a result of the analysis based on the restriction endonuclease cleavage

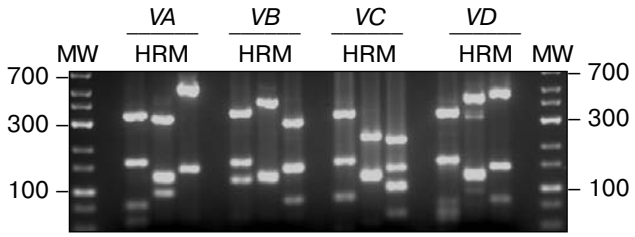

Fig. 1. HMR analysis of clones coding nanoantibodies VA (*VA*), VB (*VB*), VC (*VC*), and VD (*VD*). The results of separation of cleavage products of amplified sequences of single-domain antibodies with restriction endonucleases HinfI (*H*), MspI (*M*) or RsaI (*R*) in 4% agarose gel (NuSieve 3 : 1; Lonza) are shown. On the right and left, DNA marker (MW) fragment lengths in bp.

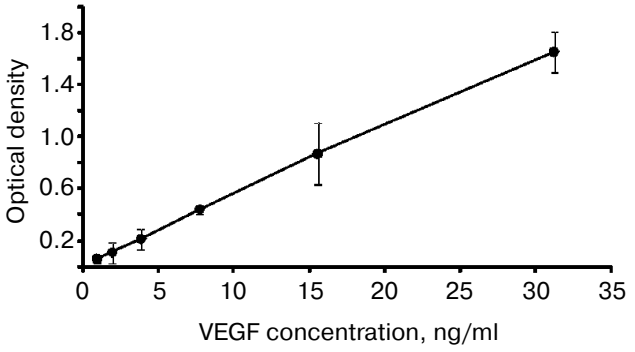

Fig. 2. Quantification of VEGF-A_{165} in ELISA using a pair of nanoantibodies VB and VC. Nanoantibody VC was sorbed overnight at 4°C (1 μg/well, 20 μg/ml). Blocking of nonspecific binding and incubation with VEGF-A_{165} protein at designated concentrations (100 μl/well) were followed by incubation with biotinylated nanoantibody VB (100 ng/well, 1 μg/ml). Detection was performed with streptavidin–horseradish peroxidase conjugate and chromogenic substrate TMB. The results of measurements of optical density of reaction products are presented as mean value for three wells ± S.D.

pattern, we selected four clones containing different cDNA of variable domains of single-chain antibodies specified VA, VB, VC, and VD (Fig. 1).

cDNA from the pHEN4 vector were cloned into modified pHEN6 vector to produce recombinant nanoantibodies containing HA- and $(His)_6$-epitopes at the C-terminus. Recombinant antibodies, expressed in *E. coli*, were purified using affinity chromatography, and their ability to interact with immobilized VEGF-A_{165} protein was studied using enzyme-linked immunoassay with HA-epitope for nanoantibody detection. For control we used immobilized nonspecific mouse immunoglobulins or the detection was performed without immobilization of the protein. All four resulting nanoantibodies were determined to be able to recognize VEGF-A_{165} protein, while the signal was absent in control wells (data not shown). This indicates the ability of nanoantibodies to bind with VEGF-A_{165} protein.

For further confirmation of the ability of the nanoantibodies to recognize VEGF-A_{165} and determination of their possible application, we studied the possibility of using the nanoantibodies for the detection of VEGF-A_{165} using ELISA and for blocking the biological activity of VEGF-A_{165}.

Nanoantibodies for VEGF-A_{165} detection using enzyme-linked immunoassay. One of the applications of antibody reagents is detection of antigen level in biological media. Therefore, we studied the possibility of using the antibodies for detection of VEGF-A_{165} in solution using sandwich-ELISA. Even though VEGF-A_{165} is a homodimer and the selection of nanoantibody couple able to simultaneously recognize different epitopes of the antigen is not critical, steric factors may have a significant influence on the ability of specific antibody to recognize antigen immobilized by binding to another antibody. The ability of different antibodies to detect VEGF-A_{165} bound to VC nanoantibodies was studied. Considering that for the analysis it is necessary to specifically detect nanoantibodies used for detection but not for occluding antigen, the detecting nanoantibodies were biotinylated, thus allowing the use of streptavidin conjugated with horseradish peroxidase for specific detection. The results revealed that only VB antibody, but not VA, VC, or VD, is able to detect VEGF-A_{165} immobilized by binding with VC protein (data not shown).

Quantitative parameters of VEGF-A_{165} detection during enzyme-linked immunoassay with the VB and VC antibody couple were studied. Figure 2 shows the results of the detection of different concentrations of VEGF-A_{165}. The specificity of detection under these conditions was about 1 ng/ml VEGF-A_{165}. Also, it reveals linear dependence of the studied parameter of optical density on the analyte concentration in the range of VEGF-A_{165} concentrations from 1 to 30 ng/ml ($r^2 = 0.9992$).

Therefore, the antibodies provide for quantitative detection of VEGF-A_{165} protein, which confirms their functional ability to recognize human VEGF-A_{165}. Moreover, these results demonstrate the possibility of using nanoantibodies against VEGF-A_{165} for a system for quantitative detection of VEGF-A_{165}.

Use of nanoantibodies for blocking biological activity of VEGF-A_{165}. Biological activity of VEGF-A_{165} is a therapeutic target in a number of oncological diseases as well as neovascular (wet) form of age-related macular degeneration. Drugs based on monoclonal antibodies whose binding with VEGF-A_{165} interferes with its interaction with the receptor are used to neutralize pro-angiogenic VEGF-A_{165} activity. We had to determine whether the nanoantibodies were able to block the biological activity of VEGF-A_{165}. To monitor VEGF-A_{165} biological activity, HUVEC cells, whose proliferative activity depends on VEGF-A_{165}, were used. In case of exerting neutralizing activity, nanoantibodies present in the culture medium would block the ability of VEGF-A_{165} to interact with cell

surface receptors and, therefore, lead to decrease in their proliferative activity. Bevacizumab antibodies that are used in clinics for neutralizing human VEGF-A_{165} activity were taken as a control. The addition of VA and VD nanoantibodies to the culture medium caused statistically significant decrease in HUVEC cell proliferation in the presence of 1 ng/ml VEGF-A_{165}. Similar results were shown upon addition of bevacizumab antibodies to the culture medium (Fig. 3a). However, VB and VC nanoantibodies had no influence on HUVEC cell proliferation in the presence of VEGF-A_{165} (data not shown). Though VA and VD nanoantibodies were able to inhibit HUVEC proliferation at VEGF-A_{165} at 1 ng/ml concentration in the culture medium, the concentration of VEGF-A_{165} 3 ng/ml or more did not have statistically significant effect on cell proliferation activity. Bevacizumab antibodies exerted VEGF-A_{165}-neutralizing activity up to VEGF-A_{165} concentration 100 ng/ml (Fig. 3b). The ability of nanoantibodies to inhibit HUVEC proliferation only at low (1 ng/ml) concentration, on one hand, reveals the limited ability of VA and VD to neutralize VEGF-A_{165} activity, which might be associated with their insufficient affinity to VEGF-A_{165}. On the other hand, the absence of influence of the nanoantibodies on HUVEC proliferation at VEGF-A_{165} concentration 3 ng/ml and higher points to the absence of nonspecific inhibition of proliferative activity by the used nanoantibody drugs. This indicates that the observed inhibiting effect of nanoantibodies on HUVEC proliferation activity in the presence of 1 ng/ml VEGF-A_{165} is due to their ability to specifically block the biological activity of VEGF-A_{165}.

DISCUSSION

Single-chain noncanonical immunoglobulins of the Camelidae family and some other species present a unique instrument for obtaining recombinant proteins with antibody properties. The antigen-recognizing determinant of these antibodies is formed by one polypeptide chain about 15 kDa long that, compared to commonly used classical immunoglobulins, allows for much easier preparation of recombinant proteins with antibody properties (nanoantibodies) and following manipulations with them aimed at raising their antigen affinity (for example, using random mutagenesis and *in vitro* evolution), obtaining multifunctional antibody reagents, etc. Moreover, due to significantly smaller size (15 against 150 kDa for immunoglobulins of G class) nanoantibodies are more stable, which is an advantage for their practical application.

Nanoantibodies are now actively studied for such applications as *in vivo* and *in vitro* diagnostics and synthesis of targeted drugs. Various molecules – from cell receptors and soluble extracellular proteins to toxins – were used as targets for nanoantibodies. In this study we describe for the first time the preparation of nanoantibodies recognizing human vascular endothelial growth factor A isoform 165.

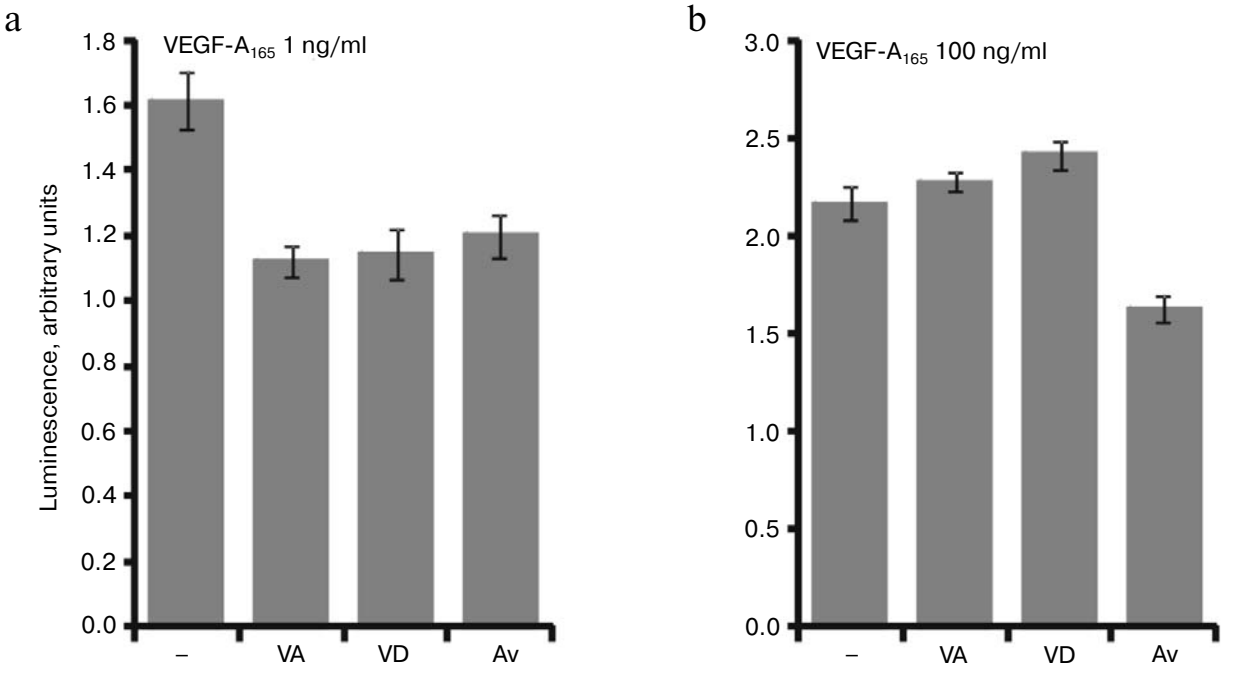

Fig. 3. Nanoantibodies VA and VD block biological activity of VEGF-A_{165} protein. The results of analysis of HUVEC cell proliferation at VEGF-A_{165} concentration in the medium 1 ng/ml (a) or 100 ng/ml (b) in the presence of the nanoantibodies (*VA* and *VD*) or antibody Bevacizumab (*Av*) and in the absence of antibody-containing reagents (–) are presented. The data are given in luminescence arbitrary units as mean of measurements from three wells ± S.D.

We showed that a pair of nanoantibodies suitable for quantification of VEGF-A_{165} by sandwich-ELISA could be selected from these antibodies. Thus, we demonstrated in principle that nanoantibodies could be used for quantitative determination of VEGF-A_{165} concentration, which provides a basis for construction of a simple (in the sense of preparing components) ELISA system for VEGF-A_{165}. Currently more attention is concentrated on blood serum VEGF-A_{165} content as a biomarker in different applications. Numerous studies established that VEGF-A_{165} level in blood, solely or in combination with other parameters, could be used as prognostic and diagnostic marker in such oncological diseases as leukemia [13], uterine cancer [14], intestinal cancer [15], non-squamous lung cancer [16], etc. Moreover, it was shown in a number of studies that VEGF-A_{165} level in blood is associated with effectiveness of antiangiogenic and other types of therapy for oncological diseases (e.g. for leukemias [13], glioblastomas [17], non-squamous lung cancer [18], head and neck cancer [19]) that provides a basis for use of this biomarker for early monitoring of drug effectiveness as well as for choosing the scheme of treatment. The VEGF-A_{165} level in blood serum can be used as biomarker in some other human pathologies, such as amyotrophic lateral sclerosis [20] and diabetic retinopathy [21]. All of this suggests that nanoantibody-containing reagents could be in high demand for VEGF-A_{165} quantification; in contrast to currently used classic monoclonal antibodies, they are easy to prepare; another merit is stability of characteristics of their producers (in contrast to hybridomas broadly used for production of classic immunoglobulins for diagnostic purposes).

The pair of nanoantibodies under study for VEGF-A_{165} quantification is characterized by good linearity parameters but has a sensitivity limit of about 1 ng/ml VEGF-A_{165}. Note that physiological VEGF-A_{165} level is within a few hundreds of pg/ml. Thus, the sensitivity of the detection system should be increased by an order of magnitude compared to the system demonstrated to detect physiological VEGF-A_{165} concentrations. This can be achieved by optimization of ELISA conditions including the use of more sensitive detection systems, selection of more suitable pairs of nanoantibodies, as well as increase in affinity of the nanoantibodies to antigen. The latter can be achieved by multimerization of nanoantibodies [22] or by maturation of nanoantibodies *in vitro* using random mutagenesis in antigen-binding region for modulation of their affinity characteristics [23].

The second field of possible application for reagents capable of VEGF-A_{165} binding is their use as medicinal drugs whose effect is based on blocking of VEGF-A_{165} biological activity. While having the abovementioned advantages compared to drugs of this type currently used in clinical practice based on monoclonal antibodies (bevacizumab) and their derivatives (ranizumab), chimeric soluble receptor (Aflibercept), and RNA aptomer (Pegaptanib), nanoantibody, in addition to its ability to bind to VEGF-A_{165}, must also be capable of blocking biological activity of VEGF-A_{165}. We showed that the two antibodies obtained in fact possess this critical property. Though qualitative characteristics of blocking of VEGF-A_{165} biological activity are insufficient for practical application of the nanoantibodies, the affinity of the nanoantibodies could be significantly increased using the abovementioned approaches. Thus, dimerization of nanoantibody against tumor necrosis factor α increased its potential for neutralization of tumor-target activity 500-fold [22]. Note that currently used in oncology antiangiogenic drugs have significant side effects due to their systemic influence on the human body [24]. One of the ways to avoid side effects (with proved efficiency) is to provide local action of a drug that could be achieved by introduction into the molecule of interest of determinants providing accumulation of the drug in tumor via interaction with tumor-specific proteins [8]. Due to unique characteristics of nanoantibodies, it is easy to attach such a determinant to the nanoantibody molecule (e.g. via preparation of heterodimers of nanoantibodies with different specificity) with minimal effect on the antigen-recognizing properties of the nanoantibody. Thus, the use of the nanoantibody platform is promising for invention of new drugs with decreased side effect complications frequently observed in systemic antiangiogenic therapy.

Thus, we were the first to prepare nanoantibodies against human vascular endothelial growth factor A isoform 165. Our experiments on application of these nanoantibodies for ELISA-based detection of VEGF-A_{165} suggest that this panel of nanoantibodies can be used for development of a diagnostic system for VEGF-A_{165} quantification, whose level has been increasingly considered as a biomarker in different pathologies. The demonstrated ability of the two nanoantibodies to neutralize VEGF-A_{165} biological activity suggests that these nanoantibodies could be basis for creation of a new drug to control pathological neoangiogenesis.

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