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Generation of a Mouse Monoclonal Antibody Recognizing Both the Native and Denatured Forms of Human VEGF

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Vascular endothelial growth factor (VEGF) and its related member, placental growth factor (PlGF), play important roles in stimulating vascular growth (angiogenesis) in both physiological conditions such as embryonic development and pathological conditions such as inflammation and tumor growth. Development of monoclonal antibodies (MAbs) capable of blocking the interaction of VEGF and its receptors, which in turn block VEGF-mediated angiogenesis, has become a novel and very powerful approach for cancer management. Here we report the generation of a mouse monoclonal antibody M23, which binds to both the natural and denatured forms of human VEGF165, as well as to two other major VEGF isoforms (VEGF121 and VEGF189) being tested. MAb M23 does not bind to other VEGF-related proteins such as PlGF. This MAb will have great future potential in VEGF-related research, diagnosis, and treatment.

Introduction

Vascular endothelial growth factor (VEGF)[1,2] and placental growth factor (PlGF)[3,4] are two well-known angiogenic factors that play important roles in stimulating vascular growth (angiogenesis), both in normal physiological conditions such as embryonic development and wound healing, and in pathological conditions such as tumor growth and inflammation.[4–6] Both VEGF and PlGF mediate their biological effects through high-affinity binding to their common receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR).[7–11] VEGF protein has at least six different forms generated by alternative exon splicing of a single VEGF gene,[2] while three of them, denoted as VEGF165, VEGF121, and VEGF189, are considered to be major forms due to high expression levels.[2,6,7] These three VEGF isoforms bind to VEGFR-1 and VEGFR-2, and they share the same N-terminus, which is the VEGF receptor binding domain. The importance of VEGF-related molecules and their receptors in angiogenesis has been clearly demonstrated in gene knock-out mice experiments, as mice embryos lacking even only one copy of VEGF gene (heterozygous) die at embryonic days 10–11,[12,13] and mice with homozygous knock-out in either VEGFR-1[14] or VEGFR-2 gene die before embryonic day 10.[15]

A large body of reports have demonstrated that VEGF and PlGF expressions are increased in a wide variety of tumors.[7,16,17] The increased levels of VEGF expression in tumors appear to be closely correlated with the aggressive nature of the cancer and poor prognosis of cancer patients.[18,19] Development of means or drugs capable of blocking VEGF and its receptor interaction, and which in turn block VEGF-mediated angiogenesis, has become a novel and a very powerful approach toward cancer management. Monoclonal antibodies (MAb), due to their high-specificity and high-affinity binding to their target antigens, have gained much attention in the fields of anti-angiogenesis and anti-tumor growth.

Bevacizumab (also called Avastin [Genentech, Fremont, CA]), a humanized version of mouse anti-human VEGF monoclonal antibody,[20–23] has obtained approval from the U.S. Food and Drug Administration Department for first line treatment of colon, lung, and breast cancers in combination with chemotherapy,[23,24] further demonstrating the importance of developing MAb-based agents in anti-angiogenesis and cancer treatment.

In this paper, we report that by fusing splenocytes from mice immunized with highly purified yeast-derived recombinant human VEGF165 protein with mouse myeloma cells, a panel of VEGF-binding MAbs were obtained. One of these MAbs, M23, was shown to be capable of binding to both the natural and denatured forms of human VEGF165, as well as VEGF121 and VEGF189, but not PlGF. This MAb may become a valuable tool in angiogenesis-related research, and it may also have future potential use in diagnosis and cancer treatment.

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Materials and Methods

Cell lines

Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), whereas mouse P3X63.Ag8.653 myeloma cells (obtained from the Institute of Biochemistry and Cell Biology, China Academy of Sciences, Shanghai) were cultured in RPMI-1640 supplemented with 10% FCS. All cell cultures were maintained at 37°C in 5% CO_2 under a humidified atmosphere in tissue culture incubators (Thermo Forma, Waltham, MA).

Expression and production of recombinant VEGF proteins (antigen preparation)

Recombinant human VEGF165 or PlGF proteins were produced by using yeast expression system (Invitrogen, Carlsbad, CA). The expression of recombinant protein in transformed *Pichia pastoris* cells was induced by 1% methanol.

The induced protein was then purified using an in-house method as described elsewhere (China patent pending). The quality and quantities of purified proteins were analyzed on SDS-PAGE gel, as well as by Western blot using rabbit anti-human VEGF polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Biotin labeling of VEGF165 protein and antibodies

Biotin labeling of VEGF165 and Bevacizumab (Avastin) were carried out using a sufto-NHs-LC-Biotein kit (Pierce, Rockford, IL). Briefly, about 1 mg purified VEGF165 or Avastin were mixed with 10−20 μL of 10 mM sufto-NHs-LC-Biotein (a molar ratio at 1:20) in a total volume of 0.5 mL at room temperature (RT) for 1–2 h. At the end of reaction, the labeled proteins were separated from free biotins by passing through de-salt columns. Avastin was obtained from a clinical treatment leftover sample.

Immunizations and generation of hybridomas

Female C57BL/6 mice (8 weeks old) were immunized with 10 μg purified human VEGF165 protein in complete Freund's adjuvant by subcutaneous (s.c.) injections. Mice were subsequently re-immunized with 10 μg VEGF protein in incomplete Freund's adjuvant by s.c. injections at 2-week intervals three times. Mice were bled from the caudal vein, and serum samples were tested for titers of antibody activity against VEGF by enzyme-linked immunosorbent assay (ELISA). Ten days after the third immunization, the mice with the highest anti-VEGF antibody titers were boosted once with 10 μg VEGF protein in incomplete Freund's adjuvant by intraperitoneal (i.p.) injection. Three days later, these mice were sacrificed, and splenocytes were isolated and fused with mouse P3X63.Ag8.653 myeloma cells in 50% PEG-1500 (Sigma, St. Louis, MO). Hybridomas were selected in RPMI-1640 medium with 10% FCS and 1× HAT (hypoxanthine-aminopterin-thymidine, Sigma). Supernatants from growing hybridomas were collected and tested for antibodies against VEGF using ELISA.

ELISA screening of antibodies

96-well flat-bottom plates were coated with yeast-derived VEGF165 (at 5 μg/mL in 0.1 M $NaHCO_3$ buffer [pH 9.6]) at 4°C overnight. After being blocked with 2% BSA in PBST buffer (1× PBS containing 0.05% Tween-20), the VEGF165-coated plates were incubated with hybridoma supernatants or mouse sera (2-fold serial diluted) at RT for 1 h. After being washed with PBST-buffer three times, plates were further incubated with horseradish peroxidase (HRP)-conjugated Affini-Pure goat anti-mouse IgG (H + L) antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at RT for 1 h. After being washed with PBST buffer three times, the substrate o-phenylenediamine dihydrochloride (OPD, with H_2O_2) was added to each well and allowed to incubate for 5–10 min. Then 1 M HCl stop solution was added to stop the reaction. The ELISA plates were then inserted into a MK3 Multiskan microplate reader (Thermo Scientific) and the optical density (OD) at 492 nm absorbance was measured and recorded.

Transient and stable transfection of CHO cells with plasmid DNA encoding human VEGF165, VEGF121, or VEGF189 proteins

Plasmid DNA encoding three major isoforms of human VEGF (VEGF165, 121, and 189) proteins[2] were constructed by inserting a cDNA corresponding to human VEGF165, VEGF121, and VEGF189 into mammalian expression vector pCDNA3.1 (Invitrogen). Plasmid DNA was introduced into CHO cells using the Fugene-6 mediated transfection method (Roche, Shanghai, China). Stable transfected cells were established by culturing cells in medium containing G418 (1 mg/mL) for 2–3 weeks.

Immunohistochemistry analysis of CHO transfectants

CHO cells transfected with either pCDNA3.1-VEGF cDNA or control DNA (empty vector) were seeded in 24-well plates at 1×10^5 cells per well. Cells were fixed with 2% paraformaldehyde (PARA) and permeabilized with 90% methanol. After being washed with 1× PBS once, cells were incubated with 200 μL of MAb supernatants at RT for 1 h. After three washes with 1× PBS, cells were further incubated with HRP-conjugated goat anti-mouse IgG antibodies (1:200 dilutions) at RT for 1 h. After four washes with 1× PBS, diaminobenidine (DAB) substrate (with H_2O_2) solution was added. The staining results were examined under inverted light microscope and photographed with a digital camera.

Dot-blot analysis of the binding of VEGF to immobilized antibodies

Hybridoma supernatants (10 μL of undiluted culture supernatants) were dropped onto the PVDF (polyvinylidine fluorides) membranes (Millipore, Billerica, MA). The membranes were then blocked with 5% milk in PBST buffer at 4°C overnight. After three washes with PBST buffer, the membranes were incubated with biotin-labeled VEGF165 protein (at 1:500 dilution) at RT for 2 h. After three washes with PBST buffer, the membranes were further incubated with diluted HRP-avidin (Vector Laboratories, Burlingame, CA) at RT for 1 h.

After four washes, the membranes were incubated with the substrate DAB (with H_2O_2) solution for 5–10 min.

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hV165   APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGC  60
        AP  EG  Q  HEV+KFMDVYQRSYC PIETLVDIFQEYPDEIEYIFKPSCVPLMRC GC
mV164   APTTEGE-QKSHEVIKFMDVYQRSYCRPIETLVDIFQEYPDEIEYIFKPSCVPLMRCAGC  59

hV165   CNDEGLECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQENPCGPC  120
        CNDE LECVPT ESNITMQIMRIKPHQ QHIGEMSFLQH++CECRPKKDR + EN C PC
mV164   CNDEALECVPTSESNITMQIMRIKPHQSQHIGEMSFLQHSRCECRPKKDRTKPENHCEPC  119

hV165   SERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRR  165
        SERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRR
mV164   SERRKHLFVQDPQTCKCSCKNTDSRCKARQLELNERTCRCDKPRR  164
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FIG. 1. Protein sequence comparison of human VEGF165 protein (hVEGF165) with mouse VEGF164 protein (mVEGF164). The different amino acid residues between these two proteins are highlighted. The blocked area represents a 44 amino acid long domain that is deleted in human VEGF121 or mouse VEGF120, respectively, due to alternative exon splicing. Two cysteines (at positions 51 and 60 in human VEGF165; and at positions 50 and 59 in mouse VEGF164) believed to form interchain disulfide bridge are underlined.

Western blot analysis

For Western blot analysis, insect cell−derived recombinant human VEGF165 protein was purchased from R & D Systems (Minneapolis, MN). VEGF proteins, either under DTT-denatured or non-denatured conditions, were separated on 15% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked with 5% milk in PBST buffer at 4°C overnight. After three washes with PBST buffer, the membranes were incubated with hybridoma supernatants at RT for 2 h. After three washes with PBST buffer, the membranes were further incubated with HRP-conjugated goat anti-mouse IgG (H + L) antibodies at RT for 1 h. After four washes, the membranes were incubated with substrate DAB (with H_2O_2) solution for 5–10 min, and the positive bands were visualized.

Competition ELISA assay

96-well plates were coated with purified VEGF165 protein (at 1 μg/mL) at 4°C. After blocking with 2% BSA in PBST buffer, plates were co-incubated with fixed amount of biotin-labeled Avastin (125 ng/mL) and a 2-fold serial diluted M23 MAb supernatant or supernatant from an irrelevant hybridoma. After being washed with PBST buffer three times, plates were further incubated with HRP-avidin solution at RT for 1 h. After being washed with PBST buffer three times, the substrate OPD (with H_2O_2) was added to each well. OD at 492 nm absorbance was measured and recorded.

Results and Discussion

Protein sequence comparison of human VEGF165 and its mouse homolog VEGF164

Protein sequence comparison of human VEGF165 and its mouse homolog VEGF164 revealed that these two proteins have 88% overall identity in their amino acid sequences. There are differences in the amino acid sequence (Fig. 1) in the N-terminus and in the middle region of the proteins. This difference in the amino acid sequence between human VEGF165 and mouse VEGF164 provides the theoretical basis for generating anti-human VEGF165 antibodies in mouse.

Generation of recombinant VEGF165 protein for immunization

To obtain a relatively pure preparation of proteins for immunization, we chose a *Pichia pastoris* yeast cell expression system for producing recombinant human VEGF165 protein. A PCR-amplified cDNA with a sequence encoding human VEGF165 was inserted into a yeast expression vector. The recombinant VEGF165 proteins were purified to homogeneity using an in-house method. The quality and quantity of the purified VEGF165 protein were examined by SDS-PAGE and Western blot using commercially available rabbit anti-human VEGF polyclonal antibodies (Fig. 2A).

As shown in Figure 2A, under non-reducing conditions (no DTT), purified recombinant VEGF165 protein migrated as a dimer with the expected molecular mass of about 43–44 kDa (Fig. 2A, lane 1). In the presence of DTT, this form of VEGF165 was largely separated into two subunits, each with a molecular mass of about 21–22 kDa (Fig. 2A, lane 2). Both dimer and monomer forms of VEGF protein can be specifically recognized by a commercially available rabbit anti-human VEGF antibody (Fig. 2A, lanes 3 and 4).

Generation of mouse anti-human VEGF monoclonal antibodies

For immunization, purified recombinant human VEGF165 protein in its non-denatured form was mixed with adjuvant and injected into C57BL/J6 mice by s.c. injections. Spleen cells from immunized mice were fused with P3X63 .Ag8.653 myeloma cells. After several fusions and ELISA screenings, we obtained a panel of more than 10 hybridomas that secreted MAbs capable of binding to human VEGF165 protein. One of these clones, M23, was found to have the highest titer against VEGF165 as demonstrated by ELISA (Fig. 2B). The relative binding affinity of this antibody for human VEGF165 protein was almost equal to that of Avastin (data not shown). Antibody isotyping showed that M23 MAb is of mouse IgG1 (kappa) type. This hybridoma was then subcloned twice by limiting dilution, and all subclones were confirmed to secrete antibody specifically against VEGF165.

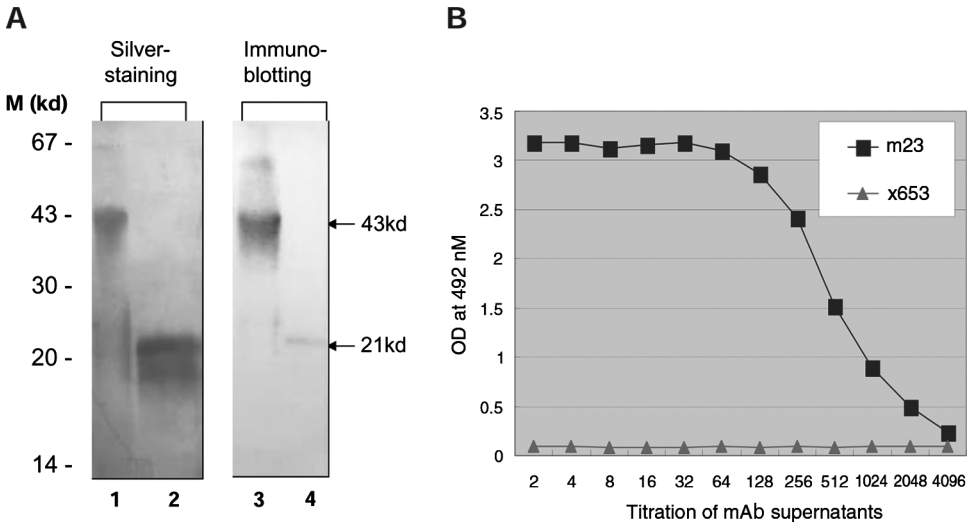

FIG. 2. Generation of mouse MAbs against human VEGF protein. (**A**) Silver staining and immunoblotting analysis of yeast-derived recombinant VEGF165 protein. Yeast-derived VEGF165 proteins were separated by 15% SDS-PAGE gel under either non-reducing (lanes 1 and 3) or reducing conditions (lanes 2 and 4). The proteins in the gel were either subjected to silver staining or transferred into PVDF membrane for immunoblot analysis using commercially available rabbit anti-human VEGF polyclonal antibodies. Positions and sizes of the dimer and monomer forms of VEGF165 protein were marked. (**B**) Identification of MAbs against human VEGF165 by ELISA assays. VEGF165-coated plates were incubated with 2-fold serial dilutions of hybridoma supernatants. The bound mouse antibodies were detected with HRP-conjugated goat anti-mouse IgG (H + L) antibodies. M23, supernatants from M23 hybridoma; X653, supernatants from mouse P3X63.Ag8.653 myeloma.

Determination of immunoreactivities of M23 MAb against VEGF165 protein

To determine whether M23 MAb can capture soluble human VEGF protein, a dot-blot assay was performed. As shown in Figure 3A, dot-blots immobilized with M23 MAb supernatant (dots 1 and 2, duplicated samples) showed capturing and binding of VEGF165 protein. The specificity of the dot-blotting results was demonstrated by the complete absence of binding in dot-blots immobilized with a control, unrelated MAb 1E4 supernatant (dots 3 and 4, duplicated samples). To further verify the immunoreactivity of M23 MAb against VEGF165 protein, recombinant human VEGF165 protein was purchased from R & D System. The protein was then separated in SDS-PAGE under either denatured or non-denatured conditions, and used for immunoblot analysis (Fig. 3B). As shown in Figure 3B, M23 MAb can recognize both the non-denatured (lane 1) and the DTT-denatured (lane 2) human VEGF165 protein. This immunoblot pattern was very similar to that when Avastin was used (Fig. 3B, lanes 3 and 4). Thus, the immunoblot results clearly demonstrated that, like Avastin, M23 MAb can recognize both the native and denatured forms of human VEGF protein.

Immunohistochemistry analysis of CHO cells transfected with human VEGF cDNA

To further confirm the specificity of M23 MAb, supernatants collected from M23 hybridoma and from P3X63.Ag8.653 myeloma cells were used as primary antibodies for immunohistochemistry (IHC) staining of CHO cells transfected with plasmid DNA encoding various isoforms of human VEGF proteins. As shown in Figure 4, incubating of CHO cells transfected of VEGF165 cDNA with MAb M23 supernatants (Fig. 4A) or Avastin (Fig. 4B) showed positive staining, while incubating the same transfectants with P3X63.Ag8.653 myeloma supernatant showed no staining (Fig. 4C).

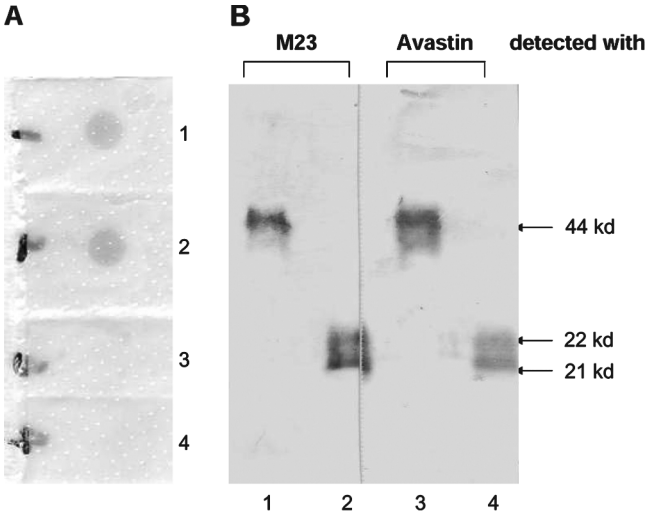

FIG. 3. M23 MAb binds to both the native and denatured forms of VEGF. (**A**) Immunodot-blot analysis of the binding of biotin-labeled VEGF165 protein. M23 MAb supernatants (dots 1 and 2, duplicated) or irrelevant control MAb 1E4 supernatants (dots 3 and 4, duplicated) were immobilized on PVDF membranes; the membranes were then incubated with biotin-labeled VEGF165, followed by avidin-HRP and DAB substrate. (**B**) Western blot analysis of VEGF165 protein. 10 μL of recombinant VEGF165 protein (suspended at 50 μg/mL in 1x PBS containing 1% BSA) were separated by 15% SDS-PAGE in either non-denatured (lanes 1 and 3) or DTT-denatured (lanes 2 and 4) conditions and transferred to PVDF membranes. The membranes were incubated with M23 MAb followed by HRP-conjugated goat anti-mouse IgG (H + L) antibodies (lanes 1 and 2) or with Avastin followed by HRP-conjugated goat anti-human IgG-Fc antibodies (lanes 3 and 4). Positions and relative molecular weights of both the dimer and monomer forms of VEGF165 proteins were marked.

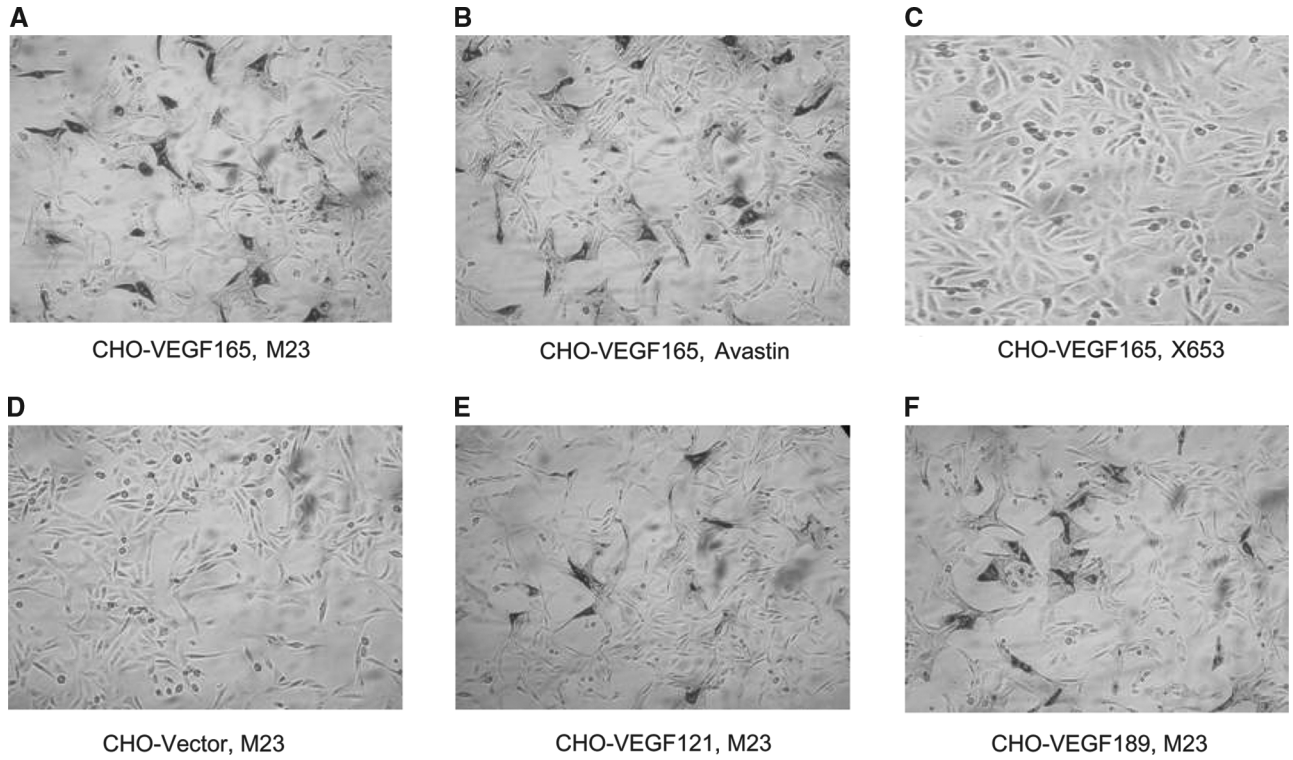

FIG. 4. M23 MAb binds to different isoforms of human VEGF. CHO cells transiently transfected with plasmid with cDNA encoding either VEGF165, 121, or 189 isoform or control vector DNA were incubated with either M23 MAb supernatants (M23) or P3X63.Ag8.653 myeloma supernatants (X653), followed by HRP-conjugated goat anti-mouse IgG antibodies. The bound antibodies were detected with addition of DAB substrate.

As expected, incubating CHO cells transfected of control vector DNA with either M23 MAb (Fig. 4D) or Avastin (data not shown) did not produce any positively stained cells. Interestingly, positive staining was also observed when M23 MAb was added to CHO cells transfected with either VEGF121 cDNA (Fig. 4E) or VEGF189 cDNA (Fig. 4F). Thus, the IHC staining results demonstrated that M23 MAb recognizes not only VEGF165, but also two other major isoforms of VEGF, namely VEGF121 and VEGF189.

M23 MAb competes with Avastin for binding to human VEGF

To examine if M23 MAb competes with Avastin for binding to human VEGF, a competition ELISA assay was carried out. For this purpose, a biotin-labeled Avastin was prepared and the optimal concentrations of biotin-labeled Avastin used for VEGF binding assays were determined (Fig. 5A). As shown in Figure 5A, when VEGF-coating plates were incubated with 2-fold serial-diluted biotin Avastin (starting at 1:1000 dilution or at a concentration of 1 μg/mL), a clear titration curve was observed (with a straight linear curve falling into a range between 1:2000 and 1:16,000 dilution). Based on this experiment's results, a 1:8000 dilution (or a concentration of 0.125 μg/mL) of biotin-labeled Avastin was chosen as the fixed amount to be used for subsequent competition ELISA assays (Fig. 5B). As shown in Figure 5B, when VEGF-coated plates were co-incubated with biotin-labeled Avastin plus 2-fold serial diluted M23 supernatants (labeled 23&Biotin-avastin), a significant dose-dependent inhibition on the binding activity of biotin-Avastin to VEGF was observed, while only a very mild competition was observed when a control unrelated MAb 1E4 supernatant was used (labeled 1E4&Biotin-avastin). Thus, these results demonstrated that M23 MAb competes specifically with Avastin for binding to human VEGF165 protein, and the two antibodies may share the same binding epitopes.

M23 MAb binds to VEGF but not PlGF

Having established that M23 MAb binds to multiple isoforms of human VEGF, we then examined whether M23 MAb can also recognize other VEGF-related proteins such as PlGF. It is well-known that VEGF and PlGF proteins share several common features: they have more than 50% identity in their amino acid sequences,[3,4] they have very similar 3-D structures,[25,26] and they both bind to the same receptor VEGFR1, or Flt-1.[4,7] To determine if M23 MAb also binds to PlGF protein, ELISA assays were performed (Fig. 6). As can be seen in Figure 6, M23 MAb can bind to VEGF-coated plates, but not to PlGF-coated plates. The absence of M23 MAb binding to PlGF protein in these assays is not due to the lack of functional antigens in sample preparations, because positive immunoreaction could be seen by adding VEGFR1-Ig (a recombinant chimeric immunoglobulin fusion protein with the extracellular domain of human VEGFR-1 joining with a human Ig-Fc region) into the same PlGF protein-coated plates (data not shown).

Thus, based on these ELISA assay results, we have concluded that M23 MAb recognizes human VEGF but not human PlGF protein.

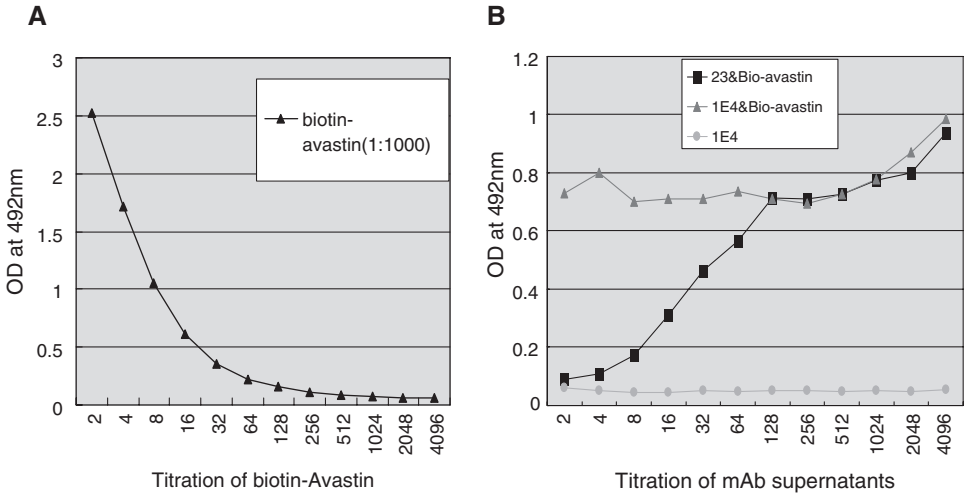

FIG. 5. M23 MAb competes with Avastin for binding to human VEGF165 protein. (**A**) Determination of optimal concentrations of biotin-labeled Avastin to be used for VEGF165 binding assay. VEGF165 protein (1 μg/mL) coated plates were incubated with 2-fold serial dilution of biotin-labeled Avastin (starting at 1:1000 dilution or at 1 μg/mL). The amount of bound biotin-labeled Avastin was detected with HRP-avidin. (**B**) M23 MAb but not control MAb competes with Avastin for binding to human VEGF165. The VEGF165-coated plates were either co-incubated with a fixed amount of biotin-labeled Avastin plus a 2-fold serial dilution of M23 MAb supernatant (23&Bio-avastin) or co-incubated with same fixed amount of biotin-labeled Avastin plus a 2-fold serial dilution of supernatant from an irrelevant control hybridoma 1E4 (1E4&Bio-avastin). The amount of bound biotin-Avastin in these wells was detected with avidin-HRP. As a negative control, some rows of the same VEGF-coated plates were also incubated with 1E4 MAb supernatant without addition of biotin-Avastin, and results from those wells were also displayed (1E4).

Conclusion

By fusing splenocytes from mice immunized with highly purified yeast- derived recombinant human VEGF165 protein with mouse myeloma cells, a panel of VEGF-binding MAbs were obtained. One of these MAbs, M23, has the highest binding affinity to VEGF165. In addition to recognizing both denatured and natural forms of VEGF165, this antibody can also recognize two other major VEGF isoforms (VEGF121 and VEGF189), but not PlGF. The M23 MAb reported here will have great future potential in VEGF-related research, diagnosis, and cancer treatment.

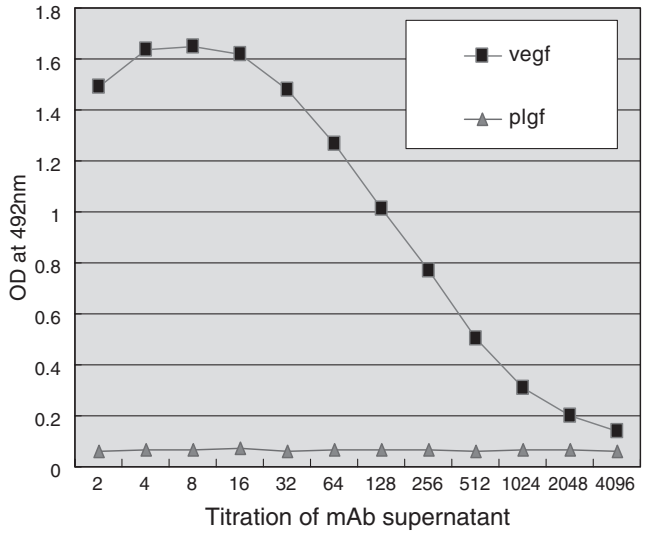

FIG. 6. M23 MAb binds to VEGF165 but not PlGF. VEGF165 or PlGF (both at 5 μg/mL) coated 96-well plates were incubated with 2-fold serial dilution of M23 MAb. The bound antibodies were detected with HRP-conjugated goat anti-mouse IgG antibodies.

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