Inhibition of Tumor Angiogenesis by a Single-chain Antibody Directed against Vascular Endothelial Growth Factor

Alessandra Vitaliti, Mariana Wittmer, Rudolf Steiner, Lorenza Wyder, Dario Neri, and Roman Klemenz

Division of Cancer Research, Department of Pathology, University Hospital, CH-8091 Zurich [A., V., M. W., R. W., R. K.]; Central Biological Laboratory, University Hospital, CH-8091 Zurich [R. S.]; and Department of Applied Biosciences, ETH Zurich, CH-8092 Zurich [D. N.], Switzerland

Abstract

Monoclonal antibody (Ab) directed against the vascular endothelial growth factor, one of the major inducers of angiogenesis, can inhibit tumor growth in mice. Treatment of cancer patients with monoclonal Ab requires large-scale production of the clean Ab and frequent application of the Ab. This might be improved by using single-chain Ab fragments (scFvs), which can be produced in large quantities in bacteria and are attractive for gene therapeutic approaches. Here we describe anti-vascular endothelial growth factor scFvs derived from a human phage-display library able to block the vascularization of the choriodal membrane of chick embryos and reduce the growth of s.c. tumors in nude mice. This work opens the way to develop gene therapy-based strategies using a scFv to treat angiogenesis-dependent diseases.

Introduction

Angiogenesis plays a fundamental role in the expansion of solid tumors beyond a minimal size of a few cubic millimeters and in metastasis formation (1). This process is mediated by different angiogenic factors, which are released by tumor or stromal cells. VEGF, a 42,000 homodimeric glycoprotein, has been identified as the major angiogenic factor stimulating endothelial cell proliferation and migration (2). In contrast to other angiogenic growth factors, VEGF acts almost exclusively on endothelial cells, where its high-affinity tyrosine kinase receptors KDR/Fk-1 and Flt-1 reside (3, 4). VEGF, besides being an angiogenic factor, is also a survival factor for newly formed blood vessels (5) and is a highly potent direct mediator of microvascular permeability (6), a process that is essential for vessel sprouting. An important role for VEGF as a mediator of tumor angiogenesis is suggested by the observation that VEGF is abundantly expressed and secreted by several tumors (7). The multifunctional role of VEGF in promoting tumor angiogenesis and metastases renders VEGF unique, and not all of its functions may be substituted for by other angiogenic factors. Therefore, VEGF is an attractive target for antiangiogenic therapeutic intervention. Several strategies to block VEGF signaling and expression in tumor-bearing animals have resulted in an impressive reduction or even inhibition of tumor growth. One of the most promising approaches to inhibit VEGF activity is the use of anti-VEGF antibodies (8, 9). The major limitations for successful clinical application of murine mAbs have been the development of an antiglobulin immune response and poor diffusion of mAbs from the vasculature into the tumor (10). In addition, production of the high amounts of mAb necessary for the treatment of human patients is technically very demanding and expensive. To circumvent the problem of the immunogenicity of the mouse Ab in human therapy, Presta et al. (8) have humanized an anti-VEGF Ab and shown that it inhibits tumor growth as efficiently as the original murine Ab. However, the other problems associated with mAbs are still unsolved.

Recent progress in phage-Ab display technology has improved the ability to select for human-derived scFvs that have desirable properties for immunotherapy. The use of scFv rather than mAb to inhibit VEGF offers several advantages. The Ab is much smaller in size and is therefore more likely to penetrate into the tumor mass. Modification of the Ab, such as fusion with specific stabilizing or toxic protein sequences, can be performed easily by gene technology methods. Moreover, gene therapeutic studies are more accessible because in vivo production of a scFv is much more efficient than the synthesis and correct assembly of a heteromeric mAb.

In this study, we describe the selection of anti-VEGF scFvs from a human-derived semisynthetic phage-display library. Two of these antibodies showed antiangiogenic activity in the chick CAM assay, and one of them inhibited the growth of a s.c. tumor derived from H-ras-transformed rat fibroblasts in mice. This Ab may be developed into a useful tool for antiangiogenic gene therapy to treat solid tumors and other angiogenesis-dependent diseases in humans.

Materials and Methods

Production of the mVEGF<sub>164</sub>-His<sub>6</sub> Recombinant Protein. Full-length mVEGF<sub>164</sub> was synthesized by PCR using plasmid pVEGF<sub>1</sub> received from G. Breier (Max Plank Institute, Bad Nauheim, Germany), as a template. The upper primer (5′-GTCGCCCTTATTGGCCATTGAAGGTCGTGGACACAGAAA-3′) introduced a SfiI cloning site (underlined) and a Factor Xa cleavage site (italic). The lower primer, (5′-GACGCCGGCGCTACGAGGGATTAT-3′), contains a Norf restriction site (underlined) and a stop codon (italic) for the termination of translation. Thirty PCR cycles consisting of a denaturing step at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min were performed. The resulting fragment was cloned into the modified expression vector pDSS6/RBSII 6xHis. Purification of mVEGF<sub>164</sub>-His<sub>6</sub> on a Ni<sup>2+</sup>-NTA agarose column (Qiagen, Hilden, Germany) was performed as described previously (11).

Selection of Anti-VEGF scFvs. The selection of scFvs from the Nissim library (12) was performed as described previously (13). Soluble scFvs produced from single colonies (see “Production of Soluble scFvs”) obtained after selection were screened by ELISA (see “ELISA”) for binding to recombinant mVEGF<sub>164</sub>-His<sub>6</sub>.

Production of Soluble scFvs. Single bacterial colonies were grown at 37°C in 2× TY medium containing 100 μM ampicillin and 0.1% glucose. When the suspension reached an A<sub>600</sub> of 0.9, the production of soluble scFv was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM, and the bacteria were incubated overnight at 30°C. ELISA. Immunoplates (Maxisorb, Nunc, Live Technology) were coated overnight at 4°C with the following antigens: (a) 10 μg/ml mVEGF<sub>164</sub>-His<sub>6</sub>; (b) 10 mg/ml lysozyme; (c) 2× TY and 4% milk powder; and (d) 10 μg/ml recombinant endoglin. Nonspecific binding was blocked with PBS and 2%
milk powder for 2 h. Bacterial supernatants containing soluble scFvs were diluted 1:1 with PBS and 4% milk powder. After 2 h, the wells were incubated with an Ab against the tag peptide (anti-c-myc Ab 9E10; 1 μg/well in PBS/2% milk powder; American Type Culture Collection, Rockville, MD) or anti-flag (M2; 1 μg/well in PBS and 2% milk powder; Kodak, New Haven, CT) for 1 h and then incubated with horseradish peroxidase-conjugated antimouse mAb (diluted 1:4000 in PBS and 2% milk powder; Southern Biotechnology, Birmingham, AL) for an additional hour. The ELISA was developed using 1,2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate) as the substrate (ABTS; Boehringer Mannheim, Mannheim, Germany), and the A Latina nm was measured.

Purification of scFvs. The cDNA encoding the selected scFv was subcloned into the Neo/Nor restriction sites of the pDN268 expression plasmid (14), which encodes a flag tag and a His6 tag at the 3' side of the cloning sites. The scFvs present in the supernatant were precipitated with 80% ammonium sulfate; resuspended in 50 mM NaH2PO4 (pH 7.5), 500 mM NaCl, and 20 mM imidazole; and dialyzed against the same buffer. The scFvs were purified over a Ni2+-NTA agarose column (Qiagen) and eluted with 50 mM NaH2PO4 (pH 7.5), 500 mM NaCl, 100 mM imidazole, and 20 mM EDTA (pH 8).

RNA Preparation and Northern Blot Analysis. Total RNA was isolated with the RNeasy kit (Qiagen), denatured with glyoxal, fractionated on 1% agarose gels, transferred onto a nylon membrane (Genescreen Plus; New England Nuclear Life Science Products, Boston, MA), prehybridized, and hybridized to a VEGF cDNA probe labeled by random oligomeric primer extension (15).

CAM Assay. Fertilized chicken eggs were kept at 37°C with 70% relative humidity in an egg breeder. On incubation day 3.5, the embryos were poured into plastic cups and incubated at 37°C with 95% relative humidity. At incubation day 8.5–9, sterile methylcellulose discs were applied to the CAM and loaded with 1–4 μg of scFv or with an irrelevant scFv (NL) as control. After 24 and 48 h, the angiogenic response was evaluated semiquantitatively under a stereomicroscope and recorded photographically.

Cell Culture. NIH3T3 and 208F cells were purchased from American Type Culture Collection. FE-8 cells were obtained from R. Schafer (Humboldt University, Berlin, Germany). All cell lines were grown in DMEM containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Animal Experiments. FE-8 cells (1 × 106) were injected into each flank of 6-week-old CD1 nude mice (Charles River, WIGA, Sulzfeld, Germany). Starting 1 day after tumor cell injection, the animals received a daily i.p. injection of anti-VEGF scFv V65, irrelevant scFv NL, or PBS. V65 was injected at 15 or 50 mg/kg, and NL was injected at 50 mg/kg. Ten days after tumor cell injection, the animals were sacrificed, and the tumors removed and weighted.

Results

For the selection of specific antibodies against mVEGF164, a human scFv phage-display library was used. Recombinant mVEGF164 containing a His6 tag at the COOH terminus was produced in Escherichia coli, purified over a Ni2+-NTA agarose column, and used as antigen for the isolation of anti-VEGF scFvs. After three rounds of panning with this protein, single colonies of bacteria, which were infected with the phages, were induced to produce and secrete scFv fragments. The binding of scFvs to mVEGF164 was monitored by ELISA. Several scFvs were identified that bound to mVEGF164, but not to different control proteins, including recombinant endoglin, which contains the same His6 tag as recombinant VEGF, lysozyme, bacterial culture medium, and milk proteins (Fig. 1). Sequence analysis of the cDNAs encoding the three scFvs revealed that each of them encoded a different Ab. These scFvs were identified that bound to mVEGF164, but not to different control proteins, including recombinant endoglin, which contains the same His6 tag as recombinant VEGF, lysozyme, bacterial culture medium, and milk proteins (Fig. 1). Sequence analysis of the cDNAs encoding the three scFvs revealed that each of them encoded a different Ab. These three scFvs were subcloned into the expression vector pDN268, which allowed the synthesis and secretion of flag- and His6-tagged antibodies. These were purified from the bacterial supernatant over a Ni2+-agarose column. To test whether the selected scFvs exhibit neutralizing activity against VEGF and, consequently, can block angiogenesis in vivo, they were applied to methylcellulose discs on the CAM of 8.5–9-day-old chick embryos ex ovo. Endogenous neovascularization of the CAM is maximal at this developmental stage. The effect of the Abs on blood vessel formation was monitored at different time points by microscopic inspection. Two of these scFvs, V14 and V65, showed a strong and reproducible antiangiogenic effect in the CAM assay (Fig. 2B; data not shown). No blood vessels penetrated into the region where the Ab was applied; instead, sprouting vessels turned around when they grew near the area where the Ab was applied, forming the arc-like structures indicated by arrows in Fig. 2B. The maximal inhibitory effect was observed with 4 μg of V65 scFv after 24 h. The established vessels, which had formed before the application of the Ab, were not affected. In contrast, a third anti-VEGF scFv, V80, and an irrelevant scFv (NL) isolated from the same phage library did not affect the normal vascularization process of the CAM (Fig. 2A; data not shown). This result indicates that V65 and V14 are able to recognize chick VEGF and neutralize its angiogenic activity. Next we tested whether V65 could affect tumor growth in mice. To this end, purified V65 was injected into nude mice bearing FE-8 tumors. FE-8 cells are tumorigenic H-ras-transformed rat fibroblasts (16). ras oncogene expression has previously been shown to induce the VEGF gene. Using Northern blot analysis, we verified that FE-8 cells do indeed express VEGF mRNA. A strong signal corresponding to VEGF mRNA was observed for FE-8 cells, whereas a much weaker signal was found for the parental cell line 208F (Fig. 3). The expression of VEGF by these tumor cells makes them a good model system to test the antiangiogenic effect of anti-VEGF scFvs. The s.c. injection of FE-8 cells into the flanks of nude mice results in the formation of well-vascularized tumors of reproducible size within 10 days. Daily i.p. injection of 50 mg/kg V65, beginning 1 day after the implantation of FE-8 cells, inhibited tumor growth by a factor of 1.8–2.7 (Fig. 4, A and B). The injection of the irrelevant scFv NL did not affect tumor

Fig. 1. Specificity of the anti-VEGF scFvs. The binding specificity of purified anti-VEGF scFvs was tested in ELISA using recombinant mVEGF164, His6 (mVEGF), recombinant endoglin (rEnd.), lysozyme (Lys.), bacterial culture medium (2×TY), and milk as antigens.
A weaker inhibitory effect was observed in response to only 15 mg/kg V65 (Fig. 4B). The antiangiogenic effect of V65 that was observed in the CAM assay and the ability of this Ab to reduce the growth of s.c. tumors support the concept that it is able to inhibit neovascularization of tumors.

Discussion

In this work, we demonstrate that a scFv selected from a human-derived phage-display library has an antiangiogenic effect in the CAM assay and is able to partially inhibit the growth of s.c. tumors derived from H-ras-transformed fibroblasts. Several approaches are being considered to improve this effect, as outlined in the following paragraphs.

Most scFvs selected from phage-display libraries have a moderate binding affinity to their antigen in the range of $10^6$–$10^7$ M$^{-1}$ (12). Viti et al. (17) showed that a high-affinity Ab against the ED-B domain of fibronectin, which is present exclusively in the extracellular matrix around tumor blood vessels, targets the tumor vasculature significantly better than a low-affinity Ab against the same epitope. Therefore, we are considering increasing the affinity of V65 to VEGF by introducing random mutation in the CDR3 region of the variable light chain domain, which is constant for all scFvs selected from the Nissim phage-display library.

The small size of scFvs enables good tumor penetration and fast blood clearance, improving tumor:normal organ uptake compared with whole antibodies. However, rapid blood clearance, which is common to such small molecules, with a half-life shorter than 30 min for the $\alpha$ phase of the blood clearance profile (17, 18) results in low quantitative tumor retention. It was shown that several anticarcinoma scFvs derived from mAbs could indeed be targeted to tumors. However, accumulation in the tumor was low, presumably due to the brief...
presence in the circulation of these small proteins (18, 19). One way to increase the half-life of the scFvs in the blood is to generate a minibody consisting of the human IgG CH3 domain fused with anti-VEGF scFv V65. Because the interaction between CH3 domains promotes and stabilizes dimerization, minibodies are assembled into a homodimeric protein of intermediate molecular weight (20).

VEGF is permanently secreted by tumors, and an effective inhibition of VEGF binding to its receptors on endothelial cells requires a sufficiently high Ab concentration around blood vessels to sequester most or all of this growth factor. An attractive way to achieve this goal is the sustained in vivo production of an anti-VEGF scFv. Gene therapeutic strategies are currently being tested using the V65 Ab. This approach should circumvent the problem of large-scale Ab production and the frequent injection of patients with this Ab.

In conclusion, we showed that a scFv directed against VEGF inhibited the growth of tumors in nude mice. With the introduction of further modifications that increase its stability in the blood and its affinity to VEGF, this scFv opens the way for a gene therapeutic treatment of solid tumors.

References

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