SPANDIDOS PUBLICATIONS

Evaluation of anti-VEGFR-3 specific scFv antibodies as potential therapeutic and diagnostic tools for tumor lymph-angiogenesis

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Abstract. Vascular endothelial growth factor receptor-3 (VEGFR-3) plays a major role in lymph-angiogenesis, tumor growth and metastatic tumor cell dissemination. The receptor is over-expressed on lymphatic vessels in the vicinity of tumors and on the tumor vasculature and therefore may be an excellent target for an effective cancer intervention. We generated and characterized single chain antibody fragments (scFv) recognizing VEGFR-3 by phage display technology and expression in *P. pastoris* and analyzed selected antibodies *in vitro* and *in vivo*. The scFvs were functionalized by the introduction of cysteines at the C-terminal end of the proteins. The scFvs are species cross-specific and bind to recombinant human and mouse VEGFR-3. ScFv AFC5 showed specific tumor accumulation in an hVEGFR-3 expressing F9 teratocarcinoma mouse model, which was also used for tumor visualization by combined single proton emission computed tomography (SPECT/CT) and immunohistochemical analysis. This antibody also inhibited binding of hVEGF-C to its receptor and reduced proliferation of human lymphatic endothelial cells. Thus, the generated VEGFR-3 specific scFv antibodies represent a valuable tool for novel cancer therapies and diagnostic applications.

Introduction

The role of tumor lymph-angiogenesis in metastatic tumor cell dissemination has been elucidated in recent studies which evaluated the molecular regulation of lymph-angiogenesis in tumor growth and invasion (1 and reviewed in 2). Lymphatic tumor spread to regional (sentinel) and distant lymph nodes represents an important pathway for early stage tumor dissemination. The lymphatic system consists of lymphatic capillary networks, which absorb tissue fluids and the collecting lymphatics, which drain the lymph. The lymphatic capillaries consist of single-layer, partially overlapping, thin-walled endothelial cells, lacking pericytes. Several molecules specifically expressed and localized in lymphatic endothelial cells such as 5'-nucleotidase, lymphatic vessel endothelial receptor-1 (LYVE-1), podoplanin, Prox-1 and vascular endothelial growth factor receptor-3 (VEGFR-3) have been identified and characterized (2,3). The vascular endothelial growth factors C and D (VEGF-C, VEGF-D) bind and activate VEGFR-3 by triggering receptor phosphorylation. Proteolytically processed forms of VEGF-C and -D also bind to VEGFR-2 whose primary ligand is VEGF-A (4). The role of the induction of lymph-angiogenesis by VEGF-A has recently been described by Hirakawa *et al* (5) and Bjorndahl *et al* (6). VEGFR-3 is a key receptor in lymph-angiogenesis and is predominantly expressed on lymphatic endothelial cells in adults. An increased expression of VEGF-C and its receptors in tumors is related to an increased density and size of lymphatic vessels as well as an increase in metastatic tumor cell dissemination. Recent studies have shown that VEGF-C and VEGFR-3 are expressed in malignant tumor cells such as breast, papillary thyroid carcinoma, prostate, Kaposi sarcoma, gastric carcinomas and others (7-11). Several studies have demonstrated that the blocking of VEGFR-3 delayed lymph-angiogenesis, for example lymphatic development was delayed in a transgenic mouse model expressing soluble VEGFR-3 (12) and in VEGF-C over-expressing tumors lymphatic drainage was reduced by treatment with soluble VEGFR-3 (13). A rat monoclonal antibody to mVEGFR-3 which antagonizes the binding of VEGF-C to VEGFR-3 was shown to block VEGFR-3, preventing both physiologically normal and tumor VEGF-C enhanced lymph-angiogenesis (14). He *et al* blocked lymph-angiogenesis and lymph node metastasis by the inhibition of VEGFR-3 signalling by systemic delivery of a soluble VEGFR-3-Ig fusion protein via adenoviral or adeno-associated viral vectors (15). Moreover, Crnic *et al* showed that the repression of VEGF-C and -D function by adenoviral expression of a soluble form of VEGFR-3 results in reduced tumor lymph-angiogenesis and lymph node metastasis (16).

These findings suggest that VEGFR-3 represents a promising target for therapeutic inhibition and diagnostic

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examination of tumor-induced lymph-angiogenesis and metastatic dissemination (17). Thus, the goal of our study was to produce and characterize human/mouse cross-reactive VEGFR-3 specific single chain antibodies (scFvs) as diagnostic and therapeutic tools. ScFv antibodies were selected with human and mouse VEGFR-3 from a human recombinant V-gene phage display library (18). The antibodies showed a strong binding to VEGFR-3 *in vitro* as well as *in vivo*. The diagnostic potential of the scFv AFC5 was evaluated in a separate experiment by single proton emission computed tomography (SPECT) analysis in a mouse teratocarcinoma model over-expressing VEGFR-3. Correspondingly, similar antibodies were generated against $VEGF_{164}$ (19), VEGF receptor 2 (20), the tumor endothelial marker TEM-1 (21) and the ED-B isoform of fibronectin (22,23).

Materials and methods

Cell lines. The adenovirus-transformed human epithelial kidney cell line HEK 293T was obtained from F. Wurm (EPFL, Lausanne, Switzerland), the mouse teratocarcinoma cell line F9 (CRL-1720) and the human erythroleukemia cell line U937 (ATCC CRL-1593) were from the American Type Culture Collection (ATCC, Rockville, MD). The HEK 293T and F9 cells were maintained in DME medium (Sigma, St. Louis, MO) without non-essential amino acids and pyruvate, supplemented with 10% heat-inactivated FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml nystatin (Gibco, Basel, Switzerland). The U937 cells were maintained in RPMI medium with 10% FBS and antibiotics as above. The stably transfected mouse teratocarcinoma cell line F9/VEGFR-3 was obtained by the transfection of F9 cells ($5x10^5$ cells) with 5 μg either circular or linearized pCDNA vector containing the extracellular domains 1-7 and the transmembrane domain of hVEGFR-3 (pCDNA/hVEGF-3D1-7TM) (24) by the calcium-phosphate method as described before (25). Briefly, 10 days after transfection, subclones were selected and further subcloning was carried out six times over a 4-month period until a clonal cell population was obtained. The *in vivo* transfection stability was evaluated by injecting the F9/VEGFR-3 cells *s.c.* in Sv129 mice and collecting the tumors after 10-14 days. Single tumor cell suspensions were prepared and the receptor expression was analyzed by Western blots of cell lysates and by flow cytometry.

Phage display, selection, modification, production and purification of scFv antibodies. Immunotubes (Maxisorp, Nunc, Wiesbaden, Germany) were coated with the extracellular domain of rhVEGFR-3/Flt4 (Reliatech, Braunschweig, Germany) at a concentration of 25 μg/ml. Phage display using the ETH-2 phage library (kindly provided by the D. Neri, Institute of Pharmaceutical Sciences, ETH Zürich, Switzerland) was carried out as previously described (18). Briefly, the phage library was incubated with an immobilized receptor protein for 1 h at room temperature (RT). The tubes were then washed to remove unbound or weakly bound phages. Specific phages were eluted and used for infection of the *E. coli* strain Tg1 (Invitrogen, Leek, The Netherlands) for amplification. The phages were harvested and used for subsequent rounds of selection. Two selection rounds each were carried out against hVEGFR-3 and subsequently mVEGFR-3 (Sigma-Aldrich, Buchs, Switzerland). Fifty clones were selected and scFv production in 96-well plates was induced with IPTG (isopropylthio-galactoside; Fermentas, Ontario, Canada). The supernatants of these clones were tested for binding to immobilized VEGFR-3 (10 μg/ml coated overnight at RT) by ELISA as described below. The sequence of each selected scFv was modified with C-terminal cysteines for functionalization as described before (22) and cloned into the yeast vector pPICZαA for secreted expression in *P. pastoris* (X-33, Invitrogen, Paisley, UK). Single clones were grown in 5 ml BMGY (buffered glycerol complex medium; Beckton Dickinson, Basel, Switzerland) for 12 h at 30˚C and stirring at 250 rpm. These cultures were used to inoculate 1 liter of BMGY in a baffled shaking flask and grown for 12 h (30˚C, 250 rpm) followed by centrifugation (2000 rpm, 15 min) and re-suspension of the pellet in BMMY (buffered methanol complex medium; Beckton Dickinson) for induction of protein expression. During further incubation (27˚C, 250 rpm, 48-72 h) cultures were supplemented with methanol at a final concentration of 0.5% (v/v). The supernatant of the yeast cultures was concentrated and dialyzed against 20 mM Tris, pH 8.0. The scFvs generated from the ETH-2 library contain the VH DP-47 germline segment sequence recognizing protein A, thus allowing purification on a protein A affinity column (5 ml column, GE Healthcare Europe GmbH, Otelfingen, Switzerland). The protein was eluted with 0.1 M glycine at pH 2.5. Fractions were neutralized to pH 7.4 and dialyzed against phosphate buffered saline (PBS, pH 7.4) at 4˚C. Due to the C-terminal cysteine modification $(scFv')_2$ antibody dimers were formed spontaneously under non-reducing conditions (22,23).

Determination of binding properties of the scFvs by competitive ELISA. The dissociation constants of the scFv antibodies AFA2 and AFC5 bound to immobilized VEGFR-3 were determined by competitive ELISA as described by Friguet *et al* (26). Varying concentrations of rhVEGFR-3 or rmVEGFR-3 ranging from 0-10 μg/ml were incubated in a solution with 1 nM scFv antibodies in PBS containing 0.02% BSA at RT overnight. Ninety-six-well plates (Nunc) were coated with 10 μg/ml receptor in PBS at RT overnight. The coated plates were washed 3 times with PBS and treated with 0.2 ml of blocking buffer (0.2 ml PBS, 2% (w/v) milk powder, RT, 2 h). The pre-incubated antibody/ antigen solutions were added to the plates and incubated at RT for 45 min followed by extensive washing and sequential incubation with anti-flag M2 (15 μg/ml) and alkaline phosphatase-conjugated goat anti-mouse antibodies (70 ng/ml, 45 min, RT). The plates were washed with PBS-Tween and with PBS prior to the addition of 0.1 ml NPP (3 mM 4-nitrophenyl phosphate, 50 mM $NaHCO_3$ and 50 mM $MgCl_2$) and reading of the absorbance at 410 nm. All experiments were carried out in triplicates. Cell lysates of transfected HEK 293T and U937 cells were prepared by re-suspending cell pellets in 6X SDS loading buffer and boiling for 10 min prior to three cycles of freeze-thawing. Samples were run on SDS-PAGE and subsequently blotted onto a polyvinylidene difluoride (PVDF, Millipore) membrane. The scFvs (1 nM) were incubated with a four-fold molar excess of rhVEGFR-3

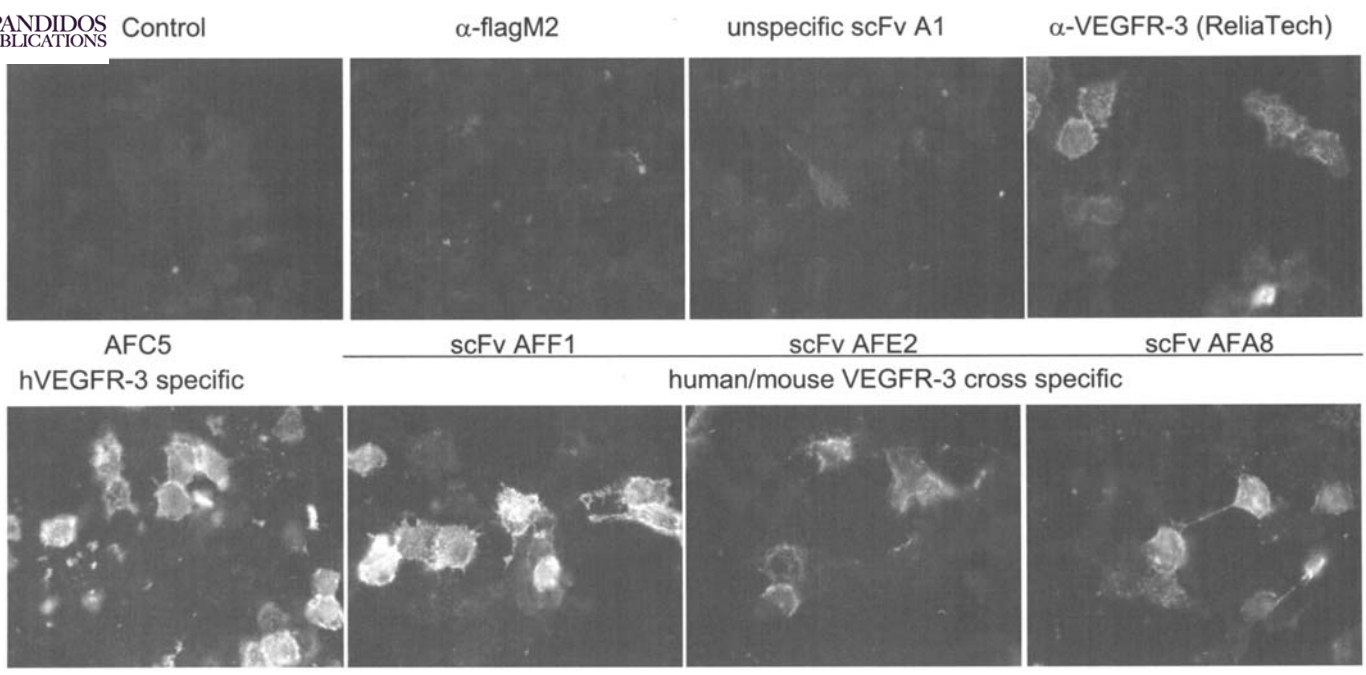

Figure 1. Immunohistochemical visualization of scFv binding to VEGF receptor-3 on transiently transfected HEK293T/VEGFR-3 cells. Controls are shown on the upper panels. The lower panels show binding of hVEGFR-3 specific scFv AFC5 (left) and binding of the cross-specific scFvs AFF1, AFE2 and AFA8 to hVEGFR-3 expressed on HEK293T cells (right). A goat anti-mouse-FITC antibody was used for staining.

for 30 min at RT before incubation for 1 h with the PVDF membrane. The blot was subsequently incubated with a mixture of anti-flag M2 (15 μg/ml) and goat anti-mouse-AP antibodies (70 ng/ml, 45 min, RT) followed by development with the color substrates 5-bromo-4-chloro-3'-indolyl-phosphate p-toluidine salt and nitro-blue tetrazolium chloride (BCIP/NBT; 1:100 v/v) for alkaline phosphatase.

Immunofluorescence. Glass coverslips were coated with poly-L-lysine-A (Sigma-Aldrich) and placed in 24-well plates (Nunc) prior to seeding HEK 293T cells (1x10^6 cells/well) for 24 h in an incubator at 37˚C, 5% CO_2. Transient transfection with a pCDNA3 vector encoding extracellular Ig domains 1-7 and the transmembrane domain of hVEGFR-3 (pCDNA3/hVEGF-3D1-7TM) was carried out by the calcium-phosphate method (25). After 4 h incubation with the DNA transfection mixture, a fresh medium was added and cells were incubated for 24 h. Coverslips were washed and incubated with 300 nM scFv for 1 h at 4˚C, washed in PBS and incubated with anti-flag M2 antibody (15 μg/ml) for 1 h at 4˚C. Washing was repeated and the coverslips incubated with 20 μg/ml goat anti-mouse-FITC antibody (Southern Biotechnology Associates Inc., Birmingham, AL, USA) for 1 h at 4˚C. Finally, the cells were fixed in 3.7% formaldehyde for 30 min at RT prior to mounting on slides with Celvol (Celanese, BL Emulsions, Frankfurt/Main Germany) and left to dry in the dark overnight.

Flow cytometry. U937 cells were grown in suspension for 48-72 h (37˚C, 5% CO_2). Cells (1x10^6 per tube in 2% FBS and 0.02% NaN_3) were sequentially incubated with scFvs and the secondary antibodies anti-flag M2 (30 μg/ml) and anti-mouse-FITC (2 μg/ml) for 30 min each on ice. The washed cells were fixed in 3.7% formaldehyde, analyzed in a FACS-Scan flow cytometer (Becton Dickinson, Mountain View, CA) and quantified using the CellQuest software (Becton Dickinson). Correspondingly, F9 and F9/VEGFR-3 cells were trypsinized (Trypsin/EDTA, Gibco), washed and fixed. All experiments were carried out in triplicates.

Biodistribution. The scFvs (AFC5, control antibody A1, 0.5 mg/ml) were labelled with ^{125}Iodine (GE Healthcare) at an activity of 50-60 μCi/ml. Cultured F9 or stably transfected F9 (F9/VEGFR-3) cells were harvested and resuspended in PBS. Female Sv129 mice (Charles River, Sulzfeld, Germany) were injected s.c. with 10^7 cells in 50 μl PBS on the lower back. When the tumors had reached volumes of 0.5-1.5 cm^3, mice were injected i.v. with 5 μg of ^{125}I-scFv (1x10^7 cpm/100 μl PBS). At 5, 15, 30, 60, 120, 180, 240, 300 and 360 min and 24 h after injection the animals (3 per time point) were anaesthetized with isofluorane and immediately sacrificed for the removal of blood, heart, liver, kidney, spleen and tumor. The organs were weighed and the radioactivity was measured using a gamma counter (Packard-Perkin Elmer, Downers Grove, IL).

Single proton emission computed tomography (SPECT) analysis. The scFv AFC5 (1 mg/ml) was labelled with ^{131}Iodine (GE Healthcare) at an activity of 920 μCi/ml. F9 or F9/VEGFR-3 cells (5x10^6 cells in 50 μl) were injected s.c. between the shoulders on the back of Sv129 mice. When the tumors had reached volumes of approximately 1.5 cm^3, mice were injected i.v. with ^{131}I-scFv AFC5 (100 μg in 200 μl PBS). Two hours after injection the animals were sacrificed in a CO_2 chamber and the scFv biodistribution analyzed post mortem with an X-SPECT™ instrument (Gamma Medica Inc., Northridge, CA). SPECT data were acquired and reconstructed using the LumaGEM software (Gamma Medica). CT data

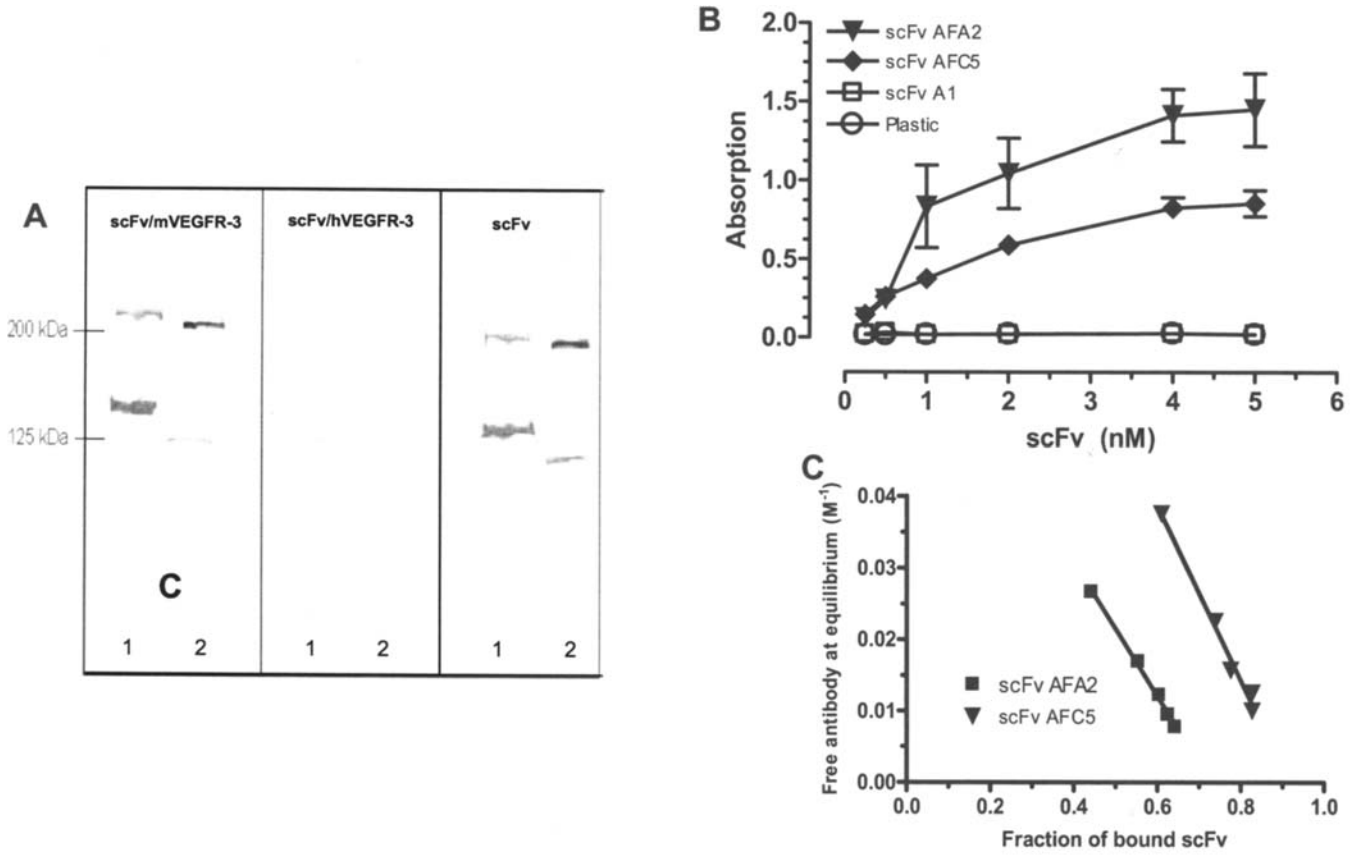

Figure 2. Western blot analysis of scFvs pre-incubated with human or mouse VEGFR-3 in solution. (A) SDS gel of cell lysates of transfected HEK293T/hVEGFR-3 (lane 1) and U937 cells (lane 2). Left panel: Binding of scFv AFA2 pre-incubated with mouse VEGFR-3. Middle panel: Binding of the human specific scFv AFA2 pre-incubated with a four-fold molar excess of hVEGFR-3. Right panel: Uninhibited binding of scFv AFA2 to hVEGFR-3. (B) binding curves of scFvs to immobilized hVEGFR-3. As controls binding of the scFvs was measured on uncoated plates. Binding of the unspecific scFv A1 to the receptor was below the detection limit.

were acquired with the X-Ray CT-system software and reconstructed with the Cobra software (v4.5.1, detector size: 1024x1024 pixels, acquired projections: 256 and smoothing filter 1, Gamma Medica). Overlays of SPECT- and CT-data were made with the IDL Virtual Machine (V6.0) software and images were generated with Amira™ (V3.1.1) software.

Immunocytochemistry. F9 and F9/VEGFR-3 cells were grown to confluency on culture slides. FBS was removed by washing twice with PBS followed by air drying of the slides for 3 h. The cells were fixed for 10 min in anhydrous acetone and dried for 1 h to eliminate any residual acetone prior to freezing the slides wrapped in aluminum foil at -80˚C. The cells were stained with scFvs AFC5 and A1 (20 μg/ml) for 4 h at RT prior to staining with anti-flag Ab and AP-conjugated anti-mouse Ab for 1 h at RT.

Human lymphatic endothelial cell proliferation assay. A Bio-one 96-well plate (Greiner Bio-One, Essen, Germany) was coated with 10 μg/ml fibronectin for 30 min. Human lymphatic endothelial cells (HLECs) were plated at 1500 cells per well in EBM (Endothelial Basal Medium, Cambrex, Verviers, Belgium) supplemented with 20% FBS, antibiotic/antimycotic solution (Fluka, Buchs, Switzerland), L-glutamine (2 mM; Fluka), hydrocortisone (10 μg/ml; Fluka) and N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (25 μg/ml; Fluka) for 24 h at 37˚C. The medium was changed to 2% FBS after 24 h. Then, the medium was removed and 150 μl EBM containing 2% FBS and 1 μg/ml of rhVEGF-C (R&D Systems Europe Ltd., Abingdon, UK) and different scFv concentrations (25-100 nM) were added and incubated for 72 h at 37˚C. The rhVEGF-C contains a Cys156Ser mutation and is a selective agonist for VEGFR-3 and does not bind VEGFR-2 (27). The wells were washed twice with PBS before adding 0.1 mg/ml 4-methylumbelliferyl heptanoate (MUH) substrate for intracellular esterases in viable cells in PBS and incubating the plate for 1 h at 37˚C. The fluorescence produced was measured at 355 nm excitation and 450 nm emission wavelengths. The experiment was carried out with 8 replicates. Results were analysed statistically using the Student's t-test.

Results

Selection, production and purification of scFv antibodies. ScFvs specific for human and mouse recombinant VEGFR-3 were generated with the ETH-2 phage library (18). After four rounds of selection, positive clones were chosen at random for further analysis, each one for human and the mouse isoform of the receptor. Selected clones were tested for specificity of VEGFR-3 binding by ELISA on hVEGFR-3, mVEGFR-3, VEGF, BSA and plastic. All scFvs were specific for VEGFR-3 and were shown to bind the receptor on transiently transfected HEK293T/VEGFR-3 cells as visualized in Fig. 1 by immunofluorescence. The scFvs displayed no unspecific

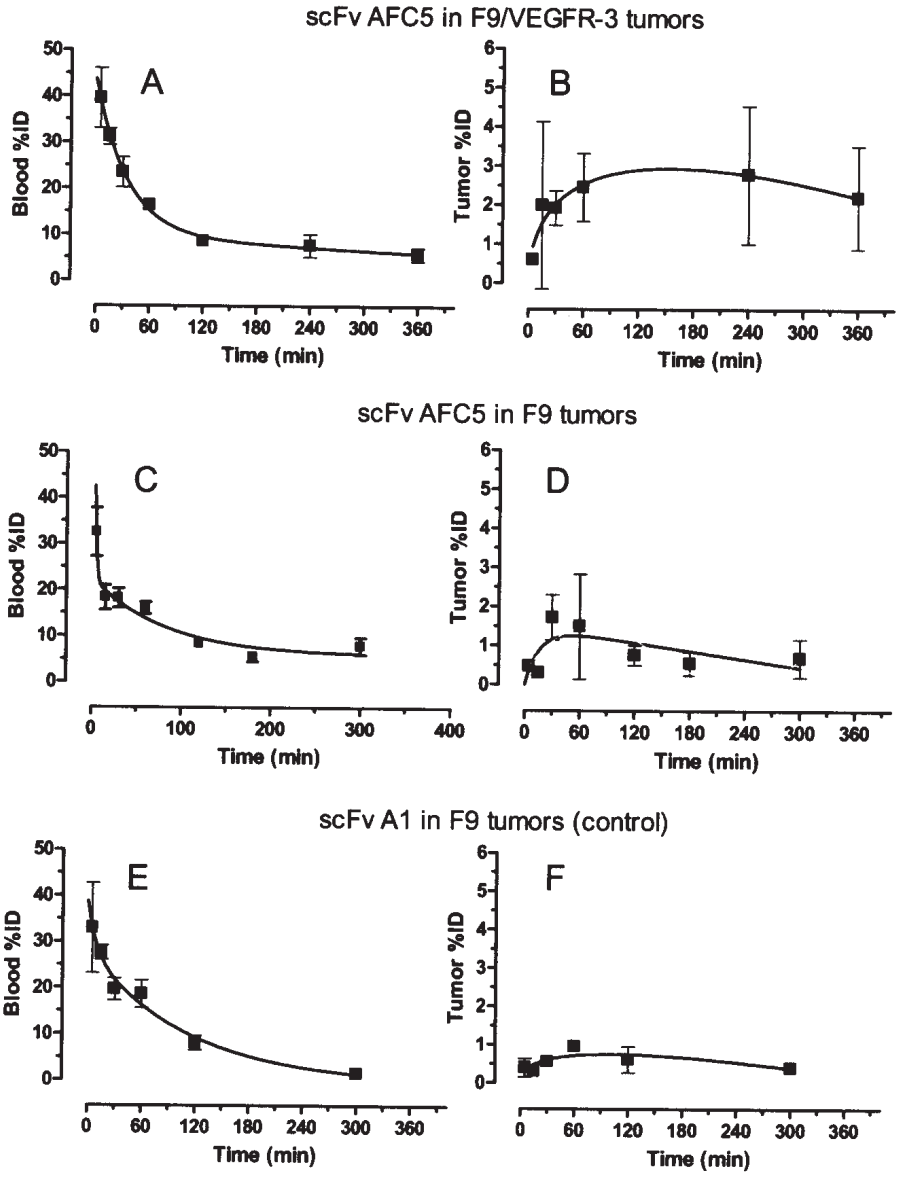

Figure 3. Blood elimination (A, C, E) and tumor accumulation (B, D, F) curves of scFv AFC5 in tumor bearing mice. ScFv AFC5 in F9/VEGFR-3 tumor bearing mice (A, B); scFv AFC5 in F9 tumor bearing mice (C, D) and scFv A1 in F9 tumor bearing mice (E, F). Three mice per group were injected with I^{125} labeled single chain antibodies and sacrificed at different time points.

binding to non-transfected cells, nor did an unspecific control antibody (scFv A1) bind to hVEGFR-3 transfected cells. As shown in Fig. 1 fluorescence was confined on the cell surface. Binding of the scFvs AFA2, AFC5, AFF1 and AFA8 to VEGFR-3 expressing U937 cells resulted in an increase of cell associated fluorescence in comparison to the negative control in the cytofluorometric analysis (data not shown). The single chain antibodies AFA2, AFC5, AFF1 and AFA8 were selected for further characterization, since they exhibited the strongest binding to VEGFR-3. The expression of scFvs in *P. pastoris* was tested on a small scale and analyzed by Western blots prior to choosing the optimal antibody producer for large scale production. By using a maximal concentration of 0.5% (v/v) methanol and by carrying out the production in baffled shaking flasks, which provides the essential aeration, a yield of 3-4 mg/l of scFvs was obtained after 72 h of expression. Subsequent protein purification over protein A allowed simple and reliable isolation of the scFvs at high purity (>95%).

Binding of scFvs to recombinant and endogenous VEGFR-3. The binding specificity of the scFv AFA2 and AFC5 to human or mouse VEGFR-3 was analyzed on Western blots. To demonstrate species-specific binding of the scFvs, Western blots were made by pre-incubating the scFvs with human or mouse VEGFR-3 in solution. As shown in Fig. 2A, cell lysates of transfected HEK293T/hVEGFR-3 (lane 1) and U937 cells (lane 2) were analyzed by SDS PAGE, followed by Western blotting. Binding of scFv AFA2 pre-incubated with mVEGFR-3 (left panel) and with a four-fold molar excess of hVEGFR-3 (middle panel) documents the species specificity of the antibody. As a control, the uninhibited binding of scFv AFA2 to hVEGFR-3 is shown in the right panel of Fig. 2A. The two bands seen on the Western blots correspond to a 175 kD precursor of VEGFR-3 which, after maturation to a 195 kD variant, is proteolytically cleaved into a 125 kD fragment (27). Due to glycolysation the proteins have a slightly higher apparent molecular weight.

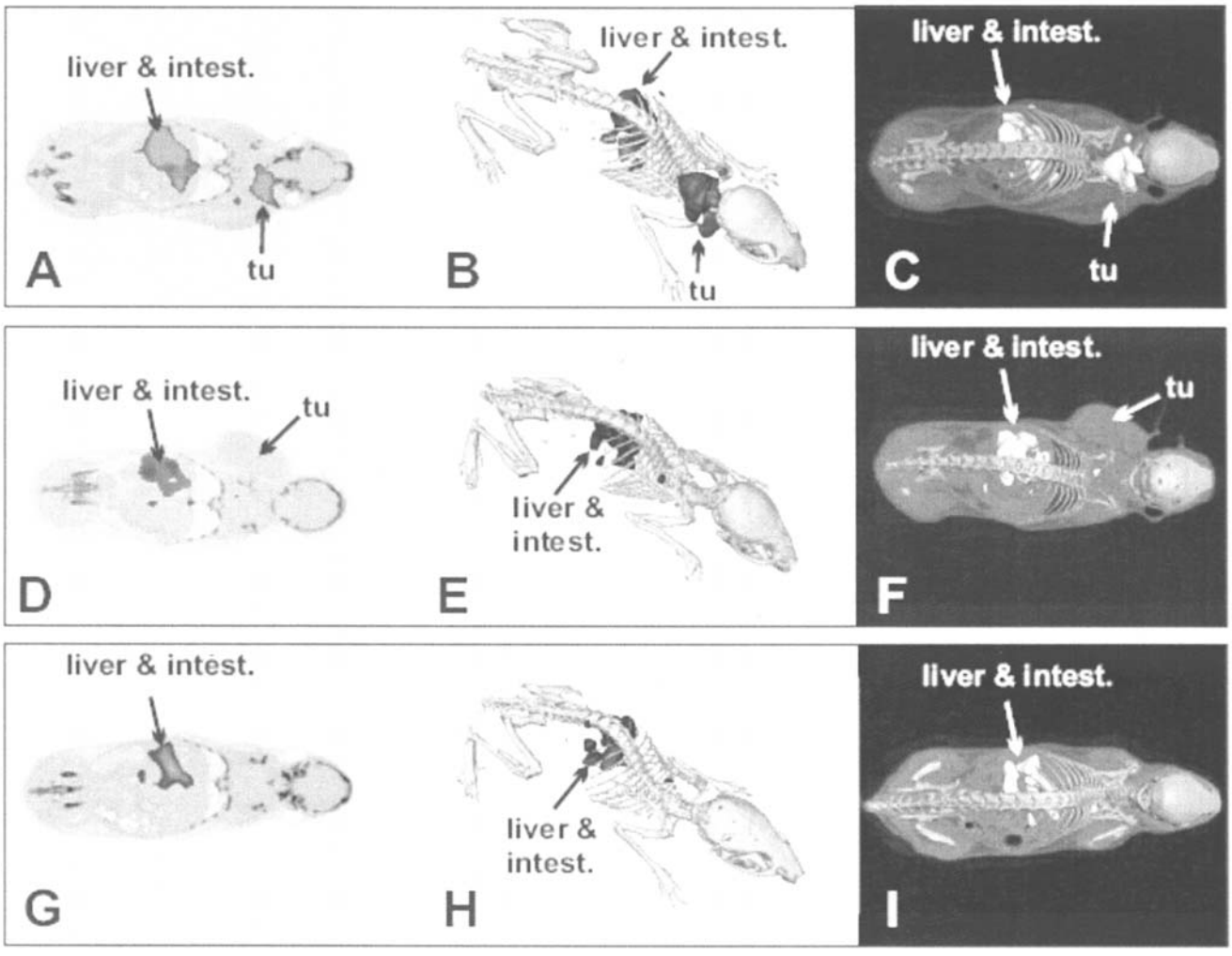

Figure 4. SPECT/CT analysis of ^{131}I-labelled scFv AFC5. Mice were either injected s.c. with F9/VEGFR-3 (A-C) or with F9 tumor cells (D-F). Control mouse without tumor (G-I). A/D/G: coronal SPECT/CT sections at the level of the tumor, liver and intestines; B/E/H: whole body SPECT/CT images; C/F/I: Combined coronal SPECT/CT and whole body SPECT/CT. Tu, tumor, intest, small and large intestines.

Binding curves and affinities of the scFvs were determined by competitive ELISA (Fig. 2B, C). Analysis of the binding curves of scFv AFA2 and scFv AFC5 indicated that more than 4 nM scFvs are required to saturate 1 μg of immobilized antigen. The calculated K_d values (Fig. 2C) were 10.6 nM and 8.9 nM for scFv AFA2 and scFv AFC5, respectively. They are in the range as determined for other scFv antibodies isolated from similar phage libraries (28). HEK 293T/hVEGFR-3 cells exposed to varying concentrations (1-5 nM) of the scFvs showed that 1 nM scFv is sufficient to visualize receptor binding (data not shown).

Biodistribution of scFv in F9/VEGFR-3 tumor bearing mice. To assess the potential of the scFv antibodies for *in vivo* targeting, F9/VEGFR-3 or F9 tumors were grown in Sv129 mice. As shown in Fig. 3A, C and E, the I^{125}-labeled scFv antibodies had initial distribution half-lives in blood of 60-80 min, followed by elimination half-lives of 6-24 h, respectively. The fast blood clearance observed is typical for single chain antibodies. In the VEGFR-3 transfected F9 tumors, antibody accumulation remained between 2 and 2.75% of the injected dose (%ID) for up to 6 h after injection (Fig. 3B) and was reduced in the F9 tumors (Fig. 3D). However, paired Student's t-test analysis between curves B and D resulted in a non-significant difference (P=0.137) between antibody uptake into the two tumors. In mice bearing F9 tumors scFv AFC5 showed similar results, not significantly different tumor accumulation as the unspecific scFv A1 in VEGFR-3 expressing tumors, probably reflecting the antibody concentration in the blood pool of the highly vascularized F9 tumors (Fig. 3D, F; P=0.22). The unspecific control antibody scFv A1 showed similar blood clearance, but lower accumulation in the tumor (Fig. 3E, F). After 30-60 min 1.75 %ID had accumulated in the tumor, whereas after 3 h only 0.5%ID was detected which was significantly different from the accumulation of scFv AFC5 in F9/VEGFR-3 tumors (Fig. 3B, F; P=0.001). ScFv AFC5 rapidly accumulated in the kidneys (18%ID after 15 min) but was cleared almost completely within 2 h, whereas liver accumulation was less than 2%ID after 2 h (data not shown).

Diagnostic potential of scFv AFC5. To visualize tumor targeting specificity and biodistribution of scFv AFC5 a whole body SPECT/CT analysis was carried out in a separate experiment with mice bearing no tumor, an F9 control tumor or the receptor expressing F9/VEGFR-3 tumor. The tumors

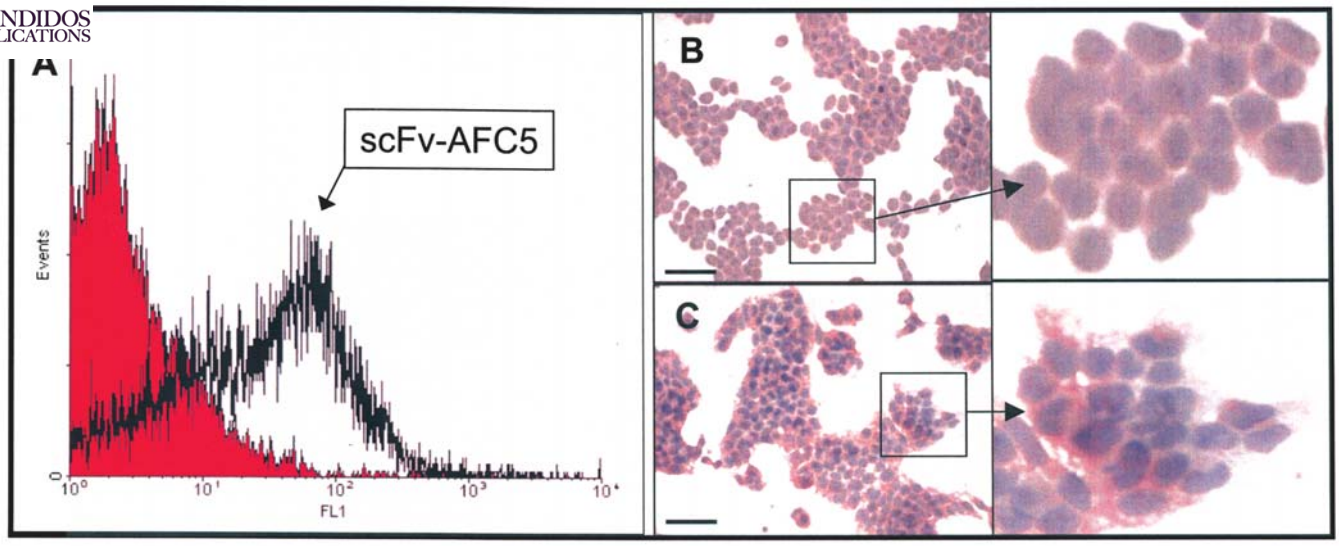

Figure 5. Cytofluorometric and immunohistochemical binding analysis of antibodies on F9/VEGFR-3 cells. Cells were incubated with 5 nM scFv AFC5 and binding was visualized by flow cytometry (A) and immunohistochemistry (B, C). In B, cells were incubated with the unspecific antibody scFv A1 and in C with scFv AFC5. Staining of VEGFR-3 expressing F9 cells is best seen in the magnified insets of the micrographs shown in the right panel. Bar: 50 μm.

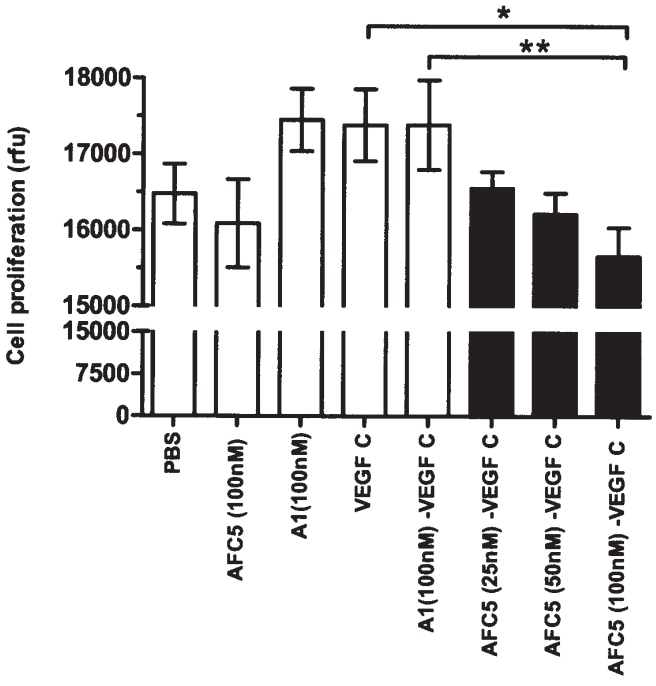

Figure 6. Analysis of proliferation of human lymphatic endothelial cells stimulated with mutant VEGF-C (rhVEGF-C Cys156Ser) and incubated with specific scFv AFC5 or unspecific scFv A1 at concentrations ranging from 25-100 nM (*P<0.01; **P<0.05).

were positioned on the back behind the neck of the mice in order not to interfere with other organs. The distribution of I^{131}-labelled scFv AFC5 was analyzed post mortem 2 h after injection. The receptor specific scFv AFC5 showed strong accumulation in the F9/VEGFR-3 tumor (Fig. 4A-C) but no accumulation in the F9 tumor (Fig. 4D-F), whereas the unspecific control antibody scFv A1 predominantly accumulated in liver and intestines of a mouse bearing no tumor (Fig. 4G-I). The diagnostic potential of AFC5 was further investigated by flow cytometry and immunocytochemistry on F9/VEGFR-3 cells as summarized in Fig. 5. Specific binding to F9/VEGFR-3 cells obtained from *in vivo* grown tumors measured by flow cytometry is shown in Fig. 5A and the immunohistochemical analysis of F9/VEGFR-3 cells with the unspecific scFv A1 as a negative control is shown in Fig. 5B. The specific binding of scFv AFC5 to VEGFR-3 is visualized as the purple staining of cells shown in Fig. 5C and the magnified insets.

Functional inhibition of VEGFR-3 with scFv AFC5. Proliferation of lymphatic endothelial cells is a key step in lymph-angiogenesis. We therefore investigated the potential of scFv AFC5 to inhibit this process by blocking VEGFR-3 signalling *in vitro*. The proliferation assay was carried out on human lymphatic endothelial cells (HLEC) obtained from neonatal foreskin. A mutated version of recombinant human VEGF-C (rhVEGF-C Cys156Ser), only recognizing VEGFR-3 and not VEGFR-2 was used (25). During the 72 h incubation period, rhVEGF-C induced cell proliferation (Fig. 6). The blocking of rhVEGF-C by different concentrations of scFv AFC5 (25-100 nM) reduced HLEC proliferation significantly ($P<0.05$, scFv AFC5 vs. A1; 100 nM). The reduced cell proliferation found after the addition of the scFv AFC5 (100 nM) suggests that binding of this antibody to VEGFR-3 may inhibit cell proliferation.

Discussion

It is now widely accepted that lymph-angiogenesis plays a major role in the spread of tumors and that VEGFR-3 and its ligands VEGF-C and -D play key roles in this process (1,2,29,30). Inhibiting signalling via this receptor is therefore considered to be a good targeting strategy that might be useful to develop new therapies. We therefore produced functionalized VEGFR-3 specific single chain antibodies for diagnostic applications and as potential therapeutic tools.

To this end, we generated VEGFR-3 species cross-specific scFv antibodies from a human phage display library. We

demonstrated a specific binding of selected scFvs *in vitro* to both purified recombinant VEGFR-3 as well as in cell lysates of receptor expressing transfected HEK 293T cells and in endogenously VEGFR-3 expressing U937 cells. Specific binding of the scFvs was observed at concentrations as low as 1 nM. ScFv binding to the receptor expressed in U937 cell lysates confirmed the findings of Schoppmann *et al* (31), who showed that the U937 erythroleukemia cell line endogenously expresses VEGFR-3. In our initial screen we selected scFvs AFA2 and AFC5 that did not recognize the mouse isoform of VEGFR-3. Blocking experiments showed that the scFvs AFA2 and AFC5 do not recognize mVEGFR-3. Therefore, additional rounds of phage display were carried out to obtain cross-reactive antibodies. The scFvs AFF1, AFE2 and AFA8 showed cross-reactivity to both the human and mouse VEGFR-3, which makes them valuable tools for investigating receptor expression in mouse tumor models and in human tissue. The K_d values of our scFvs binding to hVEGFR-3 were in the range of the dissociation constants that have been reported for several therapeutic antibodies (28). The specific binding of the scFv antibodies was visualized on transfected cells by immunofluorescence (Fig. 1) and on F9/VEGFR-3 and U937 cells by flow cytometry (Fig. 5). *In vivo*, the scFv AFC5 showed specific accumulation and prolonged retention in VEGFR-3 expressing tumors. The rather low %ID accumulation found in the tumor is probably due to the mouse model used in which the stably transfected F9/VEGFR-3 cells may only express a low number of receptor molecules on their surface. However, specific tumor accumulation could be visualized by SPECT/CT analysis as shown in Fig. 4 and the diagnostic potential of the scFvs was confirmed by immunocytochemistry on F9/ VEGFR-3 cells (Fig. 5B, C). In addition, competition assays on HLECs showed a statistically significant inhibition of hVEGF-C induced proliferation by the scFv AFC5 compared to the unspecific scFv A1 or PBS controls (Fig. 6). Since proliferation represents an important step in lymph-angiogenesis, we propose that the new anti-VEGFR-3 scFv antibodies are useful both for therapeutic and diagnostic applications, warranting their evaluation in tumor models to analyze their potential to inhibit growth and the metastatic spread of tumors.

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