Involvement of the VEGF receptor 3 in tubular morphogenesis demonstrated with a human anti-human VEGFR-3 monoclonal antibody that antagonizes receptor activation by VEGF-C

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Summary

In this report we utilize a novel antagonist antibody to the human VEGFR-3 to elucidate the role of this receptor in vitro tubular morphogenesis of bovine and human endothelial cells (EC cells) induced by VEGF-C. The antibody hF4-3C5 was obtained by panning a human phage display library on soluble human VEGFR-3. The binding affinity constant of hF4-3C5 significantly exceeds that of the interaction of VEGFR-3 with VEGF-C. hF4-3C5 strongly inhibits the binding of soluble VEGF-3 to immobilized VEGF-C and abolishes the VEGF-C-mediated mitogenic response of cells that expresses a chimeric human VEGFR-3-cFMS receptor. In fluorescence experiments, hF4-3C5 reactivity is observed with human lymphatic endothelial cells (LECs) and human umbilical vein endothelial cells (HUVECs). Binding of hF4-3C5 shows that about half of bovine aortic endothelial (BAE) cells express VEGFR-3 and cells in this subpopulation are primarily responsible for the chemotactic response to the mature form of VEGF-C (VEGF-CΔNΔC). This response was strongly inhibited by the addition of hF4-3C5. In vitro tube formation by BAE cells induced by VEGF-CΔNΔC was reduced by greater than 60% by hF4-3C5 whereas the response to VEGFΔN was unaffected. Addition of hF4-3C5 together with an antagonist antibody to VEGF-R2 completely abolished the response to VEGF-CΔNΔC. Similar results were obtained with HUVECs. Together, these findings point to a role for VEGFR-3 in vascular tubular morphogenesis and highlight the utility of hF4-3C5 as a tool for the investigation of the biology of VEGFR-3.

Supplemental data available online

Key words: VEGFR-3, Flt-4, VEGF-C

Introduction

VEGFR-3 (also known as Flt-4), a receptor for the endothelial mitogens VEGF-C and D, is believed to play a critical role in the development of the embryonic vascular system but to be restricted postnatally to endothelial cells of lymphatic vessels and specialized fenestrated capillaries (Partanen et al., 2000; Dumont et al., 1998). Consistent with this view, mis-sense mutations that inactivate VEGFR-3 in humans and in transgenic mice primarily disrupt lymphatic but not blood vessels (Karkkainen et al., 2000; Karkkainen et al., 2001). Recent reports, based largely on histochemical analysis of tumors, have raised the possibility that VEGFR-3 may also play a role in adult vascular endothelium. VEGFR-3 expression in tumor microvasculature was demonstrated in biopsies of human breast cancer and the level of VEGFR-3 expression correlated with the level of tumor invasiveness (Valtola et al., 1999). VEGFR-3 co-localization with the vascular endothelial marker PAL-E was similarly reported in samples of adenocarcinoma of the kidney and colon, invasive breast carcinoma and hepatocellular carcinoma (Clarijs et al., 2002). Importantly, VEGFR-3 expression on blood vessels associated with human cutaneous melanoma, correlated strongly with aggressiveness of the lesion (Clarijs et al., 2002). VEGFR-3 was also detected in the intratumor blood vessels of human gliomas and on vascular endothelial cells activated during the formation of granulation tissue in the skin (Witte et al., 2001). Furthermore, experimental injection of VEGF-A into monkey eyes induced the expression of VEGFR-3 in the blood vessels of the iris suggesting an interdependence of the VEGFR-2 and VEGFR-3 in tumor angiogenesis (Witte et al., 2001).

In contrast, results of animal studies have largely supported the role of VEGFR-3 in adult lymphangiogenesis rather than angiogenesis (reviewed in Karpanen and Alitalo, 2001; Detmar and Hirakawa, 2002). Transgenic expression of the VEGFR-3-specific mutant of VEGF-C in mouse skin resulted in increased growth of dermal lymphatic but not vascular endothelium (Jeltsch et al., 1997). Similarly, orthotopic implantation of human breast cancer cells that overexpress VEGF-C induced tumor lymphangiogenesis and increased metastasis without noticeable induction of angiogenesis (Karpanen et al., 2001; Skobe et al., 2001). Administration of the adenoviral form
of mature VEGF-D produced a tissue-specific response characterized by angiogenesis in the muscle and primarily lymphangiogenesis in the skin (Byzova et al., 2002). Targeted expression of VEGF-C in pancreatic islet β-cells in the VEGF-C mice resulted in the formation of a peri-islet lymphatic space lined with endothelial cells that stained positive for VEGFR-3 and the lymphatic endothelial marker LYVE-1 (Mandriota et al., 2001). In contrast, vascular vessel density near or within the islets was unaffected. Similarly, suppression of VEGF-C and D levels in tumor-bearing mice with neutralizing antibodies or a soluble form of VEGFR-3 resulted primarily in anti-lymphangiogenic rather than anti-angiogenic effects (Karpanen et al., 2001; Makinen et al., 2001a; Stacker et al., 2001; He et al., 2002). However, in one report, inhibition of murine VEGFR-3 in tumor-bearing mice with a blocking monoclonal antibody (mAb) led to intratumor hemorrhage suggesting a disruption of tumor microvasculature (Kubo et al., 2000).

We have previously addressed the relative contributions of VEGFR-2 and VEGFR-3 to the angiogenic response using a model in which bovine endothelial cells are induced to invade a three-dimensional collagen gel by the addition of various growth factors (Montesano and Orci, 1985). Using this model, we showed that invasion and subsequent formation of tubes that resemble capillaries is stimulated by either exogenous growth factors (Montesano and Orci, 1985). Using this model, we showed that invasion and subsequent formation of tubes that resemble capillaries is stimulated by either exogenous addition of VEGF to cultures of bovine aortic or microvascular endothelial cells or by autocrine expression of VEGF induced by basic fibroblast growth factor (Tille et al., 2001). The magnitude of this response could be modulated by the addition of neutralizing antibodies to VEGF; soluble forms of VEGF receptors or VEGF-2 tyrosine kinase inhibitors (Tille et al., 2001). Expanding on this work, we reported that VEGF-C\textsubscript{ANAC}, the 21 kDa proteolytically processed form of VEGF-C, induces collagen gel invasion and tube formation by BAE cells (Joukov et al., 1997; Tille et al., 2003). This response was completely inhibited by antagonistic antibodies to VEGF-2 when the inducing agent was VEGF-A. In contrast, the response to VEGF-C\textsubscript{ANAC}, a ligand for both VEGFR-2 and VEGFR-3, could only be inhibited by maximum of 67% by antagonizing VEGFR-2 (Tille et al., 2003). One interpretation of the above findings is that VEGF-C\textsubscript{ANAC} plays a significant role in the in vitro angiogenic response of BAE cells to VEGF-C. We were unable to test this hypothesis directly because the recombinant mutant of human VEGF-C (VEGF-C\textsubscript{ANAC}155S), which activates human VEGF-C\textsubscript{ANAC} but is unable to bind to VEGFR-2, failed to activate VEGFR-3 in BAE cells (Tille et al., 2003).

Antagonist antibodies directed to VEGF and its receptors VEGFR-1 and VEGFR-2 have been used as therapeutic agents and as powerful experimental tools (Zhu et al., 2002). Accordingly, we considered that a strong and specific antagonist of human VEGF-C\textsubscript{ANAC} would be invaluable for further exploration of the role played by this receptor in normal and pathologic biology of human vascular and lymphatic endothelium. We now report the production and functional characterization of a novel fully-human monoclonal antibody, hF4-3C5, that powerfully antagonizes receptor activation by VEGF-C\textsubscript{ANAC}. In addition, we demonstrate the utility of this antibody for the delineation of VEGF-C\textsubscript{ANAC}-positive populations of vascular and lymphatic endothelial cells and for examining the contribution of VEGF-C\textsubscript{ANAC} to the chemotactic response and in vitro tubulogenesis stimulated by VEGF-C\textsubscript{ANAC}.

### Materials and Methods

#### Reagents

Recombinant human VEGF-C\textsubscript{ANAC} was provided by M. Skobe (Mount Sinai School of Medicine, New York). Anti-human VEGFR-2 antibodies (IMC-1C11 and IMC-1121) and isotype controls IMC-C225 and IMC-2F8 were produced by ImClone Systems (New York). IMC-1C11 and IMC-1121 are functionally equivalent VEGFR-2 antagonists except that IMC-1C11 is a mouse-human chimeric antibody whereas IMC-1121 is fully human (Zhu et al., 2003). Soluble human VEGFR-1 (sR1-AP) and VEGFR-2 (VEGFR-2-AP) were produced by ImClone Systems as fusion proteins with human alkaline phosphatase (AP; see below). Monoclonal anti-keyhole limpet hemocyanin antibody (anti-KLH; 03210D) and human VEGF\textsubscript{165} (165-amino acid homodimeric isoform of VEGF-A) were purchased from Peprotech (Rocky Hill, NJ) and Pharmingen (San Diego, CA), respectively. Rabbit antibody to human Prox-1 was from Research Diagnostics (Flanders, NJ).

#### Cell culture

Bovine aortic endothelial (BAE) cells, isolated as previously described (Pepper et al., 1992b), were cultured in Dulbecco's modified minimal essential medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated donor calf serum (DCS) (Diagnostics, Flanders, NJ). The cells were maintained in EBM-2 medium (Cambrex, Walkersville, MD) and used between passages 2 and 6. Lymphatic endothelial cells (LECs) were a kind gift of Mihaela Skobe (Mount Sinai School of Medicine, New York) and were cultured in EBM medium.

#### Construction and expression of VEGFR-3-alkaline phosphatase fusion proteins

The cDNA encoding full-length (Ig domains 1-7) extracellular region of human VEGF-C was prepared by the polymerase chain reaction (PCR) and cloned into the RsrII and BspEI restriction sites of the expression vector AP-Tag (Flanagan and Leder, 1990). The expression of the VEGFR-3-AP fusion protein (sR3-AP) in transfected NIH 3T3 cells was detected by measuring AP in the medium and the sR3-AP protein was purified using anti-AP antibody-affinity chromatography as previously described (Zhu et al., 1998). AP fusion protein of the extracellular portion of VEGFR-2 (sR2-AP) was used as a control in some experiments (Lu et al., 2000).

#### Generation of antibodies to human VEGFR-3

Anti-human VEGF-C\textsubscript{ANAC} extracellular domain antibodies were generated by screening an antibody phage display library (de Haard et al., 1999) with sR3-AP, sR3-AP-coated Maxisorp Star tubes (Nunc, Roskilde, Denmark) were blocked with 3% milk/PBS at 37°C for 1 hour, and then incubated with the phage preparation at RT for 1 hour. The tubes were washed 10 times with PBST (PBS containing 0.1% Tween-20) and 10 times with PBS. The bound phage were eluted at room temperature (RT) for 10 minutes with a freshly prepared solution of 100 mM triethylamine (Sigma, St Louis, MO). The eluted phage were incubated with TG1 cells at 37°C for 1 hour. The cells were grown on 2YTAG plates overnight at 30°C and individual TG1 clones were rescued with M13K07 helper phage. The amplified phage preparation were blocked with one-sixth volume of 18% milk/PBS at RT for 1 hour, added to Maxi-sorp 96-well microtiter plates (Nunc) coated with sR3-AP or AP proteins (1 μg/ml) and incubated at RT for 1 hour. The plates were washed three times with PBST and bound phage were detected with a rabbit anti-M13 phage Ab-HRP conjugate (Amersham Bioscience, Piscataway, NJ) and TMB peroxidase substrate (KPL, Gaithersburg, MD) using a microplate reader.
Preparation of soluble Fab and full-length IgG molecules
Soluble Fab fragments were expressed and purified as described previously (Lu et al., 2002). For expression of full-length phage-derived IgG, the cloned VL and VH genes were ligated into an expression vector containing human light-chain constant region and human heavy-chain constant region γ. The expression construct was transfected into NS0 myeloma cells and stable clones of antibody-expressing cells were selected. The cells were grown in serum-free media and the antibody was purified from the media by Protein A affinity chromatography (Zhu et al., 2003).

Quantitative VEGFR-3-binding assay
Fab or IgG proteins were added to 96-well Maxi-sorp microtiter plates coated with sR3-AP and incubated at RT for 1 hour. Bound Fab and IgG were detected by incubating at RT for 1 hour with, respectively, mouse anti-myc antibody-HP conjugate (BioSource, Camarillo, CA) or rabbit anti-human IgG-HRP (Jackson ImmunoResearch, West Grove, PA). The plates were washed and developed as described above. Alternately, purified receptor proteins were added to 96-well Maxi-sorp microtiter plates coated with monoclonal antibodies and incubated at RT for 1 hour. After washing, p-nitrophenyl phosphate (Sigma) was added to quantify the bound sR-AP molecules.

Competitive VEGF-C blocking assay
Various amounts of soluble phage particles or antibodies were mixed with 50 ng of sR3-AP, incubated at RT and transferred to 96-well microtiter plates coated with VEGF-CANAC (200 ng/well). After an additional 2 hours, the plates were washed five times and p-nitrophenyl phosphate (Sigma) was added to quantify the bound sR3-AP molecules. The IC50, i.e., the concentration of Fab or IgG required for 50% inhibition of sR3-AP binding to VEGF-CANAC, was calculated.

Preparation of cells expressing chimeric VEGFR-3-cFMS receptor
cDNA encoding the extracellular domain of human VEGFR-3 was fused with cDNA encoding the transmembrane and cytoplasmic domains of human cFMS in the expression vector pires (Invitrogen). The DNA was electroporated into NIH 3T3 cells and cell clones were selected by growth in G418. Plasma membrane expression of VEGFR-3-cFMS was demonstrated using indirect immunofluorescence with antibodies specific for murine VEGFR-3 and by western blotting.

Immunofluorescence
FACS analysis was performed using FACS Vantage SE (BD Biosciences, San Jose, CA). Relative fluorescence index (RFI) was calculated as the ratio of the sample mean fluorescence channel and the control (isotype-matched antibody) mean fluorescence channel. For staining with human Prox-1 antibody incubations and all washes included 0.1% saponin.

RT-PCR analysis
Total cellular RNA from LECs and BAE cells was prepared using TRIzol (Invitrogen). 2 µg of each RNA were reverse transcribed using oligo dT priming with and without reverse transcriptase (BD Clontech) and subjected to 30 cycles of amplification followed by analysis on a 1% agarose gel. The primers for amplification of bovine and human transcripts were designed in regions of sequence identity for the two species. The primer sequences and GenBank accession numbers are given below:

bovine podoplanin est. BF041289; human podoplanin, AF390106;
5’TGGGCAGAAAGGCCACGAC-3’, 5’-CCCAACAAATGATTCCACGG-3’;
bovine Prox-1 est, AW445842; human Prox-1, NM002762;
5’-CTGAAAGACTGTCATCAGCAG-3’, 5’-GGATCAACATCTTTGCTTCG-3’;
bovine G3PDH, AF077815; human G3PDH, BC026907;
5’-GGAAGTCATGGCATTGGCTTTCC-3’, 5’-GGAATGAGCTTGACAAAGTG-3’.

Receptor phosphorylation
Cells were stimulated by the addition of various amounts of VEGF165 or VEGF-CANAC. VEGF-C proteins were immunoprecipitated from cell lysates using rabbit antibodies to the extracellular domain of VEGF-C (ImClone Systems), resolved by SDS-PAGE and transferred to nitrocellulose. Phosphotyrosine residues were detected by western blotting with the PY-20 antibody (Transduction Laboratories, Lexington, KY). Total VEGF-3 was detected using the SC-321 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Mitogenic assay
VEGF-CANAC-FMS cells (5×10^3 cells/well) were plated onto 96-well tissue culture plates (Wallach, Gaithersburg, MD) in 200 µl of serum-free medium and incubated at 37°C for 72 hours. Various amounts of antibodies were added and pre-incubated at 37°C for 1 hour, after which VEGF-CANAC or VEGF165 was added to a final concentration of 20 ng/ml. After 18 hours of incubation, 0.25 μCi of [3H]-Tdr (Amersham) was added to each well and incubated for an additional 4 hours. The cells were placed on ice, washed once with serum-containing medium, incubated for 10 minutes at 4°C with 10% TCA and solubilized in 25 µl of 2% SDS. Incorporated radioactivity was determined on a scintillation counter (Wallach, Model 1450 Microbeta Scintillation Counter).

Chemotaxis assay
Chemotaxis assays were performed using a 48-well chemotaxis chamber and 8 µm polycarbonate membranes coated with fibronectin. (Neuro Probe, Gaithersburg, MD). VEGF-CANAC or VEGF165 were added to the bottom wells at 5 ng/ml and 3×10^5 BAE cells were added to the top wells with or without indicated amounts of antibodies. After 4 hours at 37°C the cells on the top portion of the filter were removed by scraping and transmigrated cells were stained with Hoechst 33342. Membranes were mounted on glass slides and the nuclei were imaged using epifluorescence microscopy with a 20× objective. Images were analyzed by automated object counting using the AlphaEase software package (Alpha Innotech Corporation, San Leandro, CA).

In vitro angiogenesis assays
The BAE cell in vitro angiogenesis assay was performed as described (Mentesano and Orci, 1985). Cells were seeded onto 500 µl three-dimensional rat type I collagen gels in 16 mm tissue culture wells (Nunc), at 1.0×10^5 cells/well in 500 µl of MEM + 5% DCS. Upon reaching confluence (3 days), DCS was reduced to 2%, and the cells were treated with cytokines and antibodies. Medium, cytokines and antibodies were renewed after 2 days, and after a further 2 days cultures were photographed under phase contrast microscopy using a Sigma Diaphot TMD inverted photomicroscope (Nikon, Tokyo, Japan). Quantitation was performed as described (Pepper et al., 1992a).
Invasion was measured after 4 days and is expressed as the percent of total sprouting induced by cytokines alone. Results are shown as the means±s.e.m. from at least three experiments per condition.

The HUVEC in vitro angiogenesis assay was performed with the In Vitro Angiogenesis Assay Kit (Chemicon International, Temecula, CA) using the conditions provided by the manufacturer. Images were captured using the Nikon TE2000 inverted microscope using a 4× objective. The location of the endothelial tubes was drawn with lines of uniform thickness and a fixed gray level over the photographic images using Corel Photopaint software (Corel, Dallas, TX). The original images were removed and the total pixels corresponding to the original gray level of the drawing were measured using the Histogram function of the program.

Results
Characterization of human sR3-AP
The establishment of an assay used for the screening of antagonist antibodies to VEGFR-3 required the production of a soluble form of this receptor. We produced a chimeric protein by fusing the extracellular domain of human VEGFR-3 with secreted alkaline phosphatase and characterized the specificity of its binding to immobilized VEGF-CΔNΔC. The half-maximal concentration of sR3-AP was approximately 10 times lower than that of sR2-AP (Fig. 1A). The specificity of the soluble receptor was measured by binding to immobilized VEGF165 and VEGF-CΔNΔC. In accordance with published results, sR3-AP bound strongly to VEGF-CΔNΔC but not to VEGF165 (Fig. 1B). The affinity constant of sR3-AP for VEGF-CΔNΔC was measured by BIAcore at 3.5 nM (data not shown). This result is consistent with the previously reported value of 4.4 nM obtained with the same technique (Makinen et al., 2001b). Thus, sR3-AP was considered an appropriate reagent for the development of monoclonal antibodies to VEGFR-3.

Fig. 1. In vitro characterization of soluble human VEGFR-3 and mAb hF4-hF4-3C5. (A) Conditioned media from cells expressing human sR3-AP (■), human sR2-AP (□) or human sR1-AP (□) were normalized for AP activity and added to 96-well plates coated with VEGF-CΔNΔC. Bound receptors were detected with a rabbit antibody to AP and peroxidase-labeled secondary antibodies. sR3-AP binds to VEGF-CΔNΔC with an EC50 about ten times lower than sR2-AP whereas sR1-AP does not show binding activity. (B) Conditioned media were normalized for AP activity and added to 96-well plates coated with either VEGF-CΔNΔC or VEGF-A. The amount of bound receptor was measured using AP activity. R3-AP (clear bars) binds strongly to VEGF-CΔNΔC but not to VEGF-A. Control media from cells expressing untagged AP shows no binding (black bars). (C) Purified sR3-AP (▲), sR2-AP (○) or sR1-AP (■) were added to 96-well plates coated with hF4-3C5. The amount of bound receptor was measured using AP activity. sR3-AP binds to the mAb hF4-3C5 whereas sR1-AP or sR2-AP show no detectable binding. (D) Inhibition of sR3-AP binding to VEGF-CΔNΔC by the mAb hF4-3C5. Saturating amounts of sR3-AP were mixed with various amounts of mAbs or Fab fragments and added to 96-well plates coated with VEGF-CΔNΔC. Blocking of the receptor binding is evident with full-length hF4-3C5 (▲, IC50=1.3 nM), and Fab fragments of hF4-3C5 (□, IC50=2 nM) but not with the irrelevant human mAb IMC-2F8 (○).
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Isolation and functional characteristics of the monoclonal antibody hF4-3C5

After three rounds of panning on immobilized sR3-AP, 98% of isolated phage clones bound to VEGFR-3. The panned phage were screened for the ability to block the binding of sR3-AP to immobilized VEGF-C\textsubscript{ANAC} and clone hF4-3C5 was found to have the highest blocking activity. Soluble Fab fragments and full-length IgG forms of mAb hF4-3C5 were prepared and tested for binding and blocking of VEGFR-3. hF4-3C5 bound to sR3-AP but not to corresponding soluble forms of VEGFR-1 (sR1-AP) or VEGFR-2 (sR2-AP) (Fig. 1C). Fab and IgG forms of mAb hF4-3C5 strongly blocked the binding of sR3-AP to immobilized VEGF-C\textsubscript{ANAC} with an IC\textsubscript{50} of 2 and 1.3 nM, respectively (Fig. 1D). By contrast, the control antibody IMC-2F8, which targets the human IGF receptor was inactive. BIAcore kinetic analysis gave a $K_d$ of 56 pM for the binding of hF4-3C5 to immobilized sR3-AP (data not shown). Thus, the affinity of this antibody for VEGFR-3 is nearly 2 orders of magnitude greater than that of sR3-AP for VEGF-C\textsubscript{ANAC}.

Inhibition of VEGF-C\textsubscript{ANAC}-stimulated mitogenic response by mAb hF4-3C5

In order to test the ability of the mAb hF4-3C5 to inhibit signal transduction mediated by VEGFR-3, we prepared an NIH-3T3 cell line expressing a chimeric form of this receptor. As shown by western blotting, clone 3.1.2 expresses a fusion protein of the extracellular domain of human VEGFR-3 fused to the transmembrane and intracellular domains of cFMS (VEGFR-3-cFMS; Fig. 2A). No endogenous expression of VEGFR-3 by the parental cells could be detected. Localization of the chimeric receptor on the plasma membrane was shown by FACS analysis (Fig. 2B). The incorporation of $[^3]$H thymidine by the NIH-3T3 that express VEGFR-3-cFMS was stimulated by at least threefold by the addition of VEGF-C\textsubscript{ANAC} (Fig. 2C) but not VEGF165 (data not shown). The mitogenic response was specifically blocked in a dose-dependent manner by mAb hF4-3C5, with an IC\textsubscript{50} value of 5 nM (Fig. 2D).

Detection of VEGFR-3 on BAE cells and human LECs with hF4-3C5

We examined the expression of VEGFR-3 by BAE cells using FACS, with human LECs serving as positive controls (Fig. 3A). LECs were uniformly positive for VEGFR-3 with a relative fluorescence index (RFI) three times lower than seen with the anti-VEGFR-2 antibody IMC-1121. BAE cells were also uniformly positive for VEGFR-2 but, surprisingly, could be resolved by the reactivity with hF4-3C5 into roughly equal, VEGFR-3-positive and VEGFR-3-negative populations (Fig. 3A). The mean fluorescence intensity of the hF4-3C5-
positive sub-population was about threefold greater than that obtained with IMC-1121, whereas the RFI values were roughly similar.

VEGFR-3-positive BAE cells are not lymphatic in origin

The heterogeneity in VEGFR-3 expression observed in BAE cells raised the possibility that these primary endothelial cells contained cell populations of mixed vascular and lymphatic origin. We addressed this issue by examining the expression of the transcription factor Prox-1, which is a specific regulator of lymphatic endothelial development (Wigle and Oliver, 1999). For this analysis, we used a rabbit polyclonal antibody to human Prox-1 that was produced to homeodomain and prospero domain in the C-terminal portion of the protein. A minor shift of the BAE cells produced with the Prox-1 antibody relative to isotype-matched antibody (RFI=5) was within the error range obtained with various control antibodies (Fig. 3A). In contrast, LECs showed a significant staining for Prox-1 (RFI=32). Since the anti-Prox-1 antibody was made to the human protein, we compared the amino acid sequence of the homeodomain and prospero domains of human Prox-1 with the corresponding region of bovine Prox-1 obtained from an expressed sequence tag (est) database. The amino acid sequences of human and bovine proteins are identical, eliminating the possibility that negative staining obtained with BAE cells resulted from failure of the antibody to recognize bovine Prox-1. GenBank accession numbers used in this analysis are given in the Materials and Methods.

To further verify that the BAE cells did not contain cells of lymphatic origin, we prepared cDNA from LECs and BAE cells and used PCR to detect transcripts for Prox-1 and a second well-characterized marker of lymphatic endothelium, podoplanin (Kriehuber et al., 2001). Using primers designed to interact with regions of identity between human and bovine sequences, we failed to detect the expression of either marker by BAE cells, whereas amplification of LEC cDNA produced bands of expected size (Fig. 3B). Taken together, the FACS and PCR analysis demonstrate that the VEGFR-3-positive sub-population of BAE cells is not lymphatic in origin.

Abrogation of VEGF-CANAC-mediated in vitro angiogenic response by inhibition of VEGFR-3 signaling

We have previously reported that VEGF-CANAC, which binds to and activates VEGFR-2 and VEGFR-3 in BAE cells, induces sprouting, i.e. collagen gel invasion and tube formation (Pepper et al., 1998; Tille et al., 2003). However, we were unable to definitively establish the relative contributions of these two RTKs to the VEGF-CANAC response since the only blocking antibodies available were those to VEGFR-2. The availability of the mAb hF4-3C5 allowed us to test the role of VEGFR-3 directly. VEGF-CANAC-induced BAE cell invasion was inhibited in a dose-dependent manner by mAb hF4-3C5 with a maximal inhibition of 68% at 5 \( \mu \)g/ml (Figs 4, 5). A monoclonal anti-human VEGFR-2 antibody, IMC-1C11, also partially blocked VEGF-CANAC-induced BAE cell invasion with a maximal inhibition of 66% at 20 \( \mu \)g/ml (Fig. 5, Fig. 6A). The control antibody for hF4-3C5, namely anti-KLH, had no effect (Fig. 5, Fig. 6A). No toxicity was observed in any of the antibody-treated cultures (Fig. 5). Simultaneous addition of
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anti-VEGFR-2 and anti-VEGFR-3 antibodies resulted in complete inhibition of VEGF-C\textsubscript{DND} -induced in vitro angiogenesis (Fig. 5, Fig. 6A). Thus, both VEGFR-2 and VEGFR-3 mediate the angiogenic response of BAE cells to VEGF-C\textsubscript{DND}. Anti-VEGFR-3 antibody had no effect on VEGF\textsubscript{165} -induced BAE cell collagen invasion (Fig. 5, Fig. 6B), whereas IMC-1C11 totally blocked VEGF\textsubscript{165} activity (Fig. 6B). The control antibody for IMC 1C11, IMC-C225, had no effect (Fig. 6B).

Similar results were obtained with HUVECs. The preparation of these cells used in our experiments expressed nearly equal levels of VEGFR-3 and VEGFR-2, as demonstrated by FACS analysis with antibodies hf4-3C5 and IMC-1121 (Fig. 7A). Addition of IMC-1C11 or hf4-3C5 alone reduced the HUVEC tube formation by 59% and 63%, respectively, and the effect of the two antibodies was additive (82% inhibition) (Fig. 7B,C). As a second means of quantitation, we counted the total branch points observed under each culture condition. In the presence of antibodies IMC-1C11 or hf4-3C5, branching was reduced by 61% and 13%, respectively. Addition of the both antibodies inhibited tube branching by 87% of control (Fig. 7C).

Inhibition of VEGFR-3 but not VEGFR-2 signaling blocks the chemotactic response of BAE cells to VEGF-C\textsubscript{DND}

Since the assay used in our study involves gel invasion prior to tube formation, we determined whether directional migration of BAE cells in response to VEGF-C\textsubscript{DND} could be blocked by the inhibition of VEGFR-3. BAE cells migrated towards VEGF\textsubscript{165} and VEGF-C\textsubscript{DND} with the maximum response reached at 2 ng/ml of either ligand (see supplemental Fig. S1, http://jcs.biologists.org/supplemental/). In Boyden chamber experiments, the chemotactic response to VEGF-C\textsubscript{DND} was reduced in a dose-dependent manner by the addition of mAb hf4-3C5 (Fig. 8A). At the maximum antibody dose (100 nM), 85% of BAE cell transmigration was inhibited. Blocking of VEGF-2 by the addition of mAb IMC-1C11 had only a minimal effect (maximum 20% inhibition) and this effect was not dose-dependent. Simultaneous addition of both antibodies showed no additive effects. The apparent lack of effect of VEGF-2 inhibition on VEGF-C\textsubscript{DND} -induced BAE cell transmigration was unexpected since VEGF-C\textsubscript{DND} is known to bind to and activate VEGFR-3 (Joukov et al., 1996). Thus, we investigated the possibility that mAb IMC-1C11 is ineffective in blocking the binding of VEGF-C\textsubscript{DND} to VEGFR-2 since this antibody was developed as an inhibitor of VEGF\textsubscript{2}--VEGF\textsubscript{165} interaction (Zhu et al., 1998). However, binding of soluble VEGF-2 to VEGF-C\textsubscript{DND} was blocked in a dose-dependent manner by mAb IMC-1C11 (Fig. 8B). Since the inability to block the chemotactic response of BAE cells to

![Fig. 4. Partial inhibition of VEGF-C\textsubscript{DND} -induced in vitro angiogenesis by blocking antibody to VEGFR-3. Confluent BAE cells monolayers on three-dimensional collagen gels were treated with VEGF-C\textsubscript{DND} (100 ng/ml). Monoclonal anti-human VEGFR-3 antibody hf4-3C5 was added at the concentrations indicated. Invasion was measured after 4 days and is expressed as percent of sprouting induced by VEGF-C\textsubscript{DND} alone. Results are shown as the means±s.d. of three wells.](image)

![Fig. 5. Inhibition of VEGF-C\textsubscript{DND} -induced BAE cell in vitro angiogenesis by blocking antibody to VEGFR-2 and VEGFR-3 simultaneously. Confluent BAE cell monolayers on three-dimensional collagen gels (Control) were treated for 4 days with VEGF-C\textsubscript{DND} (100 ng/ml) alone or in combination with a neutralizing anti-human VEGFR-3 monoclonal antibody (hf4-3C5, 5 μg/ml), a neutralizing anti-human VEGFR-2 monoclonal antibody (IMC-1C11, 20 μg/ml) or a control antibody (anti-KHL, 20 μg/ml). Cell cord formation within the collagen gel was viewed by phase-contrast microscopy. Co-addition of the neutralizing anti-VEGFR-3 or anti-VEGFR-2 antibodies alone partially inhibited invasion induced by VEGF-C\textsubscript{DND}, whereas co-addition of both antibodies completely blocked invasion induced by VEGF-C\textsubscript{DND}. Cells treated with IMC-1C11 or hf4-3C5 alone showed no signs of cytotoxicity. No invasion occurred in untreated cultures. Bar, 100 μm.](image)
VEGF-C by inhibiting VEGFR-2 was unexpected, we asked whether the migrating and non-migrating populations of BAE cells were phenotypically equivalent with respect to VEGFR-3 expression. We recovered the non-migrated cells from the upper wells of the migration chamber and the migrated cells from the lower aspects of the membranes and analyzed the VEGFR-3 expression of these cell populations by FACS. When VEGF-C\textsubscript{DND} was used as a chemoattractant, the cells that transmigrated were nearly all positive for VEGFR-3 (Fig. 9). The non-migrated population was indistinguishable from the starting BAE cell population (Fig. 3A) with about half of the cells being VEGFR-3-positive. In contrast, when VEGF\textsubscript{165} was used as a chemoattractant, the pattern of VEGFR-3 staining did not differ significantly between the migrating and non-migrating populations. In fact, we observed a slight but consistent reduction in the proportion of VEGFR-3-positive cells in the migrating population and a corresponding increase in the proportion of VEGFR-3-positive cells in the non-migrating population. VEGFR-2 expression was not significantly different in the non-migrated and migrated cell populations.

![Fig. 6. Quantitation of anti-VEGFR-3 and anti-VEGFR-2 antibody-mediated inhibition of in vitro angiogenesis in BAE cells. Confluent BAE cell monolayers on three-dimensional collagen gels were treated with VEGF-C\textsubscript{ANAC} (100 ng/ml) (A) or VEGF\textsubscript{165} (100 ng/ml) (B). Anti-human VEGFR-3 antibody (hF4-3C5), anti-human VEGFR-2 monoclonal antibody (IMC-1C11) and control antibodies (anti-KLH for hF4-3C5, IMC-C225 for IMC-1C11) were added. Invasion was measured after 4 days and is expressed as percent of sprouting induced by cytokine alone. (A) hF4-3C5 and IMC-1C11 inhibited VEGF-C\textsubscript{ANAC}-induced BAE cell invasion by 68% at 5 \(\mu\)g/ml and 66% at 20 \(\mu\)g/ml, respectively. A complete inhibition of VEGF-C\textsubscript{ANAC}-induced BAE cell invasion was obtained by the co-addition of both antibodies, whereas the control antibody (KLH) had no effect on invasion at 25 \(\mu\)g/ml. (B) When added to VEGF-A-treated BAE cells, hF4-3C5 antibody had no effect at 5 \(\mu\)g/ml, whereas IMC-1C11 at 20 \(\mu\)g/ml totally blocked invasion. The isotope control antibody for IMC-C225 had no effect on invasion. Results are shown as the means±s.e.m. from at least three experiments per condition.

![Fig. 7. Inhibition of HUVEC in vitro angiogenesis by anti-VEGFR-3 and anti-VEGFR-2 antibodies. (A) HUVECs were analyzed by FACS for expression of surface VEGFR-2 and VEGFR-3 using monoclonal antibodies IMC-1121 and hF4-3C5. Both receptors were detected, with approximately equal staining intensity. (B) HUVECs were grown for 24 hours on a solid gel of basement matrix proteins (Chemicon In Vitro Angiogenesis Kit) in the presence or absence of monoclonal antibodies. The images were captured using a 4× objective of a Nikon TE2000 inverted microscope. Blocking of tube formation is evident with either mAb IMC-1C11 or hF4-3C5. Simultaneous addition of both antibodies increases the inhibitory effect. Bar, 100 \(\mu\)M. (C) Total sprouting response was measured in replicate wells using digital imaging (see Materials and Methods). Branching was counted manually as the number of tube branch points per field. The results are shown as percent inhibition relative to the average of untreated controls. The extent of tube-forming inhibition is the same whether total sprouting or branching is measured.
migrated BAE cells irrespective of the ligand used (data not shown). We conclude that that expression of VEGFR-3 endows BAE cells with an enhanced ability to migrate in response to VEGF-C compared with cells that express VEGFR-2 alone.

**Discussion**

VEGFR-3 plays a critical role in the proliferation and survival of LECs during embryogenesis and in postnatal life (Lymboussaki et al., 1998; Veikkola et al., 2001; Makinen et al., 2001b). The role of VEGFR-3 in the development of embryonic vasculature is equally well established since homozygous null VEGFR-3 mice die in utero owing to malformations of large vessels and the heart (Dumont et al., 1998).

Whether VEGFR-3 plays a significant role in the biology of adult vascular endothelial cells has been difficult to establish experimentally in part because the mature form of its primary ligand, VEGF-C, also binds to and activates VEGFR-2. Antagonist antibodies to VEGFR-1 and VEGFR-2 have shown great utility as investigational tools in delineating the function of these receptors in the normal and pathological responses of HUVECs (Zhu et al., 2002). Extending this approach to the study of VEGFR-3, we have developed the fully human antibody hF4-3C5, which displays excellent biochemical characteristics as a VEGFR-3 antagonist. The $K_d$ of the antibody is two orders of magnitude greater than that of VEGF-C binding to VEGFR-3. hF4-3C5 strongly blocks in vitro binding of soluble VEGFR-3 to VEGF-C (IC$_{50}=1.3$ nM) and inhibits the mitogenic response to VEGF-C by NIH 3T3 cells that express chimeric VEGFR-3-cFMS receptor (IC$_{50}=5$ nM). To our knowledge, hF4-3C5 is the first antagonist monoclonal antibody developed to human VEGFR-3.

We utilized hF4-3C5 to address the role of VEGFR-3 in an in vitro assay in which addition of VEGF165 or VEGF-C to NIH 3T3 cells express chimeric VEGFR-3-cFMS receptor (IC$_{50}=5$ nM). To our knowledge, hF4-3C5 is the first antagonist monoclonal antibody developed to human VEGFR-3.

**Fig. 8.** Effect of VEGFR-3 inhibition on the transmigration of BAE cells in response to VEGF-C$_{ANAC}$. BAE cells were induced to transmigrate through 8 µm polycarbonate filters by the addition of 5 ng/ml ligand to the bottom well; transmigrating cells were stained with Hoechst stain, imaged under epifluorescence using a 20x lens and counted using automatic image analysis (see Materials and Methods). (A) Chemotaxis towards VEGF-C$_{ANAC}$ is inhibited in a dose-dependent manner by mAb hF4-3C5 (■) whereas mAb IMC-1C11 (○) has no effect. Combination of the two antibodies showed no additive effect (△). Results are shown as the means±s.e.m. (Inserts) Examples of transmigrated cells stained by Hoechst and used in automated cell counting; (A1) no antibody; (A2) 100 nM each of hF4-3C5 and IMC-1C11. (B) Saturating amounts of VEGFR-2-AP were mixed with various amounts of mAbs and added to 96-well plates coated with VEGF-C$_{ANAC}$. Bound receptor was measured using AP activity. Blocking of the receptor binding is evident with the mAb IMC-1C11 (○) but not with the irrelevant mAb IMC-C225 (□).

**Fig. 9.** The VEGFR-3 (+) subpopulation of BAE cells transmigrates more efficiently in response to VEGF-C$_{ANAC}$. BAE cells were induced to transmigrate as described in Fig. 8. Non-migrating cells were removed from the upper aspect of the membrane by scraping and the transmigrated cells were released from the lower surface with protease-free cell dissociation buffer. Expression of VEGFR-3 was assayed by FACS as described in Fig. 3, and the RFI of each peak is shown in yellow. Each panel shows the staining in the absence (green) and presence (blue) of hF4-3C5. The cells that did not migrate in response to VEGF-C$_{ANAC}$ (non-migrated) exhibit the bi-modal pattern of VEGFR-3 expression with about 50% of positive cells. The BAE cells that transmigrated in response to VEGF-C$_{ANAC}$ (Migrated) are approximately 90% positive for VEGFR-3. This shift is reversed seen when the BAE cells transmigrate in response to VEGF165 with the majority of cells being negative for VEGFR-3.
addition of hF4-3C5 blocked 65% of VEGF-C<sub>ANAC</sub>-induced BAE cell invasion but it had no effect on the VEGF165-induced response. Simultaneous addition of hF4-3C5 and IMC-1121 completely abolished invasion and tube formation demonstrating that both VEGFR-3 and anti-VEGFR-2 mediate the in vitro tubulogenic response to VEGF-C<sub>ANAC</sub>. This result agrees with the findings of Pepper et al. that showed strong synergy between VEGF<sub>165</sub> and VEGF-C in the induction of collagen gel invasion (Pepper et al., 1998). We found that only ~50% of BAE cells expressed VEGFR-3, as demonstrated by binding of hF4-3C5, whereas all the cells expressed VEGFR-2. BAE cells are a primary culture of endothelium derived from bovine aorta (Pepper et al., 1992b). Thus, the heterogeneity in VEGF-C<sub>expression</sub> could indicate that the BAE cells used in our studies are a mixture of cells of vascular and lymphatic origin. However, we ruled out this possibility by showing that these cells do not express either of the well-established markers of lymphatic endothelium, Prox-1 and podoplanin (Wigle and Oliver, 1999; Kriehuber et al., 2001; Veikkola et al., 2003).

The VEGF-C<sub>3</sub> ligand VEGF-D was previously shown to induce tube formation by HUVECs but the authors were unable to discern whether VEGFR-2 or VEGFR-3 mediated the tubulogenic response of the cells (Marcocini et al., 1999). Our studies with hF4-3C5 showed that, in contrast to BAE cells, HUVECs uniformly express VEGF-3. The effects of individually added h4-3C5 and IMC-1C11 on the tubulogenic response of HUVECs were almost identical to those observed with BAE cells cells (about 60% inhibition), although their additive effect was less pronounced (82% vs. 100%). This difference can be explained in part by the fact that, in the assay used with HUVECs, the activating growth factor mixture is supplied by the gel matrix. Under these conditions, ligands for receptors other than VEGF-2 and VEGF-3 may be mediating a minor part of the response.

Our results indicate that VEGFR-3 mediates a significant part of the in vitro tubulogenic response of HUVECs upon stimulation by VEGF-C<sub>ANAC</sub>. This finding provides a mechanistic basis for some observations from animal models. For example, rat mAb AFL-4 directed to VEGFR-3 has been shown to suppress growth of tumor xenografts principally by an anti-angiogenic mechanism since disruption of microvasculature was observed both at the microscopic and electronmicroscopic level (Kubo et al., 2000).

The invasion of the collagen gel prior to tube formation can be thought of as the in vitro equivalent of HUVEC migration that occurs during the initial stages of angiogenesis. The design of the tube-forming assay makes it difficult to discern whether the inhibitory effect of an antibody is due to the inhibition of cell migration or the assembly into tubes. Thus, we used hF4-3C5 to directly examine the role of VEGF-3 in the chemotactic response of BAE cells to VEGF-C<sub>ANAC</sub>. BAE cells migrated towards VEGF<sub>165</sub> and VEGF-C<sub>ANAC</sub> and, as expected, the migration induced by VEGF<sub>165</sub> was unaffected by hF4-3C5. The chemotactic response to VEGF-C<sub>ANAC</sub> was reduced in a dose-dependent manner to a maximum of 82%. Surprisingly, whereas inhibition of VEGFR-2 with IMC-1C11 strongly reduced BAE cell migration in response to VEGF-C<sub>165</sub>, IMC-1121 had no effect on the response to VEGF-C<sub>ANAC</sub> either alone or together with hF4-3C5.

We considered the possibility that IMC-1C11, originally developed as a blocker of VEGFR-2-VEGF<sub>165</sub> interaction (Zhu et al., 1998), was ineffective owing to the presence of distinct binding sites on VEGFR-2 for VEGF<sub>165</sub> and VEGF-C<sub>ANAC</sub>. We ruled this out by directly demonstrating that IMC-1121 effectively blocks the interaction of VEGFR-2 with VEGF-C<sub>ANAC</sub>. It also seems unlikely that VEGF-C<sub>ANAC</sub> fails to activate sufficiently VEGFR-2 on BAE cells to initiate migration since porcine endothelial cells transfected with a VEGF-C<sub>expression</sub> plasmid efficiently migrate in response to VEGF-C<sub>ANAC</sub> (Cao et al., 1998). A more likely explanation of our results is based on the heterogeneity of VEGFR-3 expression by BAE cells used in our studies. Using FACS analysis, we found that nearly all the cells that transmigrated in response to VEGF-C<sub>ANAC</sub> were VEGFR-3-positive, representing a significant enrichment over the 50% found in the starting population. This effect was specific for the response to VEGF-C<sub>ANAC</sub> as the proportion of VEGFR-3-positive cells was actually slightly reduced in the population of BAE cells that migrated in response to VEGF<sub>165</sub>. Instead, we propose that the BAE cells that express VEGFR-3 have a competitive advantage in the chemotactic response to VEGF-C<sub>ANAC</sub> over the cells that express only VEGFR-2.

The most straightforward explanation for the migratory advantage of the VEGFR-3-expressing BAE cells is that the affinity of VEGF-C<sub>ANAC</sub> for VEGF-C<sub>3</sub> is about fivefold higher than for VEGF-C<sub>2</sub> (Makinen et al., 2001b). Thus, stimulation of VEGF-C<sub>3</sub> by VEGF-C<sub>ANAC</sub> is likely to be stronger and more sustained than that of VEGF-C<sub>2</sub>. The activation of both VEGFR-2 and VEGFR-3 results in the phosphorylation of the related focal adhesion kinase RAFTK/Pyk2 that is thought to coordinate cytokine receptor and extracellular matrix (ECM) signaling through its association with the cytoskeletal protein paxillin (Liu et al., 1997; Ganju et al., 1998). It is reasonable to speculate that BAE cells that express VEGFR-3 in addition to VEGFR-2 are able to initiate more efficiently responses required for cell motility, such as adherence to the extracellular matrix and cytoskeletal reorganization. It is also possible that VEGF-C<sub>ANAC</sub>-stimulated migration is enhanced by intermolecular associations. In a recent report, Dixelius et al. demonstrated heterodimerization between VEGFR-2 and VEGFR-3 in LECs and suggested that this association alters the signaling properties of the two receptors (Dixelius et al., 2003). In addition, cross-linking of the β<sub>1</sub> integrin has been shown to induce tyrosine phosphorylation of VEGF-3 and increases cell motility (Wang et al., 2001).

In conclusion, we used a novel antagonist antibody, hF4-3C5, to directly show for the first time that VEGF-3 plays a significant role in migration and in vitro tubular morphogenesis of vascular endothelial cells in response to VEGF-C<sub>ANAC</sub>.

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