

Quantification of vascular endothelial growth factor-C (VEGF-C) by a novel ELISA

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Abstract

Lymphangiogenesis plays an important role in several normal and pathological conditions such as wound healing, inflammation or metastasis formation in several malignancies. VEGF-C and VEGF-D are important and specific regulatory factors for lymphatic endothelial proliferation and lymphangiogenesis. In order to develop a highly sensitive and specific detection system for VEGF-C, we produced soluble binding proteins and antibodies for a microtiterplate-based assay. Here we describe a specific enzyme-linked immunosorbent assay (ELISA) for the measurement of human, rat and murine VEGF-C. The different antibodies developed against human and rat VEGF-C could be combined to detect processed and partially processed VEGF-C in a specific way. The ELISA was able to detect human and rat VEGF-C with a minimum detection limit of 100 pg/ml. The assay did not show any cross-reactivity with the related protein VEGF-D. Furthermore, complex formation with its soluble receptors VEGFR-2 and VEGFR-3 did not restrict the sensitivity of the assay. Using this assay, VEGF-C was measured in supernatants and lysates of different cell types and in tumour tissue samples of murine, rat and human origin. Cell lines secrete VEGF-C in very low amounts (<1 ng/ml) whereas VEGF-C transfected cells can secrete up to 50 ng/ml VEGF-C into the supernatant. In human tumour tissue samples VEGF-C was detected in some carcinomas in the low protein range. This ELISA will be a useful tool for investigations concerning the physiological function of VEGF-C in lymphangiogenesis under normal and pathophysiological conditions.

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Abbreviations: VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor 1; KDR, kinase domain region receptor; flt-1, *fms*-like tyrosine kinase; VEGFR-3, vascular endothelial growth factor receptor 3; ELISA, enzyme-linked immunosorbent assay.

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1. Introduction

The growth of lymphatic vessels is called lymphangiogenesis and occurs after tissue injury, obstruction or damage of lymphatic vessels (Junghans and Collin, 1989). The lymphatic system serves to reduce the increased tissue pressure associated with edema and inflammation (Pullinger and Florey, 1937). In the last 5–7 years a picture has emerged, that the receptor tyrosine kinase VEGFR-3 (Flt-4) and its ligands are the key players in the molecular regulation of lymphangiogenesis (Korpelaionen and Alitalo, 1998). The two ligands VEGF-C and VEGF-D induce the phosphorylation of VEGFR-3 and regulate the growth and differentiation of lymphatic endothelium (LE) either alone or in combination with other growth factors (Joukov et al., 1996; Achen et al., 1998). Both ligands are members of the PDGF/VEGF super family and are virtually identical in their molecular properties. They are produced as precursor molecules and then progressively processed during their biosynthesis to remove the N- and C-terminal ends of the proteins (Joukov et al., 1997a,b). This processing increases the affinity of VEGF-C and VEGF-D for VEGFR-3 (Joukov et al., 1997a; Achen et al., 1998), but fully processed forms of both ligands have been demonstrated to activate also VEGFR-2. Although they are less potent activators of VEGFR-2 than of VEGFR-3, they contribute to normal angiogenesis (Achen et al., 1998; Joukov et al., 1997a,b; Stacker et al., 1999). However, after binding to VEGF-C and VEGF-D, VEGFR-3 is capable of transducing signals triggering the proliferation of VEGFR-3 expressing cells in vitro and in vivo (Jeltsch et al., 1997; Oh et al., 1997; Veikkola et al., 2001). Blocking of VEGFR-3 activation inhibits the formation of lymphatic vessels in the developing embryo (Oh et al., 1997). VEGFR-3 and its ligands may play an important role in several pathologic conditions where lymphangiogenesis occurs or the function of the lymphatics is involved. More recent studies suggest that patients with mutations in the VEGFR-3 gene develop lymphedema (Irrthum et al., 2000; Karkkainen et al., 2000). However, several normal tissues (e.g. heart and lung) are also known to express high rates of VEGF-C gene. This suggests that VEGF-C is probably necessary for tissue homeostasis but the exact physiological role is unknown (Joukov et al., 1997a,b; Kirkin et al., 2001).

Very recently, an indirect ELISA was established for the measurement VEGF-C in plasma samples from cancer patients (Duff et al., 2003). In this assay, it was not possible to use purified VEGF-C standards, and for that reason only indirect statements can be made about the amount and distribution of VEGF-C in plasma samples. According to this report, colorectal cancer patients have three-fold increased VEGF-C levels in plasma compared to controls (Duff et al., 2003).

For the detection of VEGF-C protein production and secretion in human tissue and in animal models, a reliable and sensitive assay has hitherto not been available so. The objective of this work was therefore to develop such an assay for the detection and quantification of processed forms of VEGF-C. This goal was achieved by establishing of a sandwich ELISA format for VEGF-C that can be used to detect and measure VEGF-C in human tissue, tumours and in several animal or cellular models. VEGF-C amounts in the picogram range can be measured with this new ELISA, and we present here quantitative data for VEGF-C in human tissue samples and various cell culture supernatants for the first time.

2. Materials and methods

2.1. Proteins and antibodies

The fully processed rat protein dNc-VEGF-C and dNc-VEGF-D were produced in insect cells and purified from supernatants as previously reported (Kirkin et al., 2001). The processed rat VEGF-C is virtually identical to mouse VEGF-C. Human dNc-VEGF-C and soluble VEGFR-3 production was achieved as described before (Joukov et al., 1997a,b; Hornig et al., 1999). Because all commercially available antibodies against VEGF-C were found to be unsuitable for ELISA development, a polyclonal antibody against rat VEGF-C was developed in rabbits (antibody 4080; BioGenes Berlin). The procedure was similar to that described for soluble VEGFR-2 (Röckl et al., 1998). Briefly, a total amount of 1.2 mg rat VEGF-C (containing a C-terminal 6His-tag) was used for immunization of two New Zealand white rabbits. After immunisation with 0.1 mg protein, each rabbit was boosted on days 7,

14, 28, 56 and 84 with the same amount. The dilution of the serum for half maximal titer was 1:10,000. Total IgG from rabbit serum was isolated using HiTrap Protein A Sepharose columns (Amersham Bioscience, Freiburg). The anti-VEGF-D antibody 4292 has been described previously (Kirkin et al., 2001).

2.2. Generations of cell lines, serum-free cell culture supernatants, lysates and tissue sample preparation

Experiments were conducted using three human (PC-3 cells, 293 cells, COLO 800 cells) and four rat (10AS, ARIP, BRL3A, MT-450) tumour cell lines. The COLO-800 cell line was purchased from DSMZ, Braunschweig. The PC-3, BRL3A and ARIP cell lines were maintained as monolayer cultures in Ham's F-12 medium (Flow Laboratories, Irvine, Scotland) at 37 °C in a humidified incubator with 5% CO₂. The 10AS and COLO-800 cell lines were cultured in RPMI-1640 medium (Gibco-BRL, Bethesda, MD). The wild type 293 and MT450 cell lines were cultured in DMEM medium. The 293 cells were co-transfected with a rat VEGF-C expression construct (Krishan et al., 2003) and a neomycin resistance vector using GenePORTER (Gene Therapy Systems). Stable transfected clones were selected and tested for VEGF-C expression and secretion using a polyclonal anti-VEGF-C antiserum (Kirkin et al., 2001). The transfected 293 cells were cultured in DMEM medium in the presence of neomycin. All medium was supplemented with 10% fetal bovine serum (Greiner, Germany).

The cells were grown to 80% confluence in 75-cm² tissue culture flasks (Nunc, Roskilde, Denmark). Conditioned media was collected under low-serum (2%) growth conditions. In some cases conditioned media was concentrated 10-fold by ultra-filtration using 10,000 Da cut-off membranes (Vivascience, Hannover, Germany). Tissues from transgenic RIP-Tag mice overexpressing mouse VEGF-C were a gift from Gerhard Christofori, Basel. Tissue lysates from different human tumours were either from the Metropolitan Komagome Hospital in Tokyo or from the Department of Pathology at the University of Kiel. All patients signed an informed consent according to a protocol approved by the ethics committees in each institute.

Tumour tissues were snap frozen in liquid nitrogen and homogenized in RIPA buffer (0.1% SDS, 1% IGEPAL CA-630, 0.5% Na-deoxycholate, protease-inhibitor cocktail in phosphate-buffered saline). Protein concentrations were estimated according to standard protocols (BCA assay, Pierce, Rockford).

2.3. Immunoblotting

Samples were loaded onto 12–15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and separated under reducing conditions. Immunoblotting onto PVDF membrane (Millipore, Bedford, USA) was performed for 20 min at 15 V in a semi-dry blotting chamber. The membrane was blocked with 20% non-fat-milk in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 8.0) for 1 h at room temperature. Antibody 4080 was used at 2 µg/ml IgG diluted in TBS containing 10% non-fat milk for 1 h at room temperature. The anti-VEGF-D antibody 4292 was used at 10 µg/ml IgG. The secondary antibodies and the ECL detection kit were used according to the instructions of the manufacturer (Amersham Bioscience, Freiburg).

2.4. VEGF-C sandwich-ELISA

The VEGF-C antibody 4080 was isolated from serum using HiTrap Protein-A Sepharose columns. Then, depletion of the anti-his antibody fraction was done by antigen-affinity purification using 10 mg of immobilized 6H-tagged TxnTb protein (Tryparedoxin (Txn) from *Trypanosoma brucei* (Tb)). Antigen-affinity purification for the antibody 4080 was performed by immobilising 1 mg rat VEGF-C on an NHS-activated HiTrap column (Amersham Bioscience, Freiburg). The development of a highly sensitive and specific sandwich ELISA for VEGF-C was done using standard methods (Harlow and Lane, 1988). Briefly, 10 µg/ml rabbit IgG 4080 was used for coating and the antigen-affinity purified and biotinylated antibody 4080 at 1 µg/ml was used as a detector antibody. Biotinylation of antibody 4080 was done with 6 mg IgG in 100 mM carbonate buffer, pH 8.5 at 3 mg/ml with using biotin-amidohexanoic acid NHS (Sigma, St. Louis, USA). The molecular ratio biotin:protein was 30:1. As a standard, human and rat dNdc-VEGF-C was used over a concentration range

between 0.1 and 6.25 ng/ml. For visualisation of the detector, streptavidin-enzyme conjugate was used (Endogen, Woburn, MA) followed by the addition of TMB (tetra-methyl-benzidine; Roche Mannheim, Germany). After stopping the reaction with 1 M H₂SO₄, the absorbance was measured at 450 and 620 nm with an ELISA plate reader (Labsystems, Finland). Generally, the samples were analyzed in different dilutions, measuring each dilution in duplicate. Samples were diluted at least 1/2 diluted with sample diluent (BenderMedSystems, Vienna).

2.5. RNA preparation, Northern blotting and PCR

Polyadenylated RNA was prepared from snap-frozen rat multiple tissues. Then, Northern blots using 1% agarose/formaldehyde gels and 5 µg polyadenylated RNA were performed as previously described (Hoffmann et al., 1998). Blots were performed at high stringency using VEGF-C (partial rat VEGF-C cDNA sequence, GenBank accession No. AF10302) for Northern blots of RNA from multiple tissue types. RNA gels were therefore stained with ethidium bromide and gels were inspected under UV light before and after Northern transfer to ensure that equivalent RNA was present in each lane.

2.6. RNA isolation for reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the cells with TRIzol (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. cDNAs were prepared using 5 µg of total RNA and Superscript II reverse transcriptase (Gibco-BRL) with oligo (dT)_{12–18} primers and kept frozen at –80 °C until use. The resulting cDNAs were used to amplify the VEGF-C and β-actin using 5' and 3' primers and Taq polymerase (Invitrogen). The 5' and 3' primers for human VEGF-C was 5'-AGACTCAATGCATGCCACG-3' and 5'-TTGAGTCATCTCCAGCATCC-3', respectively, and those for β-actin were 5'-ATCTCCTTCTGCATCCTGTC-3' and 5'-GACGAGGCCCA-GAGCAAGAG-3', respectively.

The conditions for the PCR were as follows: 95 °C for 5 min, 32 cycles of 30 s at 95 °C, 30 s at 53 °C, and 45 s at 72 °C, followed by a 10-min extension step at 72 °C for VEGF-C, and 94 °C for 2 min, 30

cycles of 20 s at 94 °C, 20 s at 57 °C, and 30 s at 72 °C, followed by a 10-min extension step at 72 °C for β-actin. The number of cycles in the RT-PCR was determined to yield a logarithmic range of amplification of each gene for semi-quantitative analysis of the expression levels of the two genes. The plasmid pCRII with the human VEGF-C cDNA was used as a positive control (Joukov et al., 1996). The RT-PCR

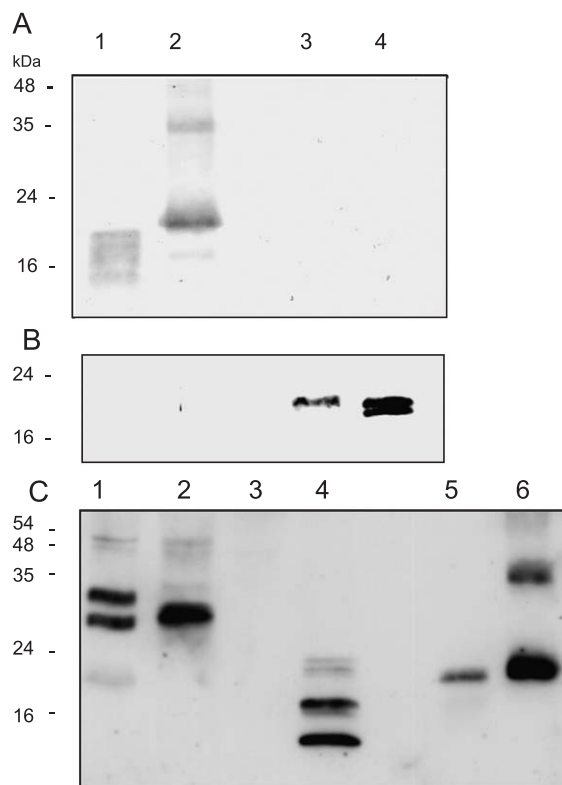


Fig. 1. Immunoblot with human and rat VEGF-C and VEGF-D using the polyclonal VEGF-C antibody 4080/his-depleted (A and C) and the anti-VEGF-D antibody 4292 (B). Proteins were separated by 12–15% SDS-PAGE and blotted on to PVDF membranes as described. After saturation, the membrane was incubated for 1 h with the AB 4080/his-depleted at 1–2 mg/ml in TBS containing 20% non-fat milk followed by incubation with a goat-anti-rabbit alkaline phosphatase-conjugated secondary antibody. A and B: Lane 1: 250 ng rat VEGF-C; lane 2: 250 ng human VEGF-C; lane 3: 250 ng rat VEGF-D; lane 4: 250 ng human VEGF-D. C: Lane 1: 150 µl 293 cell/VEGF-C supernatant; lane 2: 50 µg RIP TAG tumour lysate; lane 3: 10 µg insect cell lysate; lane 4: 10 µg rVEGF-C insect cell lysate; lane 5: 50 ng dNc-rat VEGF-C; lane 6: 50 ng dNc-human VEGF-C.

products were analysed by 2% agarose gel electrophoresis with ethidium bromide and documented.

3. Results

3.1. Characterization of the anti-VEGF-C polyclonal antibodies

First approaches to develop a VEGF-C ELISA were based on the use of commercially available antibodies but a satisfying antibody pair could not be selected. For this reason, we produced a polyclonal antibody in rabbits using the fully processed form of recombinant rat VEGF-C for the immunization. The serum was further purified by Protein A chromatography and by antigen-affinity purification to obtain pure rabbit IgG. Immunoblot analysis with human and rat VEGF-C and VEGF-D revealed that the antibody 4080 was specific for human and rat VEGF-C. Both proteins were recognized with similar sensitivities (Fig. 1A). This antibody showed no cross-reactivity with human and rat VEGF-D (Fig. 1B), the most closely related proteins to VEGF-C.

Immunoblot analysis of VEGF-C containing tissues and secreting cells demonstrated that the antibody 4080 was able to recognize all forms of the molecule. Supernatants from rat VEGF-C transfected 293 cells exhibited three bands, most likely different glycosylated and processed forms of the mouse protein (Fig. 1C). In the transgenic RIP Tag mouse tumour overexpressing VEGF-C, only one band at 28 kDa could be detected in tissue lysates, indicating that fully processed forms were not generated in this model (Fig. 1C). Baculovirus-infected insect cell lysates contained unglycosylated and glycosylated forms of processed rat VEGF-C (dNdC-VEGF-C). The human fully processed VEGF-C from *Pichia pastoris* contained a main band at 22 kDa and a second band of 35 kDa (Fig. 1C).

3.2. Sensitivity, cross-reactivity and reproducibility of the sandwich ELISA for VEGF-C

Surprisingly, a substantial part of the antiserum was cross-reactive with the C-terminal 6H-tag of the antigen. Therefore, his-tag depletion by antigen-affinity chromatography significantly reduced the

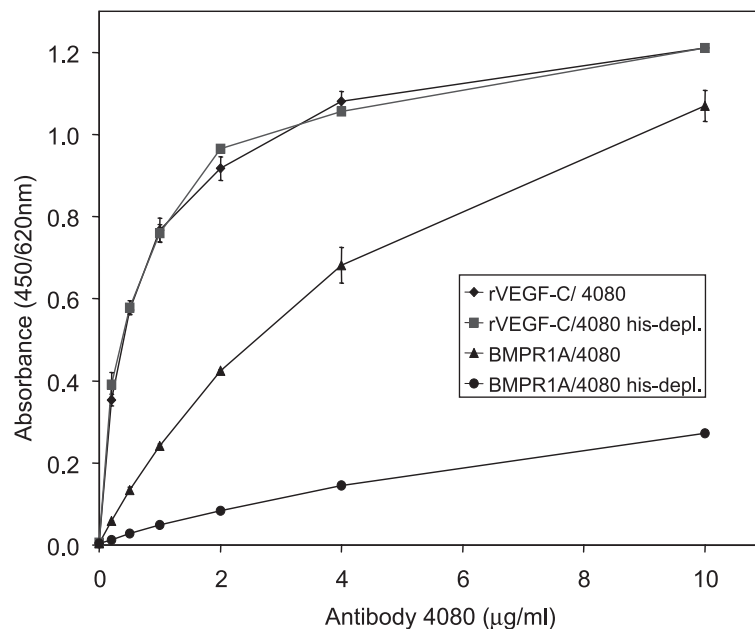


Fig. 2. Cross-reactivity of the 4080 antibody with VEGF-C and his-tagged proteins after anti-his-tag depletion. Rat VEGF-C and an unrelated his-tagged protein (BMPR1A) were coated at 2.5 µg/ml and detected by an increasing amount of the 4080 antibody before and after his-tag depletion as described. All measurements were performed in duplicate. Results show the mean absorbance at 450 nm as mean values.

cross-reactivity with other his-tag proteins, without modifying the affinity of the antiserum for VEGF-C (Fig. 2). The same polyclonal antibody 4080 was used as both capture and detector antibody and recognized different epitopes of the native, fully processed protein. The detector antibody was additionally antigen-affinity purified and biotinylated. This combination resulted in the most sensitive ELISA. A typical standard curve obtained with the ELISA is shown in Fig. 3. The minimum detection limit estimated by serial dilution was about 50 pg/ml of recombinant human and rat VEGF-C. Human VEGF-C was less readily detected than rat VEGF-C but this could be overcome by a threefold elongated incubation during colour development. If the sensitivity of the assay was determined as a significant difference from zero (two S.D. differences from the mean of the blank) using the data from Fig. 3, we calculated for rat VEGF-C a value of 6.9 pg/ml and for human VEGF-C a value of 54.5 pg/ml. As expected, VEGF-D was not recognized (Fig. 3). We also analysed the less closely related proteins VEGF-A, PlGF-1 and PlGF-2 for

Table 1

Intra-assay variations in cell supernatant samples assayed for VEGF-C

Sample	<i>n</i>	Mean (ng/ml)	SD	CV
1	6	2.005	0.176	8.75
2	5	4.004	0.119	2.99
3	5	5.646	0.250	4.43
4	5	8.371	0.482	5.76
Mean				5.48

Supernatants from 293/VEGF-C cells containing 2 ng/ml VEGF-C (sample 1) were spiked with 2 (2), 4 (3) and 6 (4) ng/ml recombinant VEGF-C.

cross-reactivity and could not detect any significant binding (data not shown). To estimate the precision and reproducibility of the ELISA, intra-assay variations were evaluated. Supernatant from VEGF-C transfected 293 cells containing 2 ng/ml endogenous VEGF-C was spiked with recombinant rat VEGF-C to final concentrations of 4, 6 and 8 ng/ml, respectively. Intra-assay variations were estimated by duplicate measurements and at least 5 independent experiments. The intra-assay coefficients of variation (CV) were below 6% (Table 1).

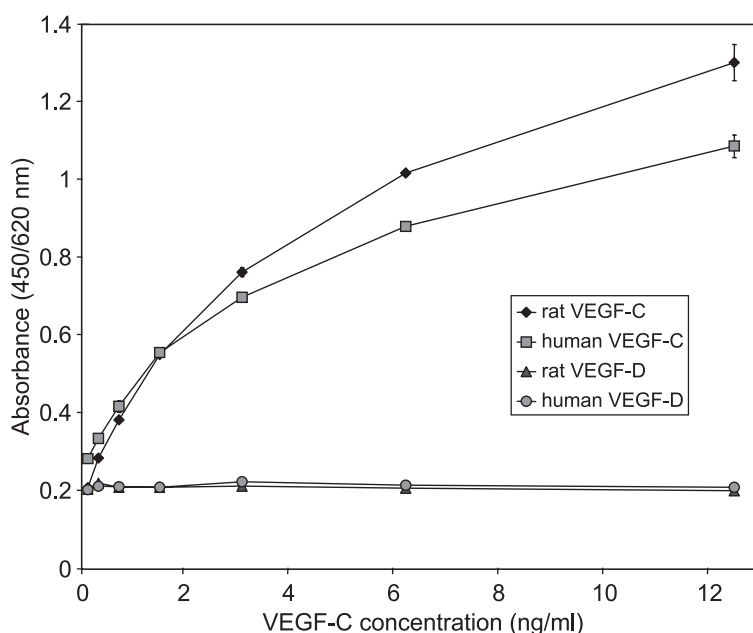


Fig. 3. Human and rat VEGF-C standard curves and cross-reactivity with the related protein VEGF-D. The different human and rat VEGF-C and -D preparations were diluted in assay buffer up to 12.5 ng/ml and a typical standard curve was measured using the ELISA procedure described. All measurements were performed in duplicate. The results show the absorbance at 450 nm as mean values. Rat VEGF-C standard was developed for 10 min, all other standards and controls for 25 min.

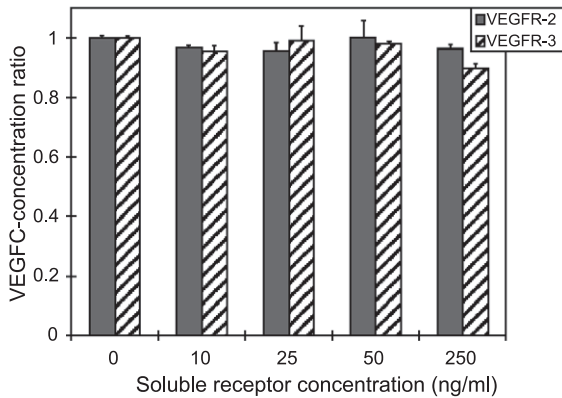


Fig. 4. Antibody 4080 binds to rat VEGF-C independently of receptor-ligand complex formation. VEGF-C (6.25 ng/ml) was added to each well coated with AB 4080 in the absence or presence of increasing concentrations of soluble VEGFR-2 and VEGFR-3 preincubated with the ligand. Biotinylated 4080 antibody was used to detect and quantify bound VEGF-C. All measurements were performed and duplicate. Results show the mean absorbance at 450 nm as mean values.

In order to exclude interactions between VEGF-C/VEGFR-3 or VEGF-C/VEGFR-2 and VEGF-C/AB 4080 binding, increasing amounts of the corresponding soluble receptors were added to VEGF-C containing supernatants. As demonstrated in Fig. 5, a 15-fold

excess of either of soluble VEGFRs did not interfere with the binding of 6.25 ng/ml VEGF-C to the capture antibody. This indicated that VEGF-C/VEGFR-3 or VEGF-C/VEGFR-2 complex formation did not adversely affect this assay (Fig. 4).

3.3. Secretion of VEGF-C from tumour cells and transfected cells under culture conditions

So far there is only limited information available about the kinetics and the amounts of VEGF-C secreted from cultured cells. Most of our knowledge about VEGF-C expression is based on gene expression studies made by Northern blot analysis and RT-PCR experiments. The rat tumour cell line BRL3A was chosen because of its relatively high expression of VEGF-C by Northern blot analysis (Krishan et al., 2003). Human 293 cells are chosen by many groups for transfection studies because they are well characterized, easy to transfect and good producers of many proteins. Although BRL3A cells express VEGF-C mRNA at a relatively high expression rate, only minor amounts of protein were secreted by these cells. After 6 days in culture only levels up to 80 pg/ml could be measured (Fig. 5). The low secretion rate of VEGF-C from other rat tumour cell lines in vitro was also noted (data not shown). However, transfected 293 cells secreted up to

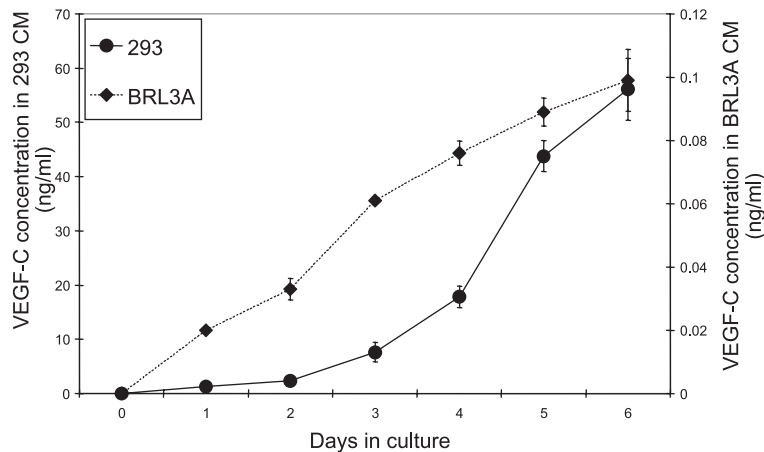


Fig. 5. Time course experiment for the secretion of rat VEGF-C from 293 cells transfected with the rat VEGF-C gene and from rat tumour BRL3A cells. Cells were cultured in 6-well plates over 1–6 days under normal culture conditions. Supernatants were collected and frozen at -20°C before measurement and supernatant from BRL3A cells was concentrated before measurement. All measurements are done in duplicate and the mean values are indicated.

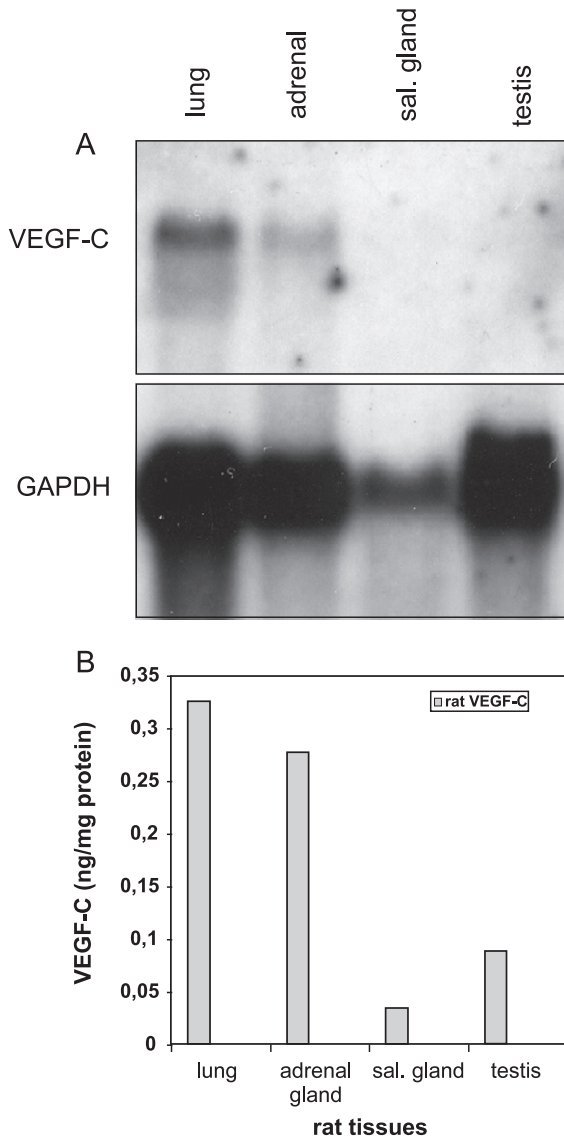


Fig. 6. Expression of VEGF-C in different rat tissues. (A) Northern blot analysis in a panel of polyadenylated RNAs derived from various rat tissues: lung, testis, salivary gland and adrenal gland. (B) ELISA measurements in samples prepared from the same rat tissues. Concentrations of rat VEGF-C are expressed as ng per mg of total protein.

50 ng/ml VEGF-C after 6 days of culture (Fig. 5). These results indicate that human 293 cells can secrete recombinant VEGF-C in relatively high amounts and that several molecular forms can be distinguished by immunoblot analysis (Fig. 1C).

3.4. Detection and quantification of VEGF-C from different murine and human tissues and correlation of VEGF-C data by using different detection methods

After the discovery of the VEGFR-3/Flt-4 ligand VEGF-C, several studies reported the expression of this gene in several tissues and organs of both human or murine origin (Joukov et al., 1996; Fitz et al., 1997; Kirkin et al., 2001). All of these studies used Northern blot analysis for the detection of human, mouse and rat VEGF-C. For the validation of our assay and to facilitate correlations with gene expression data we compared four rat tissues that are known either to express the VEGF-C gene at relatively high rates (lung and adrenal gland) or at relatively low rates such as testis and salivary gland (Kirkin et al., 2001). As shown in Fig. 6, ELISA measurements for the four different tissue lysates correlated with the Northern blot data also performed on the same (Fig. 6A and B). In 1 mg of total tissue protein, we could measure VEGF-C over the range 0.03–0.32 ng, indicating a very low abundance for VEGF-C in normal tissues. In further control experiments, we used RT-PCR with a primer pair specific for human VEGF-C. The human cDNA for VEGF-C was used as a positive control. Human PC-3 cells have been reported to exhibit high expression of VEGF-C (Joukov et al., 1996) and RT-PCR with our primer pair was able to confirm the expression of this gene (Fig. 7). On the other hand, the well characterized and frequently used human 293 cells expressed the

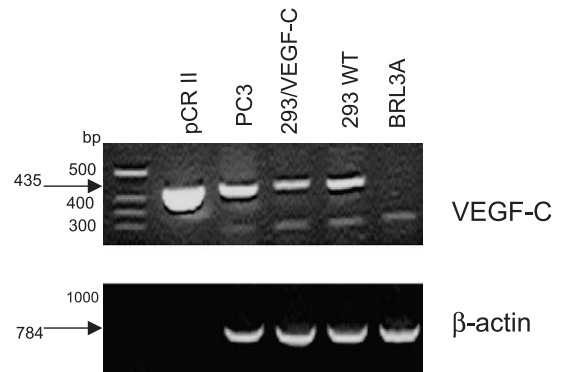


Fig. 7. Semi-quantitative RT-PCR analysis of human VEGF-C and β -actin in human and rat cancer cell lines. The sizes expected from the reported cDNA sequence for VEGF-C and β -actin are 435 and 784 bp, respectively.

gene at lower levels whether or not transfected with rat VEGF-C (Fig. 7). Because human specific PCR primers were used, the rat VEGF-C gene could not be detected in transfected 293 cells nor in the BRL3A tumour cells (Fig. 7). Our ELISA measurements with PC-3 cells and 293 wild type cells were able to confirm the results. PC-3 cells have a high VEGF-C content in their lysates (Table 2), and untransfected human 293 cells exhibited a lower expression level. In lysates from 293 cells transfected with the rat VEGF-C gene, up to 7 ng of VEGF-C per mg total protein could be detected (Table 2). Several rat tumour cell lines have been characterized previously for VEGF-C expression (Krishan et al., 2003). These cell lysates were positive for VEGF-C but contained less VEGF-C than did PC-3 cells (Table 2). Samples from the control tumour cell line MT450 (Krishan et al., 2003) were also shown to have very low VEGF-C concentrations in their lysates. Many human tumours have been characterized over the last 6 years for VEGF-C gene expression but it is not yet clear how VEGF-C and its different processed forms participate in tumour angiogenesis and lym-

phangiogenesis. When we measured several human carcinoma lysates from different sources, we found concentrations in the range of 0.6–2.2 ng/mg protein of VEGF-C (Table 2). This is comparable to other cell lysates but lower than VEGF-A in breast cancer lysates (Toi et al., 2002). However, this information is based on a very limited number of tissues. Human lymph node tissue is also a good source of VEGF-C and preliminary experiments show that VEGF-C concentrations may be higher than in several other samples (Table 2).

4. Discussion

The present study describes for the first time the establishment of a quantitative sandwich ELISA for murine and human VEGF-C. During the development of this ELISA, several polyclonal antibodies for VEGF-C were produced together with recombinant proteins for rat VEGF-C and VEGF-D. However, this approach was only successful because it was possible to use an antigen-affinity purified antibody against processed VEGF-C. Other antibodies such as anti-peptide antibodies or commercially available sources could not be used or were not sensitive enough for such an ELISA procedure.

In contrast to the other members of the VEGF family, VEGF-C and VEGF-D are produced as large precursor molecules and then processed during the release and secretion pathway (Joukov et al., 1997a,b). The largest monomeric unprocessed form is the 58 kDa protein. During the proteolytic processing of VEGF-C by proteases, major bands are seen at 30 kDa and 21 kDa in human PC-3 cells. The mature VEGF-C is the 21 kDa form but this cannot be detected in all VEGF-C producing cells and it is not clear if (partially) unprocessed forms can be secreted (Joukov et al., 1996, 1997a,b).

The antigen we have used was the rat processed VEGF-C (dNc-VEGF-C) with a high degree of homology to mouse and human mature and processed VEGF-C. Mouse and rat VEGF-C show 100% sequence identity and between rat and human there is only one amino acid difference (Kirkin et al., 2001). Therefore, human and rat processed VEGF-C protein could be easily detected in our assay as well as by immunoblotting. However, when using our antibody

Table 2
Quantification of VEGF-C in different cell culture samples and in tissue samples of different origin

Sample	<i>n</i>	Mean (ng/mg protein)	SD (%)
1. 293 cells/rVEGF-C SN (6 days)	4	11.84 (ng/ml)	0.085
2. 293 cells/rVEGF-C lysate	8	6.8	2.02
3. 293 cells/ WT lysate	2	0.43	0.131
4. BRL3A lysate	2	1.01	0.157
5. 10AS lysate	2	1.44	0.022
6. ARIP lysate	2	2.34	0.299
7. MT450 (negative control)	2	0.08	0.011
6. RipVEGF-C transgenic tumors	4	1.158	0.416
7. PC-3 cell lysate	8	6.85	2.528
8. Hum lymph node tissue	2	2.38	0.211
9. Breast cancer lysate	2	0.61	0.07
10. Pancreas cancer lysate 12,141	2	1.95	0.132
11. Gastric cancer lysate 19,907	2	2.20	0.141
12. Colon cancer lysate 2379	2	1.54	0.139

Samples were prepared from low serum containing media, cultured cells and from mouse and human tumour tissue and normal tissue samples. Cells were grown near confluency before being used for lysate preparation. Tumour samples were directly snap frozen after collection and homogenized in lysis buffer containing protease inhibitors. The VEGF-C concentrations were normalized at the concentration of the total protein in the lysate.

combination against the mature protein we cannot be certain that all VEGF-C forms are equally well detected in the assay. Using the lysate from the RIP Tag mouse tumour, we have some preliminary results that the 30 kDa protein may be less readily detectable than the 21 kDa protein.

Very recently, an ELISA for human VEGF-C has been reported (Duff et al., 2003). This ELISA used two polyclonal antibodies raised in a goat or in rabbits using the *Escherichia coli*-derived human VEGF-C peptide and a synthetic peptide corresponding to the C-terminal end of human the VEGF-C precursor. Therefore the assay recognizes the central VEGF-homology domain and carboxyl terminal domains of VEGF-C. With this ELISA, it was not possible to measure mature VEGF-C. Nor was it possible to include a dose–response curve with a recombinant VEGF-C. By immunoblotting using one or both antibodies the authors could demonstrate that plasma contained two immunoreactive 40 and 80 kDa proteins (Duff et al., 2003). From these studies it was not clear whether the two detected protein bands represented VEGF-C or other proteins cross-reacting with the antibodies. So far, 40 and 80 kDa VEGF-C proteins have not been described and mature VEGF-C could not be detected. Using plasma samples from patients with colorectal cancer, the authors could measure a threefold increase of VEGF-C in plasma. This would imply that unprocessed or partially processed forms of VEGF-C may be present in plasma samples from cancer patients. In our experiments we were not able to detect a 40 or 80 kDa band as putative unprocessed forms of VEGF-C. As discussed before, our antibody 4080 was made against the processed VEGF-C protein so that we cannot exclude the possibility that unprocessed, larger, precursor forms may not be detected. However, this is not very likely because at least partially processed forms can be detected in the RIP tag tumour mouse lysate (Fig. 1C).

We also assayed several plasma samples and found VEGF-C in some samples (data not shown). In cell and tissue lysates we were able to measure VEGF-C in relatively low amounts. Only transfected cells or PC-3 cells showed high levels of VEGF-C. Several reports in the last 6 years have claimed that many tumours express the VEGF-C or VEGF-D genes (Valtola et al., 1999; Stacker et al., 2001). With the exception of a few studies using antibodies to detect VEGF-C, most investigators have used RT-PCR or

Northern blot procedures. Our study with human tumour samples suggests that the level of VEGF-C can be slightly lower than that of VEGF concentrations, e.g. VEGF-A in breast tumour samples (Toi et al., 2002). However, these results are the first ELISA data from tumour samples and further investigations are necessary to confirm our results. The pathophysiological significance of these findings remains unclear at present and it is also not known in which way VEGF-C contributes to tumour angiogenesis, lymphangiogenesis, tumour progression and metastasis.

From the early beginnings of VEGF-C research, PC-3 cells were described as high producers of VEGF-C (Joukov et al., 1996). Compared to tumour cells, these cells generate much more VEGF-C in lysates. It was surprising that the amount of VEGF-C in conditioned media and in corresponding cell lysates did not always correlate in our assays. It may suggest that only small amounts of VEGF-C are processed and secreted from cells under culture conditions. Further studies are necessary to confirm these more preliminary results and to investigate what are the reasons for the low and differing secretion rates in cell culture.

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