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Isoform-specific Expression of VEGF-B in Normal Tissues and Tumors

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Vascular endothelial growth factor B (VEGF-B), a member of the VEGF/PDGF family, is highly expressed in many tissues with two differentially spliced transcripts generating two secreted isoforms, VEGF-B_{167} and VEGF-B_{186}. In this work, we have investigated the expression of VEGF-B in tissues and cell lines using techniques that can distinguish the two isoforms. The results showed that the VEGF-B_{167} isoform was predominantly expressed in most tissues, accounting for more than 80% of the total VEGF-B transcripts. The VEGF-B_{186} isoform was expressed at lower levels and only in a limited number of tissues. Moreover, the VEGF-B_{186} isoform was up-regulated in mouse and human tumor cell lines and primary tumors compared with their corresponding normal tissues. Taken together, our data suggest a fine genetic control of the expression of the two isoforms of VEGF-B, implying tissue- and cell-specific roles of the two VEGF-B isoforms.

Keywords: VEGF-B, isoform, regulation, tumor, cell line

INTRODUCTION

The VEGF family of growth factors has been implicated in the development and maintenance of several tissues, e.g., the cardiovascular system. Thus far, five mammalian family members have been identified (Carmeliet *et al.*, 1999; Eriksson *et al.*, 1999). Several of the members display different transcripts due to alternative splicing. Among them, the human VEGF-A gene gives rise to five different mRNA species (Neufeld *et al.*, 1999), resulting in proteins differing in their molecular mass and biological properties, as shown by the different VEGF proteins of 121, 145, 165, 189 and 206 amino acids (Carmeliet *et al.*, 2000). The VEGF-A_{165} isoform is the predominant form in most tissues, giving rise to a polypeptide with unique affinity to the neuropilin-1 receptor, besides the binding to VEGF receptors 1 and 2 (VEGFR1, VEGFR2) (Carmeliet *et al.*, 1999; Eriksson *et al.*,

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1999). VEGF-A_{121} and VEGF-A_{189} are expressed in normal tissues at lower levels. Isoform-specific VEGF-A targeting experiments in mice have shown that VEGF-A_{164} (mouse VEGF-A is one amino acid shorter than the human one) and VEGF-A_{188} are more important for postnatal growth and maintenance of normal function of cardiovascular system while VEGF-A_{120} initiates and promotes vasculogenesis (Carmeliet *et al.*, 1999). VEGF-A_{206} mainly expressed in embryonic tissues (Houck *et al.*, 1991), while VEGF-A_{145} can only be found in tumor cell lines (Poltorak *et al.*, 1997). Moreover, VEGF-A is also regulated in an isoform-specific way under pathological conditions. In lung and colon carcinomas, VEGF-A_{165} and VEGF-A_{121} are up-regulated, whereas VEGF-A_{189} is not changed, suggesting an isoform-specific role of VEGF-A in malignancy (Cheung *et al.*, 1998). The placenta growth factor (PIGF) has three different isoforms, which are expressed in a tissue- and development-specific way (Maglione *et al.*, 1993; Cao *et al.*, 1997; Persico *et al.*, 1999). However, their biological properties remain largely unknown.

VEGF-B, another member of the VEGF family, is highly expressed in heart, skeletal muscle, and at lower levels in most other tissues (Grimmond *et al.*, 1996; Olofsson *et al.*, 1996a). VEGF-B heterodimerizes with VEGF-A when co-expressed (Olofsson *et al.*, 1996a,b). Gene targeting studies have shown that VEGF-B deficiency results in a mild cardiac phenotype, and impaired coronary vasculature (Aase *et al.*, in press, 2001; Bellomo *et al.*, 2000).

VEGF-B mRNA can be detected in many tumor and tumor cell lines (Salvén *et al.*, 1998; Andre *et al.*, 2000; Eggert *et al.*, 2000; Niki *et al.*, 2000), and is up-regulated in tumor-associated macrophages and in ovarian epithelial tumors (Sowter *et al.*, 1997). Under most other conditions, however, VEGF-B expression levels are remarkably stable and not influenced by growth factors, hypoxia, hormones and oncogenes (Enholm *et al.*, 1997).

Two alternatively spliced mRNAs of VEGF-B have been found, that encode a freely secreted protein of 186 amino acids (VEGF-B_{186}), and a secreted but largely cell-associated protein of 167 amino acids (VEGF-B_{167}), respectively. The two polypeptides differ at their carboxy-termini and display different abilities to bind heparin sulphate proteoglycans and neuropilin-1 (Mäkinen *et al.*, 1999), implying distinct functional properties of the two proteins. However, the expression pattern and regulation of the two different isoforms remain obscure thus far. To understand this a quantitative RNAse protection analysis (RPA) that can specifically distinguish the two isoforms of VEGF-B, was established. The results showed that VEGF-B_{167} was the prevailing isoform in most organs, while VEGF-B_{186} was expressed at lower levels in a limited number of tissues. Interestingly, the VEGF-B_{186} isoform was highly expressed in both tumor cell lines and primary tumors. Isoform-specific VEGF-B antibodies were developed and used to confirmed the expression of the two VEGF-B isoforms in mouse tumor cells by indirect immunofluorescence staining.

MATERIAL AND METHODS

Tissues and Cell Lines

Mouse tissues and human pheochromocytomas were snap frozen upon collection, and stored in $-80\,^{\circ}C$ until used. Human melanoma cell lines (kind gifts from Dr. E. Stockert, Ludwig Institute for Cancer Research, New York Branch) were cultured in minimum essential medium, with 10% foetal calf serum and 1% NEAA.

Preparation of RNA Probes

Riboprobes were prepared using RNA polymerase (Promega) and ^{32}P-UTP (Amersham) according to the manufacturers protocol. Mouse VEGF-B (accession number MMU52820) cDNA fragment of 386 bp generated by SpeI and PstI digestion, human VEGF-B (accession number U52819) cDNA fragment of 445 bp generated by SacI digestion, human $VEGF_{165}$ isoform cDNA of 650 bp

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amplified by PCR (accession number, X62568) were purified and subcloned into pBluescript KS (Strategene) digested by the corresponding restriction enzymes, and used as templates to make RNA probes. Mouse β-actin and human GAPDH cDNA fragments (Ambion) were used as internal controls for quantification.

RNAse Protection Analysis

Total cellular RNA were prepared using the guanidinium thiocyanate/acid phenol method (Chomczynski *et al.*, 1987) and stored in −80 °C until usage. RNAse protection analysis (RPA, Ambion) was used to quantify the expression levels of different genes in different tissues. 30 or 100 µg of total cellular RNA was hybridized to the riboprobes at 45 °C overnight, followed by RNAse digestion. Digested product was separated on a 6% polyacrylamide gel, and the signals were quantified using a phosphor imager (Fujifilm Bas 1500).

Generation of VEGF-B Isoform-Specific Antibodies

The alternative splicing of VEGF-B causes a frame shift of the reading frame between the 186 and 167 isoforms in the C-terminal although exon 6B is contained in both isoforms (Olofsson *et al.*, 1996b). It is therefore possible to make isoform-specific antibodies using the peptides encoded by exon 6A-6B and exon 6B respectively. PCR fragments of either exon 6B or exon 6A-B of mouse VEGF-B encoding the C-terminal regions of VEGF-B_{167}, (Pro 138 to Arg 187) and VEGF-BA_{186} (Arg 137 to Ala 203) respectively, were generated. For VEGF-B_{167} the primers 5′-ACGTAGATCTAGCCCCAGGATCCTC (forward) and 5′-ACGTGAATTCTCAGCCCCGCCCTTGGCA (reversed) were used, and for VEGF-B_{186}, 5′-ACGTAGATCTAGGGTTGCCATACCC (forward) and 5′-ACGTGAATTCTCAGTTGACGGCGCTGGGT (reversed). The forward primers contain BglII restriction sites and the reverse primers EcoRI sites for cloning. The fragments were cloned into the pGEX-2T vector (Pharmacia). GST-fusion proteins were prepared according to the manufacturer's recommendations (Pharmacia). Purified proteins were used to immunize rabbits essentially as described (Aase *et al.*, 1999). Anti-VEGF-B_{167} Ig was affinity-purified against the GST-fusion protein, and anti-VEGF-B_{186} Ig against the baculovirus-derived protein (Aase *et al.*, 1999). The specificities of the affinity-purified VEGF-B antibodies were tested by immunoblotting using reduced baculovirus-derived mVEGF-B_{167} and mVEGF-B_{186} (Aase *et al.*, 1999) separated by 12.5% SDS-PAGE. Recombinant mVEGF-B_{167} was obtained from baculovirus-infected insect Sf9 cells. The baculovirus vector was generated by subcloning mVEGF-B_{167} cDNA (Olofsson *et al.*, 1996a) into pFASTBAC1 (PharMingen). Baculovirus was generated as described (BAC-TO-BAC™ Baculovirus Expression System, Life Technologies). The generated mVEGF-B_{167} recombinant protein was retained intracellularly, and the SDS-soluble fraction was used in the SDS-PAGE analysis.

Immunofluorescence Localization of VEGF-B Isoforms in Tumor Cells

B16 mouse melanoma cells were seeded in 6-well dishes on sterilized coverslips at a density of 50 000 cells/well one day prior to indirect immunofluorescence staining. The localization of VEGF-B was carried out essentially as described (Andersson *et al.*, 1997). The VEGF-B_{167} and VEGF-B_{186} specific antibodies were diluted to a concentration of 20 µg/ml. The ER specific marker calnexin (Santa Cruz Biotechnology) was used at a dilution of 1:50. Rabbit Ig was visualized with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibodies and goat Ig with tetramethyl isothiocyanate (TRITC)-conjugated anti-goat IgG antibodies. As negative controls, the cells were incubated with only the secondary IgGs or pre-immune rabbit IgG. In all cases no consistent positive staining was obtained. Immunofluorescence was detected using a Zeiss Axiophot fluorescense microscope.

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RESULTS

VEGF-B_{167} is the Major Isoform Expressed in Both Adult and Embryonic Tissues

A cDNA fragment of 384 bp of the mouse VEGF-B gene covering exons 3, 4, 5, 6A and 6B was cloned and used as template to make an RNA probe suitable for quantification of the two isoforms (Fig. 1A). Using this probe, the VEGF-B_{167} isoform can be distinguished from VEGF-B_{186} due to the lack of exon 6A. The RPA results showed that when using 30 μg of total cellular RNA in the analyses, only the VEGF-B_{167} isoform was detectable (Fig. 2A). The expected 384 bp fragment derived from VEGF-B_{186} was undetectable. Given the shorter length of the protected VEGF-B_{167} isoform (209 bp), and thus less radioactivity incorporated, this indicates that the VEGF-B_{167} isoform is highly expressed in many tissues, while the VEGF-B_{186} isoform is expressed at lower levels, if any. In separate experiments using 100 μg of RNA in each assay and overexposure of the gels, a protected fragment of 384 bp, corresponding to VEGF-B_{186}, was weakly detectable in some of the tissues tested (Fig. 2A). The transcript levels of VEGF-B_{167} and VEGF-B_{186} were quantified using a Phosphor imager with mouse β-actin as the internal control. The results showed that highest expression levels of VEGF-B_{167} were seen in heart, skeletal muscle and diaphragm, while lower expression levels were seen in most other tissues. VEGF-B_{186} expression was limited and found only in heart, skeletal muscle, diaphragm, colon, kidney, brain and lung, to compare the relative expression levels of the two isoforms, the ratio of VEGF-B_{186}/VEGF-B_{167} expression was calculated by comparing the intensities of the 384 and 209 bp bands. The highest ratios, approximately 0.25, were seen in heart, kidney and colon. Considering the length of the two protected fragments, which is proportional to the amount of incorporated radioactivity, the expression level of VEGF-B_{186} was about 0.1–0.2 of that of VEGF-B_{167} in the tissues with highest expression, and lower in all other tissues (see Table I).

To investigate if the expression pattern of the two isoforms of VEGF-B was developmentally regulated, the same experiment was performed using total cellular RNA (30 μg of RNA per assay) extracted from mouse embryos of different developmental stages and several extra-embryonic tissues. The data showed that the VEGF-B_{167} isoform was also here the predominant transcript and no protected fragment corresponding to VEGF-B_{186} could be visualized (Fig. 2B).

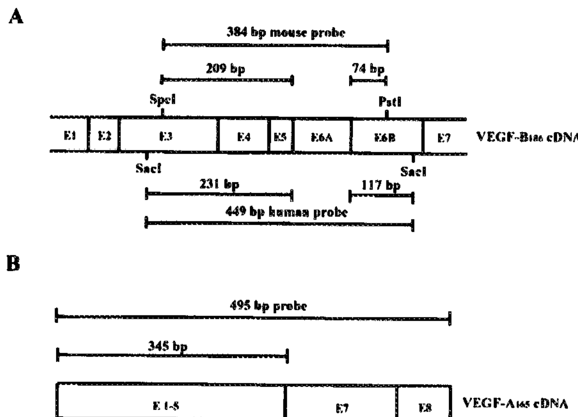

FIGURE 1 Probes used in the RNAse protection analysis. The open boxes represent the different exons of the VEGF-B and VEGF-A genes. (A) The 384 bp fragment derived from the mouse VEGF-B_{186} isoform was cloned as a template to make the RNA probe, which also stands for the protected fragment generated by the VEGF-B_{186} isoform during RPA analysis. The 209 bp and 74 bp fragments stand for the protected fragments derived from the VEGF-B_{167} isoform using this probe. The same strategy is used to generate a human VEGF-B probe. The 449 bp fragment corresponds to the VEGF-B_{186} isoform, while the 231 and 117 bp fragments corresponds to the VEGF-B_{167} isoform. (B) The 495 bp fragment derived from the human VEGF-A_{165} isoform was cloned as a template to make the RNA probe, which also stands for the protected fragment corresponding to the VEGF-A_{165} isoform. The 345 bp protected fragment is derived from the other isoforms of VEGF-A, which contain exons (E)1–5. Scales are arbitrary.

VEGF-B_{186} is Highly Expressed in Rodent Tumor Cell Lines

To investigate the expression pattern of the two isoforms of VEGF-B in tumor cell lines, total cellular RNA was extracted from different rodent

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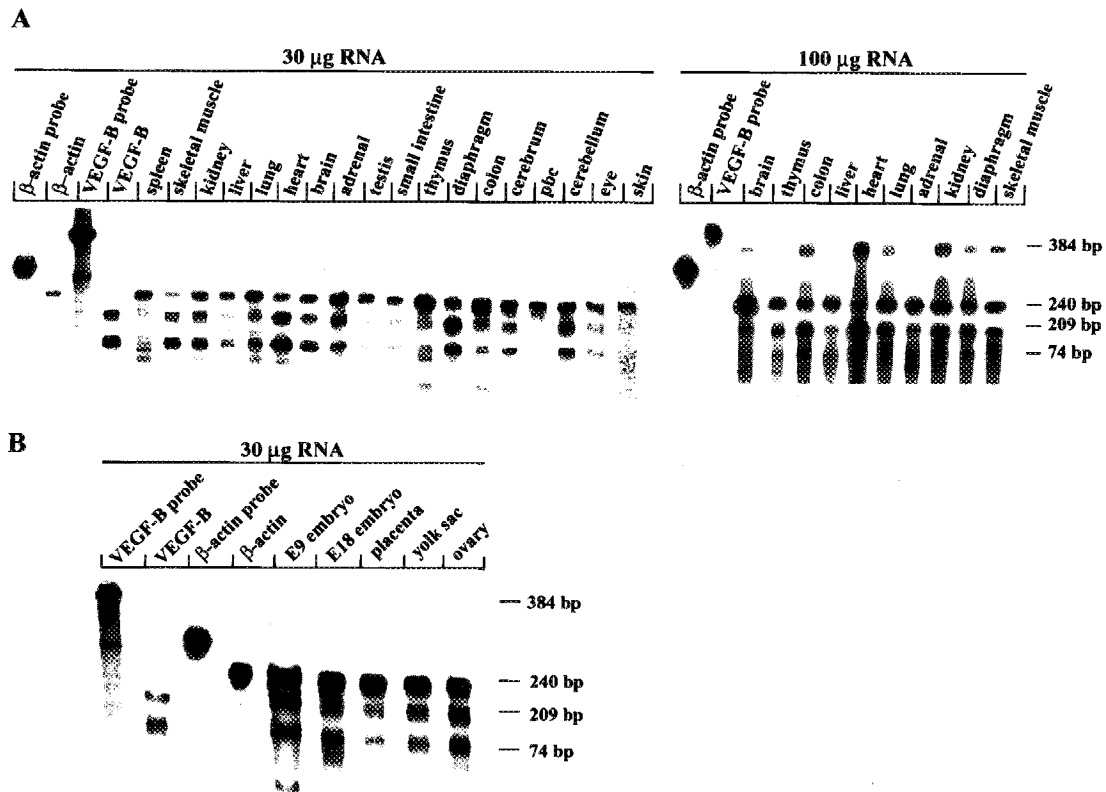

FIGURE 2 Expression of VEGF-B isoforms in mouse adult and embryonic tissues. (A) RPA of total cellular RNA (30 µg or 100 µg per assay, as indicated) showed that VEGF-B_{167} was the major isoform expressed in normal adult mouse tissues. The 209 and 74 bp fragments were derived from the VEGF-B_{167} isoform and the 384 bp fragment was derived form the VEGF-B_{186} isoform. The expression of the VEGF-B_{186} isoform was only detected using 100 µg total cellular RNA per assay. The mouse β-actin probe was used as an internal control for quantification, as indicated by the 245 bp protected fragment. (B) VEGF-B_{167} was the major isoform expressed in embryos and other tissues as indicated by the presence of the 209 and 74 bp fragments, and the absence of the 384 bp fragment. 30 µg of total cellular RNA was used per assay. pbc, peripheral blood cells.

tumor cell lines, including mouse T241 fibrosarcoma, mouse B16 melanoma, mouse Lewis lung carcinoma and rat BT4C glioma, and used in RPA (30 µg of total cellular RNA per assay). Surprisingly, the VEGF-B_{186} isoform was highly expressed in all cell lines tested, indicating a specific up-regulation of this isoform (Fig. 3). The smaller size of the protected fragment of the VEGF-B_{186} isoform in the rat glioma sample is due to sequence differences between the mouse and rat VEGF-B genes. The relative ratios of VEGF-B_{186}/VEGF-B_{167} were greater than 0.5, ratios much higher than those observed for normal mouse tissues (Table I).

VEGF-B_{186} is Highly Expressed in Human Melanoma Cell Lines and Pheochromocytomas

To further look into the expression of the two isoforms of VEGF-B in human tumor cell lines, a cDNA fragment of the human VEGF-B gene was cloned and used as a probe in RPA using the same strategy as previously outlined (Fig. 1A). Twenty two different human melanoma cell lines were investigated using 30 µg of total cellular RNA and GAPDH was used as an internal control for quantification. The results showed that in most of the cell lines tested, both VEGF-B_{186} VEGF-B_{167} were highly expressed (Fig. 4A, upper panel), displaying

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TABLE I Expression levels of VEGF-B isoforms in mouse normal adult tissues and transformed cell lines[a]

Tissues	VEGF-B_{167}	VEGF-B_{186}	VEGF-B_{186}/VEGF-B_{167}
Heart	27	7.3	0.27
Skeletal muscle	27	3.2	0.12
Diaphragm	16	3.0	0.19
Colon	7	1.7	0.24
Cerebellum	6	nd	–
Eye	6	nd	–
Kidney	4	1	0.25
Brain	4	0.6	0.15
Adrenal	4	nd	–
Lung	3	0.45	0.15
Cerebrum	3	nd	–
Liver	1	nd	–
Testis	0.5	nd	–
Small intestine	0.5	nd	–
Spleen	0.5	nd	–
Thymus	0.5	nd	–
Peripheral blood cells	0.2	nd	–
T241	12	7.4	0.62
B16	9	4.5	0.50
BT4C	8	6.3	0.79
LLC	6	3.2	0.53

[a] The expression values in columns 2 and 3 are arbitrary units of VEGF-B expression after normalization against the internal control, β-actin. The fourth column represents the ratio of the expression levels of VEGF-B_{186}/VEGF-B_{167}. nd, not detected.

an average $VEGF_{186}/VEGF_{167}$ ratio of approximately 0.4. The total VEGF-B expression levels were quantified by combining both isoforms and normalizing against the internal control. Both isoforms of VEGF-B were present in all cell lines tested with highest expression levels in samples 3, 6, 9, 10 and 22 (Fig. 4A). For comparison, VEGF-A expression was investigated using a probe derived from the VEGF-A_{165} isoform (Fig. 1B), which gives two protected bands; the fully protected 495 bp fragment corresponding to the VEGF-A_{165} isoform, and a 345 bp fragment corresponding to exons 1–5 in the other isoforms (Fig. 4A, lower panel). The results showed that VEGF-A is expressed in all the cell lines with dramatic variations of transcription levels, in contrast to VEGF-B that appeared relatively stable. VEGF-A was highly expressed in samples 5, 7, 11, 16 and 20. Notably, VEGF-B expression levels were under the average value in those samples.

The isoform-specific expression of VEGF-B and VEGF-A were also investigated in human benign and malignant pheochromocytomas. The results showed that VEGF-B_{186} was highly expressed in both benign and malignant tumors, at similar and high levels, with an average VEGF-B_{186}/ VEGF-B_{167} ratio of about 0.5 (Fig. 4B, upper panel). The total VEGF-B expression levels were similar in benign and malignant samples. VEGF-A was expressed in both groups at similar levels (Fig. 4B, lower panel).

Both Isoforms of the VEGF-B Protein are Expressed in B16 Melanoma Cells

To verify the synthesis of VEGF-B protein in tumor cells, indirect immunofluorescence microscopy analysis was preformed on B16 mouse melanoma cells. Isoform-specific Igs directed against VEGF-B_{167} and VEGF-B_{186} could be generated since the alternative splicing of exon 6A results in different amino acid sequences of the C-terminal regions of the two proteins. The specificities of the two affinity-purified Ig fractions were tested on

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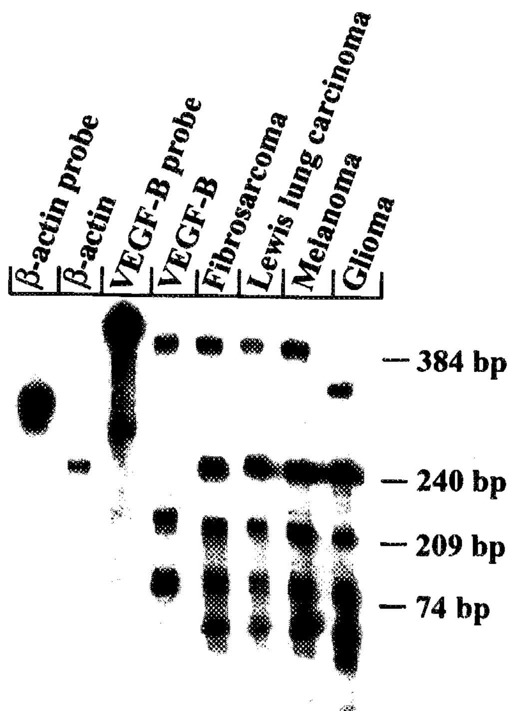

FIGURE 3 The VEGF-B_{186} isoform is highly expressed in mouse tumor cell lines. Total cellular RNA from different rodent cell lines (mouse T241 fibrosarcoma, mouse Lewis lung carsinoma, mouse B16 melanoma, and rat BT4C glioma) were used in the RPA (30 μg of total cellular RNA per assay). The VEGF-B_{186} isoform, indicated by the 384 bp fragment, was highly expressed at levels comparable with that of the VEGF-B_{167} isoform as illustrated by the 209 and 74 bp protected fragments. A smaller protected fragment of the 186 isoform was present in the rat glioma sample due to the sequence differences between the mouse and the rat VEGF-B genes.

proteins produced from baculovirus-infected Sf9 cells. The immunoblot analysis showed that the two Ig fractions only reacted with their respective isoform, and showed no cross reactivities (Fig. 5). In detergent-permeabilized B16 cells, both VEGF-B_{167} (Fig. 6A) and VEGF-B_{186} (Fig. 6E) were expressed as visualized by the strong staining using the respective isoform-specific Ig fraction. Intracellularly, the two VEGF-B isoforms co-localized with calnexin, an ER-specific marker, indicating that both proteins were predominantly localized in the ER (Fig. 6B,C,F,G). Intact, non-detergent treated cells specifically displayed cell-surface associated VEGF-B_{167} immunostaining (Fig. 6D), while no specific staining was obtained using the Ig fraction directed towards the VEGF-B_{186} isoform (data not shown). Non-specific staining was analyzed using pre-immune rabbit IgG as the primary Ig instead of the two Ig fractions directed against the two VEGF-B isoforms, or using the secondary Ig only (Fig. 6H). In conclusion, these data verify and extend previous observations showing that only VEGF-B_{167}, but not VEGF-B_{186} becomes cell-bound upon secretion (Olofsson *et al.*, 1996a,b).

DISCUSSION

In this study, we used quantitative RPA to investigate the tissue distribution and expression levels of the two isoforms of VEGF-B. Our findings showed that VEGF-B_{167} is the major isoform expressed in both adult and embryonic mouse tissues, accounting for more than 80% of the total VEGF-B transcripts. The VEGF-B_{186} isoform, however, was expressed at a much lower level and in a tissue-specific way. We also used immunocytochemical staining and confirmed that both isoforms were expressed in mouse tumor cells and that the extensive inter- and intramolecular disulphide-bonding required for correct synthesis of functional dimers are likely to retain them in the ER. Our data suggest a genetic control of the expression of the two VEGF-B isoforms, and possibly different biological functions. In our previous studies on VEGF-B expression, the two isoforms were shown to be expressed at comparable levels. However, the two isoforms were studied separately by RT-PCR and Northern blotting, and we can conclude that these data do not accurately reflect the transcription levels (Olofsson *et al.*, 1996a,b).

The two isoforms of VEGF-B encode different polypeptides with different biological properties (Olofsson *et al.*, 1996a,b). VEGF-B_{167} is heparin-binding and becomes tightly cell-associated upon secretion suggesting an involvement in autocrine or paracrine signalling pathways, possibly as a

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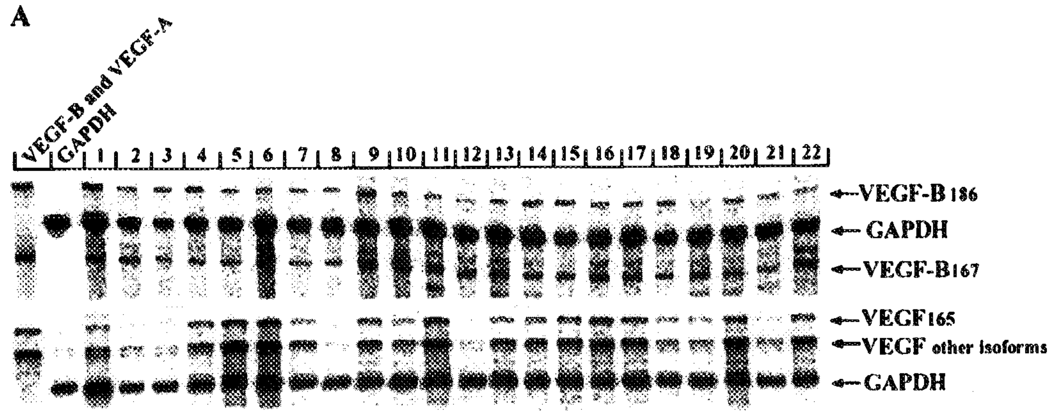
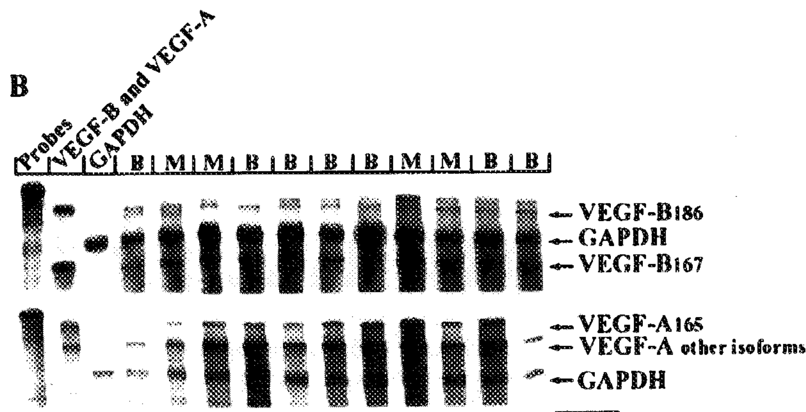
A



																							Average
VEGF-B	1	2	2.5	1	1	2.5	1.5	1.5	3	2.5	2	1.5	1	1	2	1	1	1.5	1.5	1	1	2.5	1.6
VEGF-A	1.5	3	2.5	4	19	7	9	2.5	7	3	13	2	4.5	5	5	11	7.5	4	3	10	3.5	8	6.1
VEGFB (186/167)x 100	70	35	75	85	45	20	55	40	65	15	20	25	20	50	60	20	30	80	25	25	45	20	42

B



												Average
VEGF-B	1	1	0.6	0.8	0.7	0.7	0.3	1	1.2	0.8	1.3	0.9
VEGF-A	0.7	1.3	1.4	0.9	1.1	1.2	1.1	2	1.2	1.6	0.8	1.2
VEGF-B (186/167)x 100	65	67	45	45	36	54	37	48	43	48	48	49

FIGURE 4 VEGF-B and VEGF-A expression in human melanoma cell lines and pheochromocytomas. (A) Expression of both isoforms of VEGF-B (upper panel) and VEGF-A isoforms (middle panel) in human melanoma cell lines. Compared with the stabile VEGF-B expression, VEGF-A expression levels were more variable. Using GAPDH as the internal control, both VEGF-B and VEGF-A expression levels were quantified (in arbitrary units). The table (the lower panel) shows the VEGF-B_{186}/VEGF-B_{167} ratio. (B) Both VEGF-B isoforms (upper panel) and VEGF-A isoforms (middle panel) were highly expressed in human benign and malignant pheochromocytomas. The isoform-specific expression of VEGF-B and VEGF-A was investigated using the same strategy as used above. The table (lower panel) shows the relative expression levels of VEGF-B and VEGF-A expression after normalization against the internal control, GAPDH. B, benign; M, malignant.

spatial guidance for target cells. VEGF-B_{186}, however, does not bind heparin, and is thus freely secreted, suggesting that this isoform can affect remote target cells. In other studies, quantitative RT-PCR analyses have shown that in human dermal microvascular endothelial cells, VEGF-B_{186} is

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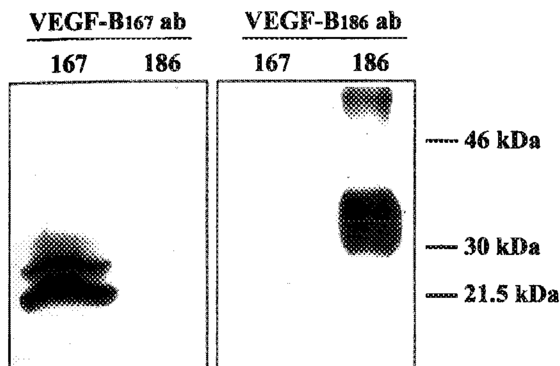

FIGURE 5 Immunoblot analysis of VEGF-B_{167} and VEGF-B_{186} recombinant proteins. The two isoforms of VEGF-B are denoted as 167 and 186 respectively. Under reducing conditions VEGF-B_{167} migrates as 21 and 25 kDa species and VEGF-B_{186} as 30–32 kDa species using the isoform-specific antibodies. The results showed no cross-reactivity between the two isoform-specific antibodies. The molecular weight standards are indicated to the right. ab = antibody.

the predominant form of VEGF-B. In contrast, VEGF-B_{167} was highly expressed in bovine retinal pericytes suggesting their different functions (Yonekura *et al.*, 1999).

VEGF-B can dimerize with VEGF-A, when co-expressed (Olofsson *et al.*, 1996a,b). Different locations of the two VEGF-B isoforms therefore may suggest a different distribution of VEGF-A, that directly or indirectly may affect angiogenic pathways. Although both isoforms of VEGF-B bind VEGFR1 and NP1, the VEGF-B_{186} isoform needs to be proteolytically processed to be able to bind NP-1 (Mäkinen *et al.*, 1999). This indicates that the activity of VEGF-B_{186} is more tightly controlled by other factors, which may be regulated in tissue- and developmental-specific ways.

The fine genetic control of VEGF-B_{186} expression suggests a tissue-specific requirement for this isoform. The regulatory mechanism underlining this tissue-specific expression and its function will be of importance for further understanding of the biological roles of VEGF-B. Previous data have shown high-level expressions of VEGF-B in different tumors and cell lines (Enholm *et al.*, 1997; Sowter *et al.*, 1997; Salven *et al.*, 1998; Andre *et al.*, 2000; Eggert *et al.*, 2000; Niki *et al.*, 2000). However, isoform-specific regulation of VEGF-B

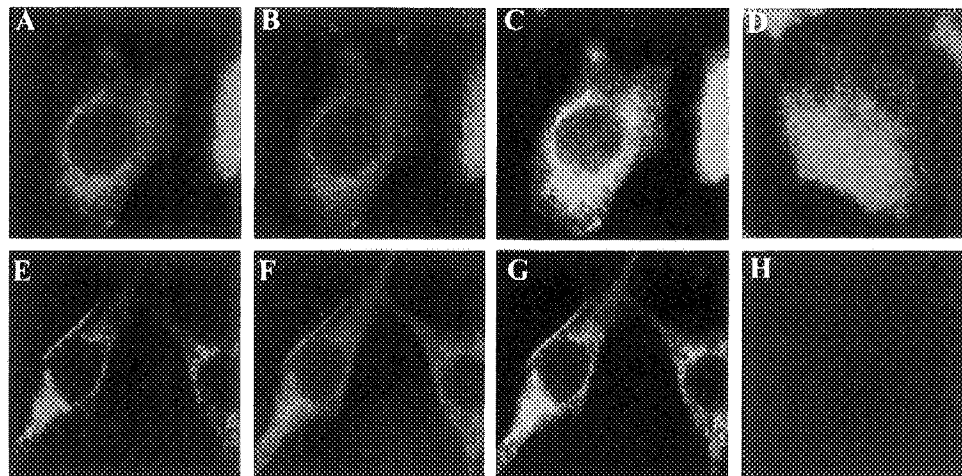

FIGURE 6 (See Colour Plate at back of issue.) Double immunofluorescence staining of VEGF-B isoforms and calnexin in mouse B16 melanoma cells. (A,C,E,G) Localization of VEGF-B_{167} (A,C,D) and VEGF-B_{186} (E,G) using isoform-specific antibodies and FITC-conjugated secondary Ig (green) in detergent-permeabilized cells. (B,F) Calnexin, a marker of the endoplasmic reticulum (ER), was similarly localized using specific antibodies and TRITC-conjugated secondary Ig (red). (C,G) Colocalization of VEGF-B isoforms and the ER-marker calnexin is shown in the merged pictures (yellow). Both VEGF-B_{167} and VEGF-B_{186} isoforms are preferentially localized to the ER. (D) Localization of VEGF-B_{167} in intact, non-detergent-treated cells showing that VEGF-B_{167} becomes cell-bound upon secretion. (H) Control staining using pre-immune rabbit IgG to verify the specificity of the technique.

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has not been investigated thus far. Giving the fact that the expression level of the VEGF-B_{167} isoform was high and relatively stable, constituting more than 80% of the total VEGF-B expression in most tissues, any changes in VEGF-B_{186} expression may well be overlooked by the predominant expression of VEGF-B_{167}. Taking together, our data suggest different roles of VEGF-B_{167} and VEGF-B_{186} considering the predominant expression of VEGF-$_{167}$ in normal tissues, and the up-regulation of the VEGF-B_{186} isoform in tumors. Detailed studies regarding the cellular expression of the two VEGF-B isoforms may be of particular importance to further understand the role of VEGF-B in both physiological and pathological conditions.

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