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EXPRESSION AND LOCALIZATION OF PLACENTA GROWTH FACTOR AND PlGF RECEPTORS IN HUMAN MENINGIOMAS

SANDRA DONNINI[1], MARCIA R. MACHEIN[2], KARL H. PLATE[2] AND HERBERT A. WEICH[1]*

[1]*Department of Gene Regulation and Differentiation, National Research Center for Biotechnology (GBF), 38124 Braunschweig, Germany*
[2]*Department of Neuropathology, Neurocenter, Freiburg University Medical School, Breisacherstrasse 64, 79106 Freiburg, Germany*

SUMMARY

It has previously been suggested that in human brain tumours, endothelial cell proliferation during angiogenesis is regulated by a paracrine mechanism involving vascular endothelial growth factor (VEGF) and its receptors (VEGF receptor 1 and VEGF receptor 2). The mechanism of growth factor up-regulation is based on hypoxic activation of mRNA expression and mRNA stabilization and genetic events, leading to an increase of growth factor gene expression. The role of the other newly discovered VEGF family members with a high specificity for endothelial cells in the pathogenesis of glial neoplasms is unknown. To investigate which other members of the VEGF family are overexpressed in human brain tumours, the mRNA levels of placenta growth factor (PlGF), VEGF-A, and VEGF-B genes were determined by northern blot analysis in surgically obtained human meningiomas. In the 16 meningiomas examined, the mRNA for PlGF was highly expressed in four tumours and VEGF-A mRNA was highly abundant in three tumour samples. There was no close correlation between PlGF mRNA levels and VEGF-A expression levels. VEGF-B mRNA was abundantly expressed in all tumour samples at uniform levels. In a PlGF-positive tumour sample, immunoreactive VEGFR-1 and VEGFR-2 were detected in endothelial cells of the blood vessels. PlGF protein was detectable in most but not all capillaries of the tumour. PlGF is thus highly up-regulated in a subset of human meningiomas and may therefore have functions, in some tumour vessels, connected to endothelial cell maturation and tube formation. These findings suggest that PlGF, in addition to VEGF-A, may be another positive factor in tumour angiogenesis in human meningiomas. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—**placenta growth factor (PlGF); vascular endothelial growth factor (VEGF); receptors; meningiomas; angiogenesis; brain tumours**

INTRODUCTION

The progression and growth of solid tumours are dependent on the formation of new blood vessels, a process called tumour angiogenesis, which is regulated by growth factors that are secreted by tumour cells and often act specifically on vascular endothelial cells.[1] We have previously reported that vascular endothelial growth factor (VEGF-A) is an angiogenesis factor in brain tumours and mediates tumour vascularization *in vivo*.[2] VEGF and its high affinity receptors (VEGFR-1 and VEGFR-2) are expressed in normal brain at low levels, but are up-regulated up to 50-fold in tumour tissues.[3,4] These observations have strongly supported the concept that vascularization in brain tumours is regulated by paracrine mechanisms, VEGF-A being a key molecule for this process.

Another member of the VEGF growth factor family is placenta growth factor (PlGF), a dimeric glycoprotein with 53 per cent homology to VEGF-A.[5,6] PlGF binds to only one of the two receptors, namely VEGFR-1.[7] PlGF is chemotactic for monocytes and therefore active in signal transduction, but it is not angiogenic in the chicken CAM assay.[8,9] However, it was very recently reported that PlGF-1, a non-heparin-binding splice form of PlGF, is also angiogenic *in vivo* in the rabbit cornea pocket assay.[10] Recently, three new members of the VEGF family were described as VEGF-B, VEGF-C, and VEGF-D (for a review see ref. 11). Whereas the role of VEGF-A in tumour development has been well documented, few data have been reported for the role of PlGF or of VEGF-B and the other new members in tumour-associated angiogenesis.

Besides our description of PlGF overexpression in some brain tumours,[4] PlGF expression has been reported in hypervascular renal cell carcinomas and in some thyroid and germ-cell tumours.[12] PlGF is also up-regulated in fetal growth retardation.[13] In contrast to VEGF-A, PlGF and VEGF-B are not regulated by hypoxia and their physiological roles are largely unknown.[11] PlGF is highly expressed in placenta[14] and its expression may be sensitive to steroid hormones. Because meningioma growth has been reported to be steroid-dependent,[15] we investigated in this study whether PlGF and other members of the VEGF family are up-regulated in human meningiomas.

*Correspondence to: Herbert A. Weich, Dept. RD1F/GBF, Mascheroder Weg 1b, D-38124 Braunschweig, Germany.
E-mail: hwe@gbf.de

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Table I—Expression of vascular endothelial growth factor and placenta growth factor genes in meningiomas

Patient No.	PlGF expression	VEGF-A expression	VEGF-B expression	Age (years)	Sex	Meningioma Histology and grade
509	+++	+++	++	66	f	M (I)
514	+	+	++	70	f	M (I)
520	+	+	++	68	f	M (I)
521	+++	+	±	68	f	F (I)
522	+	+	+++	57	f	M (I)
525	+	+++	++	47	m	T (I)
557	+++	++	+++	83	f	F (I)
602	−	−	−	51	f	M (I)
631	++	+	++	80	m	F (I)
643	+	+	+++	64	f	M (I)
647	+	++	++	58	f	M (I)
654	+	−	++	69	f	F (I)
675	±	±	±	10	f	M (I)
694	±	++	−	8	m	F (I)
759	+	+++	−	70	f	M (I)
760	+	++	+++	29	f	M (I)

VEGF=vascular endothelial growth factor; PlGF=placenta growth factor.
Quantification: − =negative; ± =hardly detectable; + =clearly detectable expression; ++ =moderate expression; +++ =strong expression.
M=meningothelial meningioma; F=fibroblastic meningioma; T=transitional meningioma. Subclassification of meningiomas according to the 1993 World Health Organization criteria.

MATERIALS AND METHODS

Tissue specimens

Sixteen cases of intracerebral meningioma classified according to the WHO classification (1993) were included in this study. Tumour specimens were received directly from the neurosurgical theatre. Part of the specimen was fixed in 4 per cent buffered formalin, embedded in paraffin, and processed for routine histological diagnosis. An adjacent part of the tissue was snap-frozen in liquid nitrogen and stored at − 70°C prior to use.

Probes, RNA isolation, and northern blotting

The human VEGF-A probe was a fragment of 0·7 kb, generated as previously described.[4] Human VEGF-B cDNA was a gift from Drs Kari Alitalo and Ulf Eriksson.[16] PlGF cDNA was cloned from human placenta as described earlier.[6] Total RNA isolation and northern blotting were performed as before.[4] The blots were analysed by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and/or exposed to Kodak XAR films with an intensifying screen at − 70°C over 2–7 days. Ribosomal RNA bands were indicated as size markers. For control of RNA loading, the blots were stripped and rehybridized with a ^{32}P-labelled GAPDH cDNA probe.

Antibodies

Affinity-purified PlGF antibodies were obtained from immune rabbit serum and from protein-A purified total IgG. The antibodies were raised against a peptide conjugated to KLH, containing the first 20 NH_3-terminal amino acids of human PlGF protein. The affinity purification of the PlGF antibodies was very similar to that described for the VEGF antibodies.[2] The generation of mouse monoclonal antibodies against VEGFR-1 and VEGFR-2 has been described in detail.[17] The mouse clones KDR-1 and FLT-19 were used in this study. The monoclonal antibodies did not cross-react with each other or with the related soluble extracellular FLT-4 protein (a gift from Dr Kari Alitalo), nor with soluble PDGF-βR proteins (a gift from Dr Michael Pech). For immunostaining, ascites fluid diluted 1:50 was used.

Immunohistochemistry

Ten micromolar acetone-fixed cryosections were incubated with 0·3 per cent H_2O_2 in methanol for 20 min to inhibit endogenous peroxidase. After several washes with phosphate-buffered saline (PBS), the sections were incubated with 2 per cent normal serum or with 1 per cent bovine serum albumin (Sigma, Deisenhofen, Germany) to block non-specific binding. Subsequently, sections were incubated overnight at 4°C with the primary antibody or control as follows: the concentration of the rabbit anti-PlGF antibody was 2·5 μg/ml IgG and ascites solution was used 1:50 diluted. The monoclonal anti-PECAM antibody (CD31) was diluted 1:30 (Dako, Hamburg, Germany). For control purposes, 500 ng of human recombinant PlGF-2 was incubated with 120 ng of PlGF IgG for 15 min at room temperature. This mixture was then applied as the primary antibody on selected sections. Sections were washed twice in PBS for

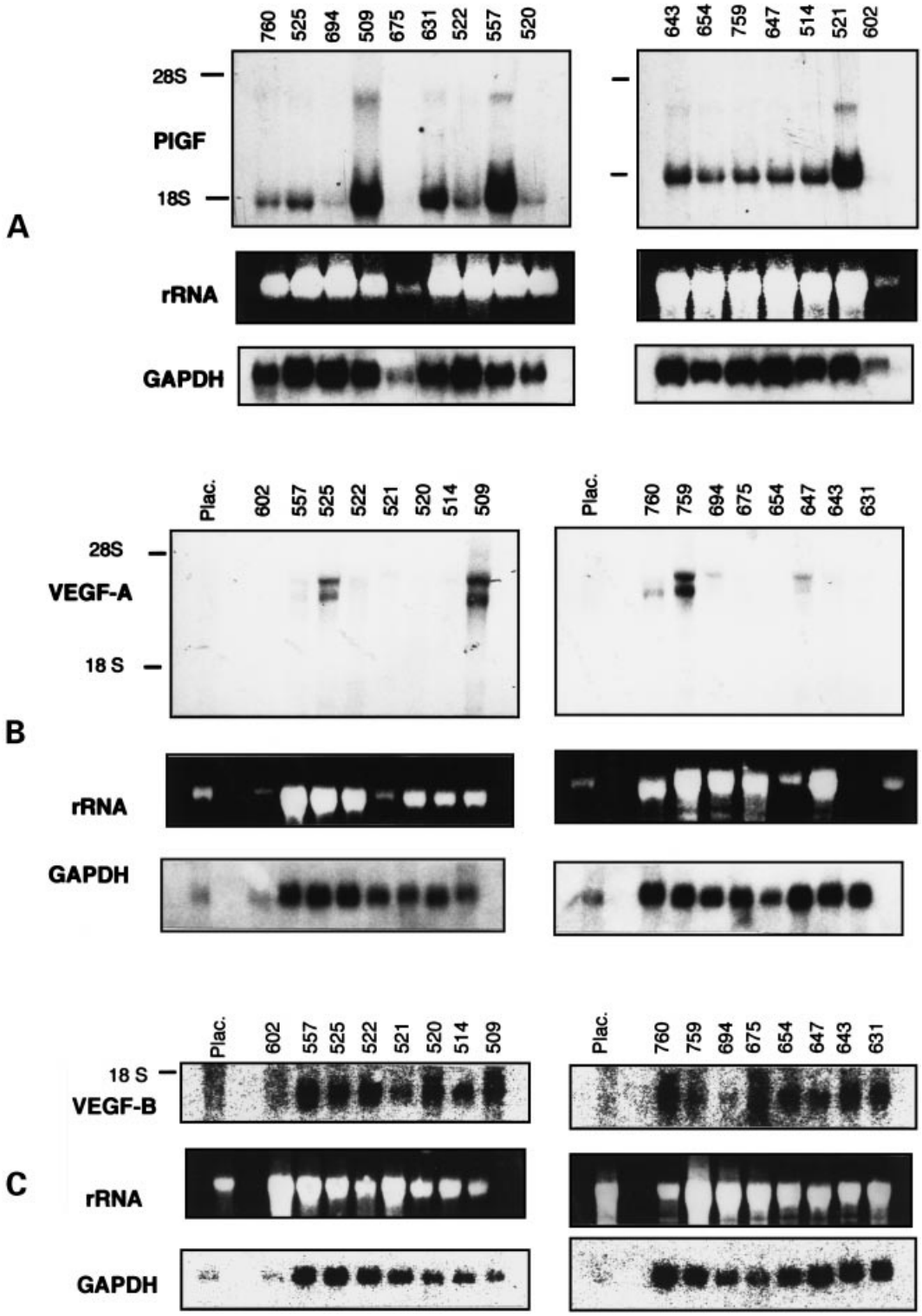

Fig. 1—Northern blot analysis of PlGF (A), VEGF-A (B), and VEGF-B (C) gene expression in human brain tumours. The lanes represent 16 different meningioma specimens as specified in Table I. Five micrograms of total RNA was electrophoresed on a 1·25 per cent agarose-formaldehyde gel and after blotting to nitrocellulose membrane hybridized to a human cDNA that had been ^{32}P-labelled by random priming. The RNA loading was controlled by ethidium bromide staining of the gel (28 S RNA) and by hybridization to the GAPDH gene. The amount of PlGF mRNA expression was quantified by a PhosphorImager or by exposure to X-ray films

15 min and incubated with a biotinylated goat anti-rabbit or goat anti-mouse IgG (0·5 μg/ml; Dianova, Hamburg, Germany). After the washes, detection of antibody binding was performed with a streptavidin–peroxidase kit (Vector, Burlingame, CA, U.S.A.) and 3,3′-diaminobenzidine (Sigma, Deisenhofen, Germany)

in accordance with the manufacturer's instructions. Sections were counterstained with haematoxylin, dehydrated, and mounted.

RESULTS AND DISCUSSION

Sixteen meningiomas were included in this study (Table I). Tumours were derived from 13 women (81 per cent) with ages ranging from 10 to 83 years and three men (19 per cent) with ages ranging from 8 to 80 years. Nine meningiomas (56 per cent) were histologically subclassified as meningothelial, six as fibroblastic (38 per cent), and one (6 per cent) as transitional meningioma. Total RNA from all meningiomas was used to estimate the PlGF, VEGF-A, and VEGF-B. mRNA levels by northern blot analysis. Little is known about the mRNA expression levels of the new members of the VEGF family in brain tumour tissues. Recent studies suggest that PlGF-1 is involved in neovascularization *in vivo*[10] and in the recruitment of monocytes.[8] Unlike VEGF-A, PlGF is not significantly up-regulated by hypoxia.[18] Most of the samples expressed PlGF mRNA (Fig. 1a). Four tumour samples showed a high expression level (24 per cent) and ten samples a weak expression level (59 per cent) of the PlGF gene. Only three samples were completely negative for PlGF expression (17 per cent). Most meningioma samples showed moderate VEGF-A gene expression, which is in agreement with our previous results on VEGF expression in meningiomas,[4] but 5 of 16 (30 per cent) meningiomas expressed high VEGF-A mRNA levels (Fig. 1B). Three samples were positive and five samples were weakly positive for VEGF-A gene (Table I). There was no clear correlation between PlGF and VEGF-A expression. However, two out of four samples with high PlGF expression also showed VEGF-A expression.

Although originally cloned from human tumour cell libraries, it has been shown that VEGF-B gene is expressed in a variety of normal human tissues[11,19] but very little is known about its expression pattern in tumours. Like PlGF, VEGF-B binds to VEGFR-1 and is not up-regulated by hypoxia.[19] In the meningiomas investigated, the VEGF-B expression levels were quite homogeneous with a relatively high basal expression level (Table I and Fig. 1C). Eleven samples out of 16 were clearly positive for VEGF-B mRNA expression (68 per cent) but the expression pattern did not correlate with VEGF-A or PlGF expression. Taken together, our results suggest that the regulatory mechanisms for the overexpression of these three VEGF family members in meningiomas are different and independent of each other. Besides the up-regulation by hypoxia for VEGF-A, other mechanisms in tumour cells must operate, resulting in different levels for mRNA for PlGF and VEGF-B.

As PlGF and VEGF-A proteins have more than 50 per cent amino acid homology, care was taken to develop an antibody which showed no cross-reactivity between VEGF-A and PlGF. The PlGF antibody used in this study recognized both PlGF forms, but not VEGF-A (Fig. 2). Tumour sample No. 557 was used for

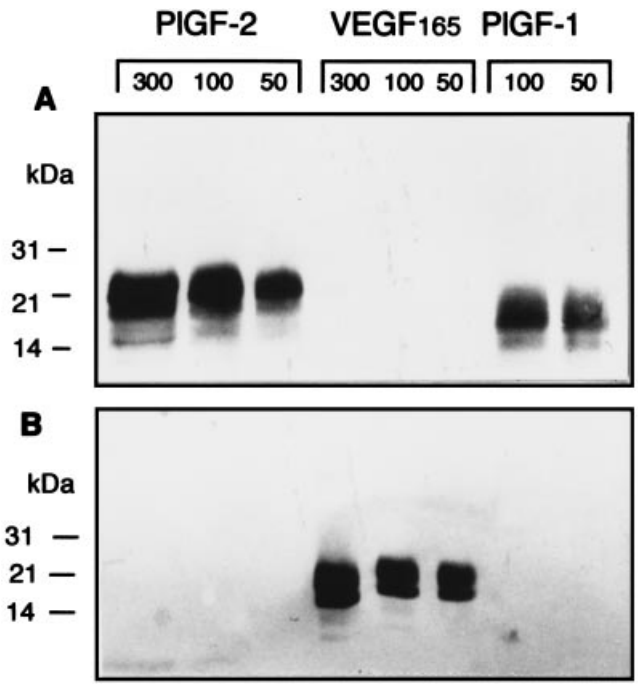

Fig. 2—Specificity of the PlGF antibody demonstrated by immunoblotting with different amounts of PlGF and VEGF-A (in ng/lane). The PlGF antibody (A) recognizes both PlGF splice forms, but not VEGF-A. The presence of the VEGF-A protein is shown by using a VEGF-specific antibody (B)

immunostaining, because this tumour showed high PlGF expression (Table I). An antibody against PECAM-1 was used as a marker for vascular endothelial cells in order to demonstrate vascular density in the tumour sample. PECAM-1 staining showed a high focal vascular density, with all endothelial cells labelled (Fig. 3). In contrast, PlGF protein was predominantly detected around blood vessels, but not in tumour cells. This finding suggests that PlGF acts in a paracrine way to activate specific receptor-tyrosine kinases localized on the surface of endothelial cells (Fig. 3).

It is interesting to note that only some of the tumour vessels were PlGF-positive and only some of the tumour vessels expressed VEGFR-1 and VEGFR-2 (Fig. 3). In addition, the amount of FLT-1 protein varied from vessel to vessel and may reflect a heterogeneous activation status or the onset of vessel regression in some regions of the tumour. These findings indicate that probably not all capillary blood vessels in solid brain tumours are positive for KDR and FLT-1 and that PlGF, as one of the ligands for VEGFR-1, is more heterogeneously expressed than would be expected from our earlier results.[4] Up-regulation of VEGFR-1 and VEGFR-2 on tumour endothelial cells may be influenced by paracrine-acting factors and may reflect dynamic processes during vessel formation and vessel regression in tumour progression. These findings therefore suggest that endothelial cell proliferation, migration, vessel maturation, and the recruitment of perivascular cells are dynamic steps which are not necessarily connected *in vivo* with the up-regulation of both VEGF receptors. However, our results are based on a

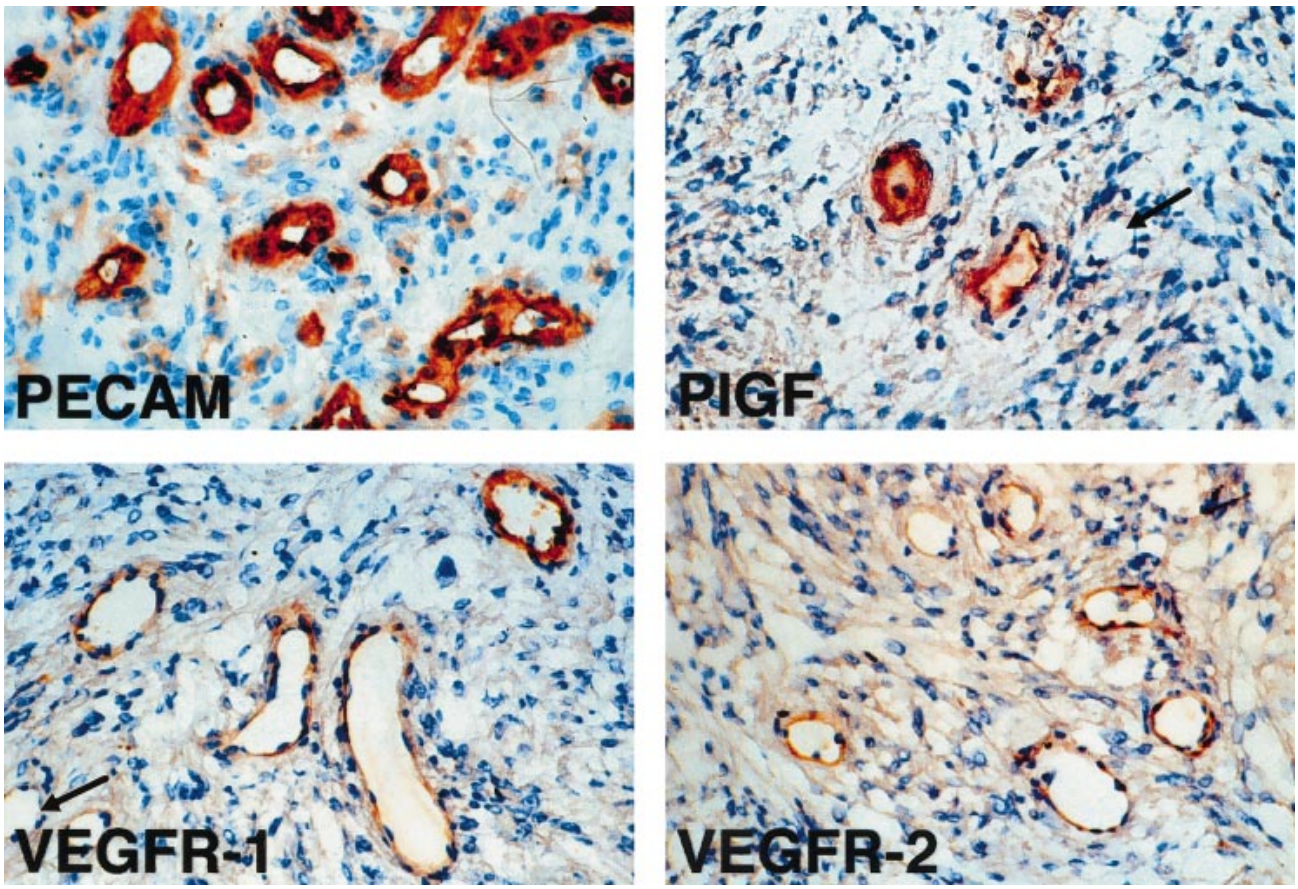

Fig. 3—Immunohistochemical localization of PECAM, PlGF, VEGFR-1 (FLT-1), and VEGFR-2 (KDR) protein in meningioma 557. PECAM-1 staining shows a high focal vascular density, with all endothelial cells labelled. PlGF is predominantly detected around blood vessels, presumably due to binding to VEGFR-1. Some vessels in the tumour tissue (arrow) were PlGF-negative. VEGFR-1 and R-2 were expressed in most, but not all vascular endothelial cells. The arrow denotes a VEGFR-1-immunonegative tumour blood vessel

very limited number of tumour samples and further studies with additional samples and antibodies specific for the two VEGF receptors are necessary to elucidate the general mode of receptor up-regulation and distribution in brain tumours.

Tumour cells were negative for VEGF receptors. The abundant vascularization of meningiomas can be visualized angiographically by means of contrast-enhanced computer tomographic scanning. So far, it is unclear whether PlGF and VEGF-B contribute to the observed vascularity and oedema formation in meningiomas. Increase in vascular permeability and angiogenic activity *in vivo* are key characteristics for VEGF-A but have not so far been reported for PlGF or VEGF-B. The hypothesis that PlGF acts as an angiogenic factor *in vivo* is based on a single publication.[10] In contrast, most other reports found no significant endothelial cell-stimulating activity *in vitro*, or angiogenic activity *in vivo*.[6,7,20,21] However, it has been reported that PlGF is chemotactic for monocytes and endothelial cells.[8,10] It could therefore be involved in the recruitment of these cells during tumour development and progression, and act as an indirect angiogenesis factor. The biological role of PlGF and the other new members of the VEGF family in meningioma development and in meningioma angiogenesis requires further examination.

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