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Angiogenic profile of childhood primitive neuroectodermal brain tumours/medulloblastomas

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Abstract

Primitive neuroectodermal brain tumours (PNET) including medulloblastomas (PNET/MB) are the most common malignant brain tumours of childhood. Similar to many other brain tumours, PNET/MB often show marked neovascularisation. To determine which angiogenic factors contribute to PNET/MB angiogenesis, we examined the expression of eight angiogenic factors (vascular endothelial growth factors (*VEGF*, *VEGF-B*, *VEGF-C*), basic fibroblast growth factor (*bFGF*), angiopoetins (*Ang-1*, *Ang-2*), transforming growth factor (*TGF-α*), and platelet-derived endothelial growth factor (*PDGF-A*)) by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in six PNET cell lines and 28 primary PNET/MB. Expression levels of angiogenic factors were compared with microvessel density, *TrkC* mRNA expression, clinical variables and survival outcomes. Our results indicate that all PNET/MB tested produce a wide range of angiogenic factors that are, individually or together, likely to play a direct role in PNET/MB tumour growth. This suggests that anti-angiogenesis approaches targeting VEGF alone may be insufficient in PNET/MB.

Keywords: Angiogenesis; Brain tumour; Medulloblastoma; Vascular endothelial growth factor; Basic fibroblast growth factor; Angiopoetin; Transforming growth factor-α; Platelet-derived endothelial growth factor

1. Introduction

Primitive neuroectodermal brain tumours (PNET) including medulloblastomas (PNET/MB) and supratentorial PNETs constitute more than 20% of all paediatric brain tumours [1]. PNETs are characterised by their aggressive clinical behaviour and high incidence of leptomeningeal dissemination. Despite 5-year survival rates for PNET/MB exceeding 80% in some studies [2], nearly half of these patients will eventually die from progressive tumours. Accordingly, the identification of novel therapeutic strategies, such as gene therapy, agents designed to interfere with signal transduction, maturation agents, immunotherapy and anti-angiogenesis drugs, remains a major goal [3].

Angiogenesis, the formation of new blood vessels from existing vasculature, is a tightly regulated process that is essential for tumour growth [4,5]. Endothelial cell activation, proliferation, migration and tissue infiltration from pre-existing blood vessels are triggered by specific angiogenic growth factors produced by tumour cells and the surrounding stroma [6–9]. The known angiogenic growth factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiopoetin-1 and-2 (Ang-1 and Ang-2), transforming growth factors (TGFs), and platelet-derived growth factors (PDGF) (reviewed in Refs. [10,11]).

VEGF is a major angiogenic effector and endothelial-specific mitogen, and comprises four isoforms that arise from alternative splicing of a single gene. The poly-

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peptides of 121 and 165 amino acids (the latter is the most abundant and best characterised form) are soluble, whereas the 189 and the rare 206 amino acid forms remain bound to heparan sulphate proteoglycans at the cell surface. The different VEGF isoforms bind to two tyrosine kinase receptors, flt-1 (fms-like tyrosine kinase, VEGFR-1) and KDR (kinase insert domain containing receptor, VEGFR-2, Flk-1), with high affinity. These two receptors are almost exclusively expressed on endothelial cells [12]. VEGF-B and VEGF-C are two members of the VEGF family that have mitogenic and/or chemotactic actions on endothelial cells, indicating that they may also contribute to the induction or maintenance of angiogenesis [13]. VEGF-B exists in two splice isoforms of 167 and 186 amino acids, respectively, and is expressed in most tissues. VEGF-C binds and activates the tyrosine kinase receptors flt-4 (VEGFR-3) that is mainly expressed in the endothelium of lymphatic vessels and KDR that is expressed in all endothelia [14]. VEGF-B and VEGF-C have been detected in a variety of human solid tumours [13].

Ang-1 is the ligand for TIE2, a receptor-like tyrosine kinase almost exclusively expressed in endothelial cells. It may be important for vessel integrity as it mediates interactions between endothelium and the surrounding matrix. Ang-2, a protein with high structural homology to Ang-1, has been implicated in loosening contacts between endothelial and periendothelial cells, thus rendering endothelial cells accessible to angiogenic inducers such as VEGF [11]. bFGF is a mitogenic, angiogenic and neurotrophic factor expressed by many tumour cells. It is a potent stimulator of endothelial cell growth *in vitro* and *in vivo* [15,16] and synergism with VEGF has been demonstrated by *in vitro* angiogenesis assays [17]. PDGF consists of two related polypeptides (A- and B-chain) [18,19]. Originally identified in the regulation of cell migration and proliferation, it has recently been found to possess angiogenic capability both *in vitro* and *in vivo* [20]. TGF-α has been shown to induce VEGF expression [21] and has also an angiogenic role *in vivo* [22,23].

Human brain tumours such as anaplastic astrocytomas and glioblastoma multiforme are among the most highly vascularised solid tumours. Similar to other brain tumours, such as meningiomas, these tumours often produce high levels of bFGF, TGF-α, angiopoetins, and VEGF [24–29]. Moreover, expression of *VEGF* mRNA was shown to correlate with vascularity in both gliomas and meningiomas [30]. Similar to many other CNS tumours, some PNET/MB show marked neovascularisation [31] and it has been reported that some of these tumours show immunoreactivity for VEGF and bFGF [32,33]. However, to our knowledge, no systematic analysis of other important angiogenic factors has been performed thus far in PNET/MB. Therefore, the purpose of this study was to determine the angiogenic profile of CNS PNET/MB. Differential mRNA expression of eight angiogenic factors was examined in 28 primary PNET/MB and in six CNS PNET cell lines and compared with intratumoral microvessel density, age, metastatic stage, neurotrophin *TrkC* mRNA expression and survival outcomes.

2. Patients and methods

2.1. Patients and therapy

78 patients were diagnosed with a PNET/MB at the Children's Hospital of Philadelphia between January 1988 and December 1998. All diagnoses were confirmed by histological assessment of a tumour specimen obtained at surgery by one neuropathologist. Frozen tumour tissue adequate to perform RT-PCR was available from 28 PNET/MB patients. Tumour samples were snap-frozen in liquid nitrogen in the operating room and then stored at -80 °C until further analysis. The demographic and treatment characteristics of the 28 study patients were comparable to those of the 50 PNET/MB patients not included in the present study due to a lack of frozen tumour tissue (data not shown). Therefore, the subset of patients included in the present study can be considered as representative. The median age at diagnosis for these PNET/MB patients was 7 years (range 0.3–14.8 years). Evidence of leptomeningeal metastasis (M1-3) was present in 8 (29%) children, whereas 20 (71%) patients had M0. Gross total resection ($\geqslant$90%) was achieved in 22 patients (79%) and partial resection ($\geqslant$50%, but $<$90%) in 6 patients (21%). 22 (79%) patients had local radiation therapy $\geqslant$50 Gy (XRT) + chemotherapy, 2 (7%) patients had XRT alone, and 4 (14%) patients had chemotherapy alone. 23 (96%) of 24 patients with $\geqslant$50 Gy local radiation therapy had $\geqslant$23 Gy craniospinal radiation. Chemotherapy was administered in 15 patients according to a previously described protocol including vincristine, lomustine and cisplatin [34], in nine younger children according to infant brain tumour protocols [35] and in 2 patients according to other regimens. Approval to link laboratory data to clinical data had been obtained by the Institutional Review Board. For comparison, we studied 11 paediatric glial CNS tumours obtained from the brain tumour bank of the Children's Hospital of Philadelphia that consisted of three pilocytic astrocytomas, four fibrillary astrocytomas, one anaplastic astrocytoma and three glioblastoma multiforme.

2.2. Human PNET cell lines

DAOY and PFSK PNET cells were purchased from the American Type Culture Collection (Rockville, MD, USA). D341, D425 and D458 PNET cells were a kind

gift from Dr Henry Friedman, Duke University, Durham, NC, USA. UW228-2 PNET cells were a kind gift from Dr John R. Silber, University of Washington, Seattle, WA, USA.

2.3. Semiquantitative RT-PCR

RNA isolation, reverse transcription and PCR were performed according to methods previously described [36–39]. PCR primers for angiogenic factors were designed to bracket cDNA sequences that cross an intron–exon boundary in genomic DNA and have been previously described [39,40]. Primers for *VEGF* detected all four splice isoforms of *VEGF*: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. Primers for *VEGF-B* detected both $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$. Primers for neurotrophin receptor *TrkC* were 5′-TTC CTC TCT TCC GCA TGA AC-3′ (sense) and 5′-AAG CCA TTG TCC TCA CTC GT-3′ (antisense). All primers were biotinylated. To correct for variations in RT-PCR and chemiluminescence detection steps, the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPD*) was used as an internal control. Expression levels of the target transcripts were expressed as target/*GAPD* signal ratios after densitometric analysis of transcript signals using the NIH Image program (US NIH; http://rsb.info.nih.gov/nih-image).

2.4. Intratumour microvessel density

Formalin-fixed paraffin-embedded tumour tissue sufficient to perform immunohistochemistry was available from 26 of the 28 PNET/MB. Endothelial cells were stained by CD34 immunohistochemistry according to established methods [41]. Intratumour microvessel density (MVD) was assessed for areas of the tumour that contained the highest MVD following the method of Weidner and colleagues [42]. In brief, the area of hot spot MVD was located at a total magnification of 50× by two independent observers. Microvessels were counted at a total magnification of 100× magnification using a Leica microscope with a Hamamatsu colour camera and a videoscreen. Any brown staining endothelial cell or endothelial cell cluster, clearly separated from adjacent microvessels, was regarded as a single countable microvessel. Neither vessel lumen nor red blood cells were used to define a microvessel. Larger vessels containing more than eight erythrocytes or a thick lamina muscularis were excluded from the count. The MVD was expressed as the number of microvessels observed in an area of 0.7 mm^2.

2.5. Statistical analysis

Clinical characteristics of included and excluded patients were compared using Fisher's Exact test. Differences in angiogenic factor expression levels between tumour types were tested using Mann–Whitney U test. Spearman rank-correlation coefficient was used to compare expression levels of angiogenic factors and MVD. Two-sample *t*-tests were used to test possible associations between angiogenic factors and clinicopathological variables. Relative risk of progression or death was calculated by univariate analysis using Cox regression models [43].

3. Results

3.1. Expression profile of angiogenic factors in PNET/MB

We used semiquantitative RT-PCR analysis to examine the expression of eight angiogenic factors in six PNET cell lines and 28 primary PNET/MB (Figs. 1 and 2). *VEGF* mRNA was detected in all six PNET cell lines and all primary PNET/MB. The PNET cell lines showed expression of all four isoforms of *VEGF*, whereas the expression pattern of *VEGF* isoforms varied in primary PNET/MB: $VEGF_{165}$ was detected in 100% (median normalised expression level 0.52, range 0.06–1.26), $VEGF_{121}$ in 89% (median normalised expression level 0.20, range 0.00–1.36), $VEGF_{189}$ in 75% (median normalised expression level 0.10, range 0.00–0.78) and $VEGF_{206}$ in 4%, suggesting that $VEGF_{165}$ is the most abundant *VEGF* isoform in PNET/MB. The levels of mRNA expression within the three most abundant isoforms were positively correlated, namely $VEGF_{165}$ with $VEGF_{189}$ ($P<0.0001$) and VEGF121 ($P=0.0001$), as well as $VEGF_{189}$ with $VEGF_{121}$ ($P<0.0001$).

VEGF-B mRNA was detected in both isoforms in all PNET cell lines. $VEGF\text{-}B_{167}$ and $VEGF\text{-}B_{186}$ correlated with each other ($P<0.0001$) and were expressed at relatively low levels in 16 (57%) of 28 primary PNET/MB. *VEGF-C* mRNA was found in 5/6 PNET cell lines and in 22 (79%) of 28 primary PNET/MB. *Ang-1* mRNA was found in two PNET cell lines at relatively high expression levels and in four at low expression levels. Twenty-four (86%) of 28 primary PNETs showed expression of *Ang-1*. *Ang-2* mRNA was detected only in one of six PNET cell lines, but in 22 (79%) of 28 primary PNET/MB samples. *TGF-α* mRNA was present in two of six PNET cell lines and in 21 (75%) of 28 primary PNET/MB. *PDGF-A* mRNA was detected in four of six cell lines at variable levels and in all of the primary PNET/MB. Expression levels of *PDGF-A* were highly variable with more than a 30-fold difference between the highest and lowest values.

Several angiogenic factors correlated positively with each other: $VEGF\text{-}B_{186}$ with $VEGF_{121}$ ($P=0.005$), $VEGF\text{-}B_{186}$ with $VEGF_{189}$ ($P=0.005$), $VEGF_{165}$ with

Ang-2 ($P=0.01$), *VEGF-B*$_{167}$ with *VEGF*$_{189}$ ($P=0.02$) and *VEGF-B*$_{186}$ with *VEGF*$_{165}$ ($P=0.05$) (Fig. 3).

3.2. Comparison of the angiogenic profile of PNET/MB with that of the glial CNS tumours

In the 11 paediatric glial tumours evaluated in this study, expression of *VEGF* correlated with the grade of malignancy; that is, high-grade gliomas (anaplastic astrocytoma and glioblastoma multiforme) had a higher expression of *VEGF*$_{121}$ and *VEGF*$_{165}$ compared with low-grade gliomas (pilocytic astrocytoma and fibrillary astrocytoma) (Fig. 2). The ranges of expression levels of the angiogenic factors in PNET/MB were similar to those of glial tumours, but there were significant differences when expression levels were compared statistically. PNET/MB had lower expression levels of *bFGF* ($P=0.002$), *VEGF*$_{121}$ ($P=0.01$), TGF-α ($P=0.03$), *PDGF-A* ($P=0.03$) and *Ang-2* ($P=0.04$) when compared with the glial tumours. In contrast, expression levels of *VEGF*$_{165}$, *VEGF*$_{189}$, *VEGF-B*$_{167}$, *VEGF-C*, *Ang-1* were similar in the PNET/MB and glial tumours.

3.3. Correlation of angiogenic factors with microvessel density in PNET/MB

The MVD of primary PNET/MB was highly variable with a range from 20 to 323 microvessels per 0.7 mm^2 (median MVD: 53 microvessels per 0.7 mm^2). Positive correlations between expression levels of *VEGF*$_{165}$, *VEGF*$_{121}$, *VEGF*$_{189}$, *Ang-2*, *VEGF-B*$_{167}$, *PDGF-A* and MVD were statistically non-significant (Fig. 4).

3.4. Correlation of angiogenic factors with TrkC mRNA expression, age, M-stage and survival outcomes

There was no significant association between the expression levels of the angiogenic factors and *TrkC* mRNA expression, age (<3 years versus >3 years) or M-stage (M1-3 versus M0). 10 of 28 PNET/MB patients died of progressive disease, 1 patient is alive with progressive disease. Median follow-up for the 17 patients who remain alive and progression-free at the time of this report was 4.8 years (range 0.6–7.0 years). Univariate analysis using Cox regression revealed that *TrkC* mRNA expression (hazard ratio 0.046, $P=0.005$), but not the expression of angiogenic factors, was a statistically significant prognostic factor in PNET/MB patients.

4. Discussion

Our results indicate that all PNET/MB tested produce a wide range of angiogenic factors that are, individually or together, likely to play a direct role in PNET/MB tumour growth. In 93% of the primary PNET/MB and in all six PNET cell lines more than 4 different angiogenic factors were detected. The most abundant angiogenic factor in the PNET cell lines was *VEGF*$_{165}$

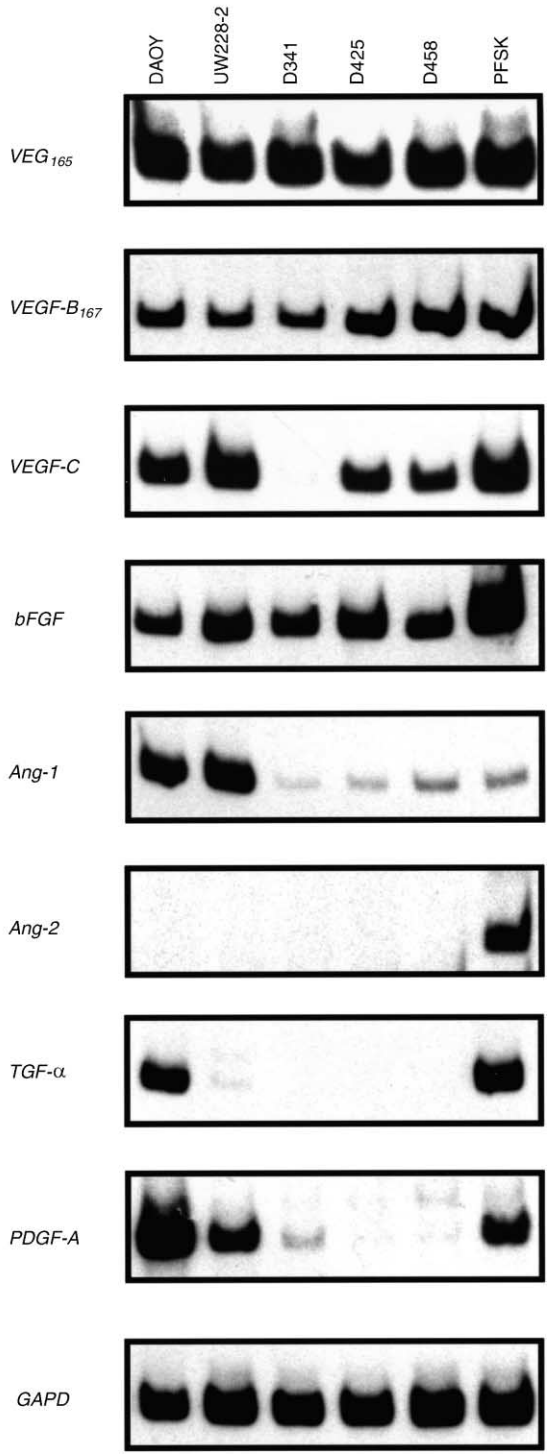

Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) of angiogenic factors in human primitive neuroectodermal brain tumour (PNET) cell lines. Representative example of semiquantitative RT-PCR showing different levels of expression of *VEGF*$_{165}$, *VEGF-B*$_{167}$, *VEGF-C*, *bFGF*, *Ang-1*, *Ang-2*, *TGF-α* and *PDGF-A* in human PNET cell lines. The expression of the housekeeping gene *GAPD* served as the internal control.

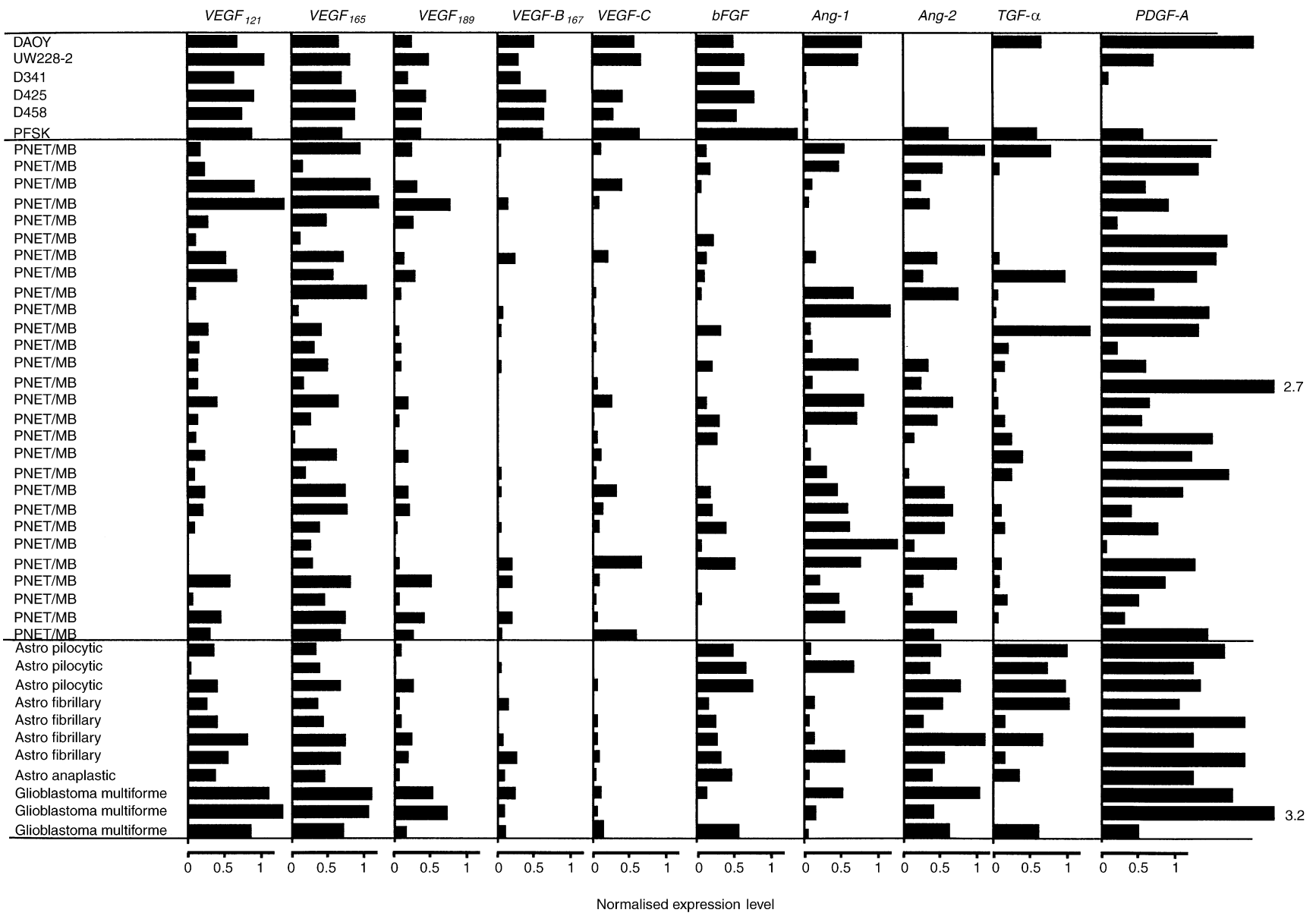

Fig. 2. Expression of angiogenic factors in primitive neuroectodermal brain tumour (PNET) cell lines and in primary PNET/medulloblastomas (PNET/MB) and glial brain tumours. mRNA expression levels of $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF\text{-}B_{167}$, *VEGF-C*, *bFGF*, *Ang-1*, *Ang-2*, *TGF-α* and *PDGF-A* were determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Expression levels of the target transcripts were normalised by use of an internal *GAPD* control. astro, Astrocytoma.

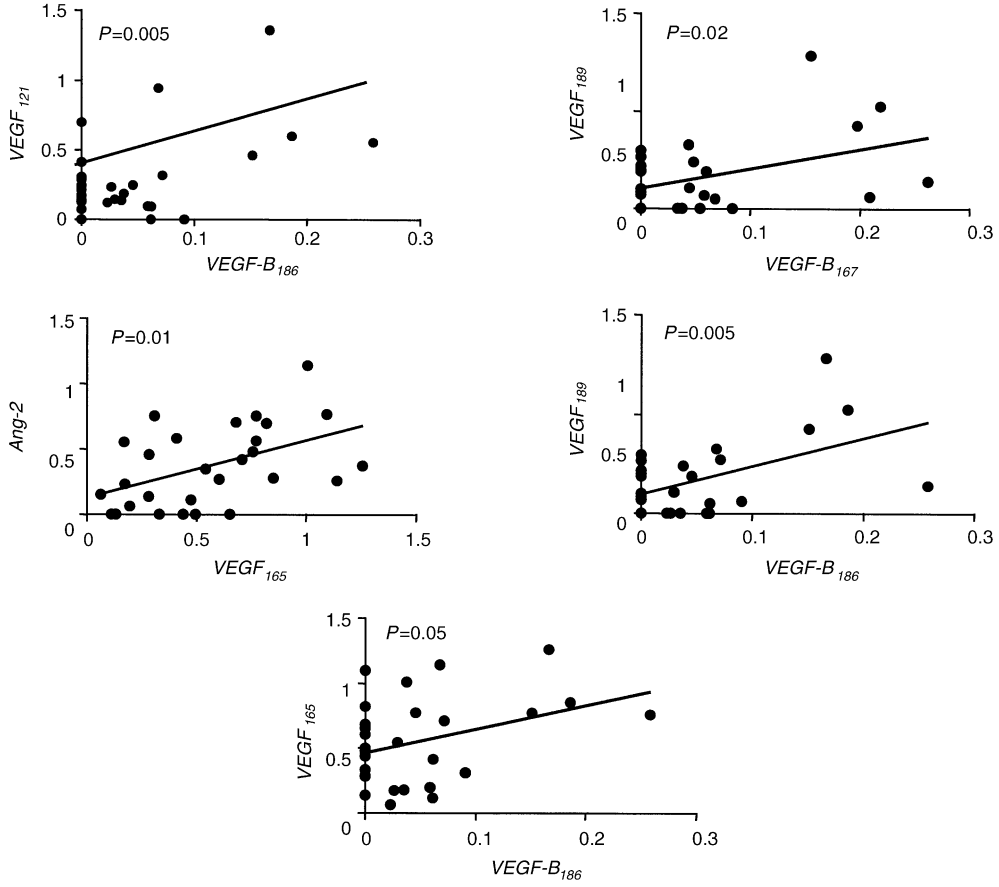

Fig. 3. Comparison of expression levels of angiogenic factors in primary primitive neuroectodermal brain tumours/medulloblastomas (PNET/MB). mRNA expression levels of angiogenic factors were determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Expression levels of the target transcripts were normalised by use of an internal *GAPD* control.

followed by $VEGF_{121}$, *bFGF*. In the primary PNET/MB, $VEGF_{165}$ and *PDGF-A* were expressed in 100%, followed by $VEGF_{121}$ (89%), *Ang-1* (86%), *Ang-2* (79%), *VEGF-C* (79%), *TGF-α* (75%) $VEGF_{189}$ (75%) and *VEGF-B* (57%).

VEGF has been recognised as being one of the most potent angiogenic factors for many solid tumour systems including malignant gliomas [45–47]. High expression of VEGF by tumours or in serum is associated with a worse prognosis in lung, colorectal, and breast cancer [48–50]. In the present study, $VEGF_{165}$ was one of the most abundant angiogenic factors in the PNET cell lines and primary PNET/MB. Moreover, $VEGF_{165}$ expression levels in the primary PNET/MB were similar to those in paediatric glial tumours. The *VEGF* receptors are endothelial-specific, and whereas they are expressed very rarely in endothelial cells of the normal vasculature, they are overexpressed in the vascular supply of neoplastic tissue [51]. Hence, it has been proposed that the inhibition of this factor may result in the inhibition of angiogenesis and a number of agents have been developed to inhibit VEGF. These include monoclonal neutralising antibodies to VEGF [52] or VEGF receptors [53], and synthetic small molecule drugs such as SU5416 that block selectively the VEGF receptor [54].

In the present study, not only *VEGF*, but also *bFGF*, *Ang-1* and *VEGF-C* were abundantly expressed in the PNET cell lines and primary PNET/MB. Moreover, expression of *VEGF*, *VEGF-B* and *Ang-2* were statistically correlated with each other in the primary PNET/MB specimens. This suggests that several angiogenic peptides act in concert in the regulation of neovascularisation in PNET/MB. VEGF-related factors might interact with the VEGF system in a number of ways, e.g. VEGF-B is known to form heterodimers with VEGF [55]. Upregulation of VEGF family members might be mediated by upregulation of common transcription factors, or some angiogenic factors may act through a second messenger system by inducing the expression of other angiogenic factors. TGF-α was found to function as a potent inducer of VEGF synthesis by transcription of the *VEGF* gene promoter via AP2 transcription factors [21] and synergies between bFGF and VEGF have also been reported [17]. However, statistically significant correlations between the expression levels of TGF-α, bFGF and VEGF were not found in the present study, suggesting that interaction of VEGF with VEGF-B, Ang-1 and Ang-2 may be more important in PNET/MB or that angiogenic

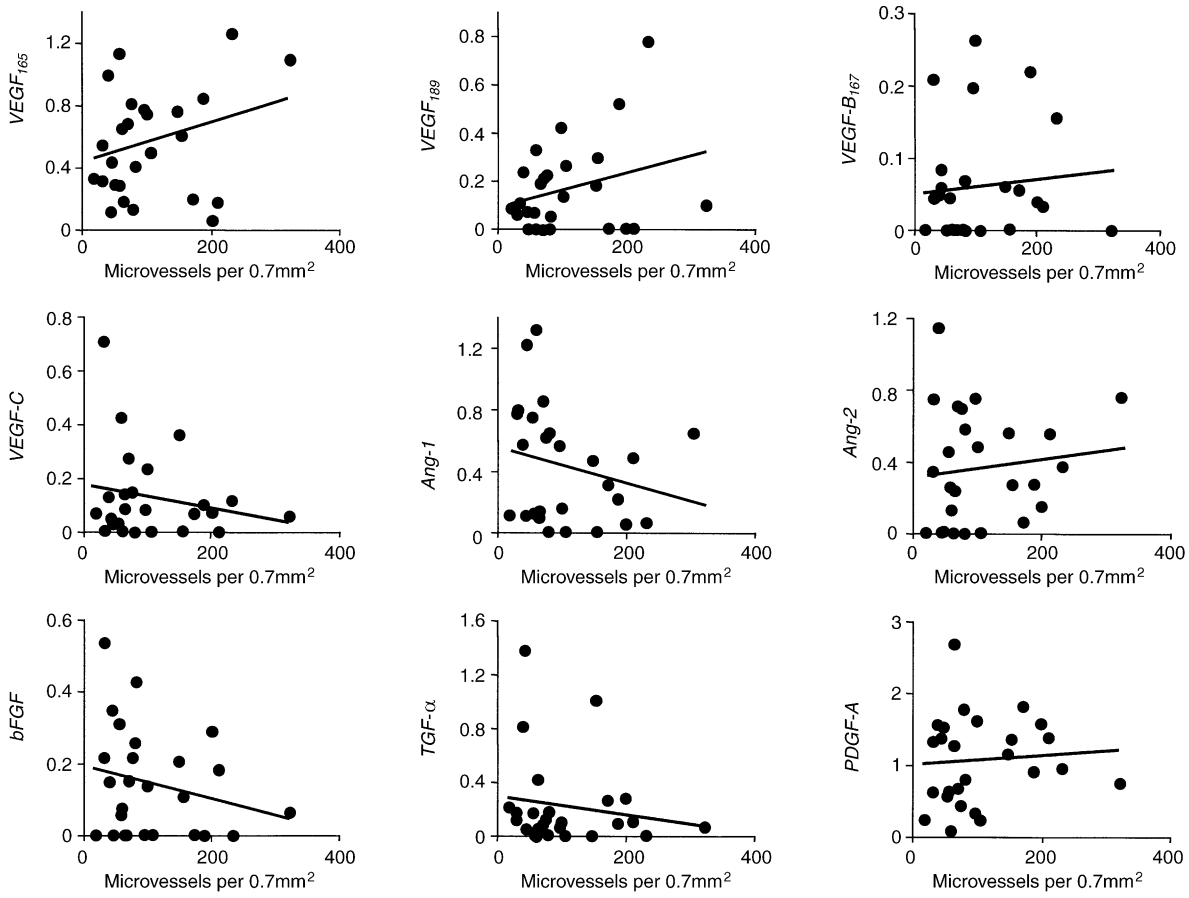

Fig. 4. Comparison of expression levels of angiogenic factors and microvessel density in 26 primitive neuroectodermal brain tumour including medulloblastomas (PNET/MB).

factors may each be expressed during a certain stage of tumorigenesis of PNET/MB.

One environmental condition known to enhance VEGF expression is hypoxia [56]. Other mechanisms that regulate expression of angiogenic factors in tumour cells are still not well understood and signal transduction pathways that regulate VEGF production have yet to be elucidated. Interestingly, we found recently that the neurotrophin receptor TrkA inhibits angiogenesis in neuroblastoma [57]. TrkA transfected SY5Y neuroblastoma cells demonstrated a clear decrease in mRNA expression levels of *bFGF*, $VEGF_{165}$, *Ang-1* and *Ang-2* in comparison to SY5Y control cells. CNS PNET and neuroblastoma share clinical and biological similarities. They are both embryonal tumours derived from primitive neuroectodermal cells. They both peak in incidence in early childhood and represent the most common intracranial and extracranial neoplasms, respectively, in this age group. Whereas high expression of the neurotrophin receptor TrkA is associated with a favourable prognosis in neuroblastoma [58], high expression of TrkC is associated with a favourable prognosis in CNS PNET [44,59]. Thus, we correlated expression levels of angiogenic factors with *TrkC* mRNA expression levels in primary PNET/MB. However, no significant correlation was found between the single angiogenic factors and *TrkC* mRNA expression. In addition, no differences in the expression of angiogenic factors were seen in the DAOY PNET cells overexpressing *TrkC* compared with control cells (data not shown). This is in contrast to neuroblastoma [57] and indicates that TrkC is not likely to influence tumour angiogenesis in PNET/MB.

In the present study, *TrkC* mRNA expression, but not the expression of angiogenic factors was a significant prognostic factor for survival outcomes in PNET/MB patients. Although the number of patients in the present study is too small to draw firm conclusions, it seems unlikely that angiogenic factors will become more predictive of survival outcome than established clinical (M-stage, residual tumour bulk, age at diagnosis) and biological prognostic factors including *TrkC* expression [44,59] and HER2/HER4 receptor coexpression [60]. However, larger studies are needed to assess (co-)expression levels of angiogenic growth factors, as well as inhibitors in order to establish their prognostic significance in PNET/MB.

Taken together, our results suggest that *VEGF*, *VEGF-B*, *VEGF-C*, *bFGF*, *Ang-1*, *Ang-2*, *TGF-α* and

PDGF-A are expressed in PNET/MB and that the expression levels of $VEGF_{165}$, *VEGF-B*, *VEGF-C* and *Ang-1* are similar to that of paediatric glial CNS tumours. The ubiquitous expression of several angiogenesis stimulators in PNET/MB suggests that anti-angiogenesis therapy may provide a novel strategy that may be particularly useful for highly vascularised tumours. While the present study suggests a biological role for VEGF in PNET/MB angiogenesis, it also indicates that anti-angiogenesis therapeutic strategies targeting VEGF alone may be insufficient. This is due to the redundant expression of other angiogenic factors making it likely that more general anti-angiogenesis approaches may be necessary, like therapy with the angiogenesis inhibitor TNP-470, a synthetic angiostatic agent derived from *Aspergillus fumigatus* that specifically inhibits endothelial proliferation independent of the angiogenic factor expression [61].

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