

Angiogenic Factors in Plasma of Brain Tumour Patients

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Abstract. *Background: Angiopoiesis and angiopoietic growth factors are of considerable importance in the development and progression of intracranial tumours. However, knowledge of the plasma detectability of distinct angiogenic factors in patients with brain tumour is very limited. This study evaluates the plasma concentrations of the angiogenic factors angiopoietin-2 (Ang-2), vascular endothelial growth factor (VEGF) and platelet-derived growth factor BB (PDGF-BB) in patients with brain tumour. Patients and Methods: Plasma samples of 78 patients suffering from various types of intracranial tumours (glioblastoma multiforme, GBM, n=22; astrocytoma, n=12; meningioma, n=16; and intracranial metastasis, n=28) were analysed. For determination of plasma concentrations of angiogenic factor, highly specific enzyme-linked immuno sorbent assays (ELISAs) were used. Results: Ang-2 plasma concentration in GBM patients was significantly lower when compared with that in patients with meningioma and intracranial metastasis. Highest levels of VEGF concentrations were detected in plasma derived from patients suffering from meningioma. Interestingly, VEGF plasma levels depended on the number of intracranial lesions, with significantly higher concentrations in patients with 3 or more lesions when compared with those with 2 or fewer lesions. However, no correlation between the survival time of the patients and the plasma levels of the tested growth factors was obtained. Plasma levels of PDGF-BB did not differ between the individual tumour groups. Conclusion: The detectability of the angiogenic factors Ang-2 and VEGF, as well as of PDGF-BB, in the plasma of patients suffering from various types of brain tumours is described. The plasma detectability of the individual angiopoietic factors seems to depend at least partly on the tumour type as well as on tumour progression. This might be of prognostic and therapeutic relevance.*

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Primary brain tumours are classified into 4 malignancy groups as defined by the World Health Organisation (WHO) (1). The most malignant tumour is the glioblastoma, also known as glioblastoma multiforme (GBM). Although chemotherapy, radiotherapy and surgical methods have progressed enormously in recent years, the prognosis of GBM patients is still very limited (2).

Angiogenesis plays a crucial role in tumour growth, particularly in GBM. The unique structure of brain vessels, consisting of endothelial cells (EC), pericytes and astrocytes, is abnormal in these tumours. There are many growth factors and cytokines involved in the complex mechanism of angiogenesis. Vascular endothelial growth factor (VEGF) is a secreted dimer with characteristic kinase domain receptor binding sites (3, 4). Based on its potential to increase vascular permeability, Senger and colleagues were the first to hypothesize an involvement of VEGF in angiogenesis (5). Development of abnormal vascular structures in mice lacking VEGF has encouraged this hypothesis (6). Endothelial expression of VEGF was detected in all angiogenesis stages, namely survival, proliferation, migration and permeability, underlining the central role of this growth factor in new vessel formation (7). Up-regulation of VEGF transcription in tumour cells due to hypoxia (8, 9) represents a stimulus for angiogenesis. Notably, in gliomas, expression levels of VEGF and its receptor highly correlate with their malignancy grade (10).

Angiopoietins have been identified as one of the most important growth factors involved in the VEGF signalling pathway (11, 12). Upon stimulation, angiopoietin-1 and -2 (Ang-1 and -2) are secreted and act *via* the Tie 2 tyrosine kinase receptor (13). In mice, alterations of the expression levels of angiopoietins and their receptor Tie 2 resulted in a high extent of vessel malformations. Thus the potential role of the angiopoietin/Tie 2 receptor system in angiogenesis has been suggested (11, 14-16). Ang-1 induces auto-phosphorylation of the tyrosine kinase-dependent Tie 2 receptor, whereas Ang-2 is a natural antagonist (16). In GBM, Ang-1 was highly expressed in tumour cells and Ang-2 was predominantly present in ECs (17-19). It is hypothesized that Ang-1 promotes angiogenesis in GBM (20, 21), whereas Ang-2 serves as an early marker of tumour angiogenesis in astrocytomas (22).

In addition to the potential permissive function between VEGF and angiopoietins in glioma progression (23), platelet-derived growth factor (PDGF) might also be relevant for vessel formation and cell migration in brain angiogenesis (24, 25).

In analogy to primary brain tumours, an adequate blood supply is of similar importance for the formation and progression of brain metastases, which represent 50% of all malignant brain tumours (1). Although the mediators of the formation of intracranial metastases are still under investigation, data already exist showing the central role of VEGF signalling in this process in mice (26, 27).

Elevated expression of vascular growth factors Ang-2, VEGF and PDGF-BB in various types of brain tumours is a well-known fact, however their plasma detectability *in vivo* has not been investigated yet. Based on the importance of angiogenesis in tumour development, the determination of their plasma levels might be a useful clinical tool. This study is aimed at the evaluation of the plasma detectability of distinct angiopoietic factors in patients suffering from brain tumours and at the comparison of their plasma levels in dependence of the individual tumour type.

Patients and Methods

Study population. The study participants were patients at the Department of Neurosurgery, Medical University of Vienna, between November 2005 and August 2007. All blood samples were taken from forearm veins prior to the resection of intracranial tumours or any other treatment modalities. A written informed consent was received from each patient prior to vein puncture. The study was approved by the local Ethics Committee. The diagnosis for each group was made by histological investigation of surgically removed tumour tissue by experienced pathologists.

Sample collection. The blood samples were collected into 7 ml vacutainer EDTA tubes and were immediately cooled on ice. Subsequently, plasma was separated from cells by centrifugation at 3200 rpm for 10 min at 4°C and immediately frozen in aliquots at -80°C for further use. The exact time points of blood sampling and freezing were documented. All blood samples were taken between 5 to 6.30 p.m., 6 to 15 days after the first diagnosis of the malignant tumours.

ELISA assays for Ang-2, VEGF and PDGF-BB. These analyses were performed with commercially available ELISA kits (Quantikine® Human VEGF, Quantikine® Human Ang-2 and Quantikine® Human PDGF-BB Immunoassay, all from R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Briefly, standards were prepared using the provided recombinant proteins. One hundred µl of assay diluent followed by 50 µl of either standard or plasma sample were added to each well and incubated for 2 h on a constant shaker at room temperature. Subsequently, wells were washed 3 to 4 times with washing buffer (R&D Systems) using the ELX Auto strip Washer (Biotex Instruments, Winooski, VT, USA) and 200 µl of conjugate were added to each well. After an additional incubation period of 2 h under constant shaking at room temperature, which was followed

Table I. Demographic data of brain tumour patients separated into four groups.

	GBM	Astrocytoma	Metastasis	Meningioma
Number of patients	22	12	28	16
Gender, n (%)				
Male	15 (68)	5 (42)	17 (60)	7 (44)
Female	7 (32)	7 (58)	11 (40)	9 (56)
Age, years (mean±S.D.)	64 (±13)	43 (±16)	61 (±9)	56 (±15)
Died	17	1	19	ND
Survival (months) (mean±S.D.)	8 (±9)	ND	8 (±8)	ND

GBM, Glioblastoma multiforme; ND, not defined.

by the described washing procedure, 200 µl of substrate solution was applied to each well and samples were incubated for 30 min under light protection. The reaction was stopped by addition of stop solution (50 µl) and the ELISA plate was read at 450 nm within 30 min using Powerwave ELISA reader (Biotec Instruments, Winooski, VT, USA). The limit of quantification was specific for each assay and given as 8.3 pg/ml for Ang-2, 5.0 pg/ml for VEGF and 15 pg/ml for PDGF-BB.

Statistical analysis. Statistical analyses were performed with SPSS Version 11.0 (SPSS, Chicago, IL, USA). Comparisons between groups were calculated using unpaired Student’s *t*-test. The differences were considered as significant when *p*≤0.05. Parameters are indicated as grouped mean values±S.D. The association between the 3 angiogenesis factors were evaluated with Pearson’s correlation coefficient. The same test was used for performing a correlation analysis between the survival rates of brain tumour patients in different groups.

Results

Patient characteristics. In this study, blood samples were collected from a total of 78 patients suffering from malignant or benign brain tumours undergoing either a neurosurgical investigation or tumour-directed intervention. The study participants suffered from one of the following brain tumours: meningioma (n=16); GBM WHO Grade IV (n=22); astrocytoma WHO II-III (n=12); intracranial metastasis (n=28). In the metastasis group, the primary tumours consisted of lung carcinoma (n=16), renal cell carcinoma (n=5), breast cancer (n=3), melanoma (n=2) and thyroid carcinoma (n=1). For detailed patient characteristics, see Table I.

Plasma concentrations of Ang-2, VEGF and PDGF-BB in the study population. In a first step, the Ang-2 concentration in peripheral blood samples was measured (Figure 1A). Although Ang-2 was detectable in all plasma samples, a strong interindividual variability was observed, which depended at least partly on the tumour type. Interestingly, the

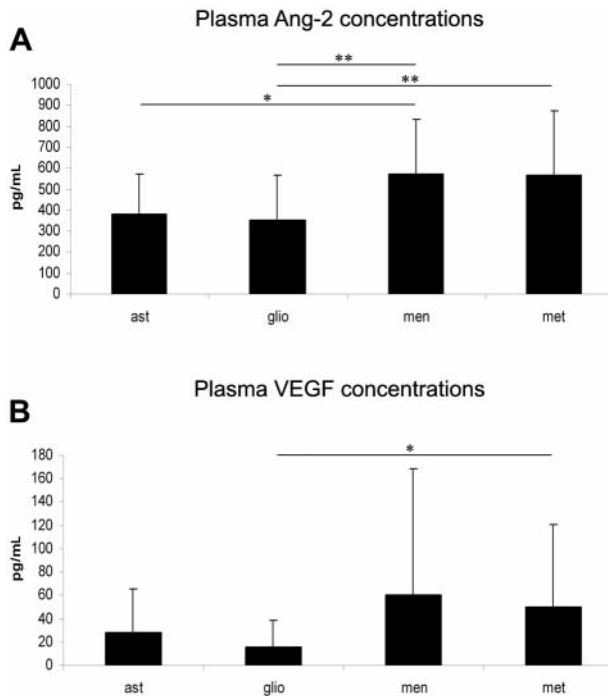


Figure 1. Ang-2 (A) and VEGF (B) plasma levels of four brain tumour patient groups. A, The Ang-2 concentration was significantly lower in the GBM group when compared with that in the meningioma (** $p < 0.007$) and intracranial metastasis population (** $p < 0.007$). In the astrocytoma group, Ang-2 levels were lower in comparison to the meningioma group ($p < 0.05$) (ast: astrocytoma, glio: glioblastoma multiforme, men: meningioma, met: intracranial metastasis). B, The VEGF plasma levels paralleled the Ang-2 plasma concentration in brain tumour patients. VEGF levels were significantly lower in GBM patients than in patients suffering from intracranial metastasis ($p < 0.03$).

mean Ang-2 concentration in the GBM group (351.95 ± 213.56 pg/ml) was much lower than that observed in patients with meningioma (571.2 ± 263.28 pg/ml, $p < 0.007$), or those with intracranial metastasis (565 ± 305.27 pg/ml, $p < 0.007$). In patients suffering from astrocytomas, the plasma concentrations (378.95 ± 193.33 pg/ml) were similar to those observed in GBM patients, which was significantly lower than the Ang-2 plasma levels of patients with meningioma ($p < 0.05$).

As expected, the concentration of VEGF paralleled those measured for Ang-2 (Figure 1B). The VEGF plasma concentrations of the intracranial metastasis group (50.3 ± 70.5 pg/ml) were significantly higher than those of patients suffering from GBM (15.50 ± 22.5 pg/ml, $p < 0.03$). However, no statistically significant difference was detected between the plasma VEGF levels of the GBM and the meningioma groups.

Although PDGF-BB was detectable in all tested plasma samples, the plasma concentrations of this growth factor differed only marginally between the individual tumour groups.

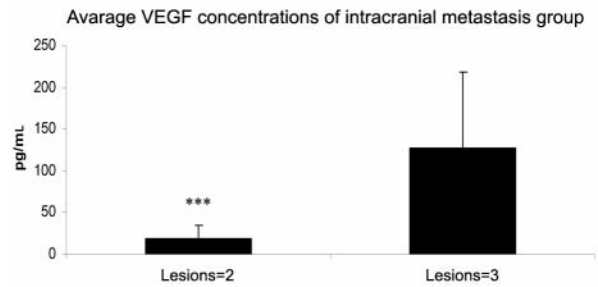


Figure 2. VEGF plasma levels with regard to the extent of intracranial metastasis. Intracranial metastasis patients were separated into those with 2 or fewer and those with 3 or more intracranial tumour lesions. The VEGF concentration of the group with two or fewer lesions ($n=20$) was significantly lower than that of the group with three and more lesions ($n=8$): *** $p < 0.00001$.

Correlation of Ang-2, VEGF and PDGF-BB plasma levels with the number of intracranial metastases. Intracranial metastasis strongly depends on the generation of new blood vessels. Therefore, the plasma levels of Ang-2, VEGF and of PDGF-BB in patients bearing 1 or 2 metastatic lesions ($n=20$) were compared with those of patients exhibiting 3 or more intracranial metastatic lesions ($n=8$). Whereas there was no significant difference between groups with respect to the Ang-2 and PDGF-BB plasma concentrations, the plasma concentration of VEGF (Figure 2) was significantly higher in patients suffering from 3 or more metastatic lesions when compared with patients suffering from fewer than 3 metastatic lesions (18.2 ± 16.5 pg/ml versus 126.9 ± 91.3 pg/ml; $p < 0.0001$).

Correlation of Ang-2, VEGF and PDGF-BB plasma levels with patient survival. The expression levels of distinct angiopoietic factors have been shown to depend on the tumour malignancy grade. Therefore, the plasma levels of Ang-2, VEGF and PDGF-BB were correlated with the patients' survival time. The individual survival data were obtained from the hospital records. Data were available for 17 of the GBM patients, of whom all had died; for 28 patients suffering from intracranial metastasis, of whom 19 had died; of 12 astrocytoma patients, of whom 1 had died at the time of this study. The mean survival periods of the GBM patients and of the patients suffering from intracranial metastasis were 8 ± 9 months and 8 ± 8 months, respectively. The survival data were compared with the plasma levels of the individual growth factors using Pearson's correlation coefficient. However, none of the 3 growth factors was found to correlate with the survival of the patients.

Discussion

Similar to other tumour entities, angiopoiesis is of central importance in brain tumour growth. The role of angiopoietic factors in the development of brain tumours was extensively studied with respect to mRNA and protein at the tissue level,

however, no data has been presented about their plasma detectability in this type of tumour patients.

This paper describes considerable plasma values of the angiopoietic factors Ang-2, VEGF and PDGF-BB in samples derived from meningioma patients as well as in the plasma of patients suffering from brain metastasis. In contrast, especially in plasma derived from GBM and to a similar extent in the plasma of patients with astrocytoma, concentrations of Ang-2 were significantly lower than those of patients with meningioma and intracranial metastasis. No significant difference was found between the individual tumour entities with respect to the PDGF-BB concentrations. Earlier studies performed on brain tumours including GBM and astrocytoma demonstrated high tissue expression levels of Ang-2 (17, 19). Additionally, there are several studies showing high tissue expression as well as high plasma concentrations of this factor in association with other extracerebral neoplasias (28, 29). Thus, the finding of low Ang-2 plasma concentration in GBM patients was surprising. One possible explanation for this discrepancy might result from the highly specific characteristics of central nervous vasculature. The varying ability of distinct substances to penetrate the blood-brain barrier is a well-known fact. Moreover, alterations of the blood-brain barrier in association with central nervous system neoplasias are frequently observed. Although little is known about the potency of Ang-2 to pass the blood-brain barrier, altered blood-brain barrier permeability for these factors might explain the difference between the reported tissue expression levels and the plasma concentrations of Ang-2. In this context, the high expression levels of angiopoietic factor receptors, which have been observed especially in GBMs, are of additional interest (17). Rapid receptor binding and the resulting absence of these factors in the plasma might also contribute to the phenomenon of low plasma concentration of Ang-2 in the circulation of patients suffering from high Ang-2-expressing GBM.

As reported earlier (12), Ang-2 and VEGF are synergistically involved in the formation of new blood vessels. Thus, a correlation of the plasma concentrations of VEGF and Ang-2 in GBM patients was expected. However, the data did not show any significant correlation. This might be due to high interindividual differences of VEGF concentrations within the same group in the study population. Interestingly, the VEGF concentration was significantly higher in patients with intracranial metastasis when compared with patients suffering from GBM. Previously, VEGF was found to be highly detectable in the plasma derived from various kinds of primary solid tumours (30-33). Thereby the high VEGF levels detected within the intracranial metastasis group might reflect the high tumour load of these patients. Moreover, a limited permeability of the blood-brain barrier as well as rapid intracerebral degradation at the site of secretion might additionally contribute to the relatively low VEGF concentrations in the plasma derived from GBM patients.

A correlation of VEGF and PDGF-BB expression levels was demonstrated in distinct tumours, which led to the hypothesis that PDGF-BB represents one of the key mediators in angiogenesis (34). Secretion of VEGF in angiogenic and metastatic procedures may be an indirect effect of PDGF-BB stimulation (35). Therefore, the absence of a significant correlation between the plasma levels of VEGF and PDGF-BB in our study population was surprising. On the one hand, this might be due to autocrine stimulatory effects on the mRNA, which has been observed for VEGF and PDGF-BB, as well as for Ang-2 and VEGF (36-38). On the other hand, in analogy to the observation concerning the difference between Ang-2 expression and plasma detectability, this finding might be due to additional factors influencing the release of PDGF-BB from tumour tissue into the circulation.

Malignant brain tumours have a poor prognosis, which is in part due to delayed diagnosis but also due to their aggressive growth potential with limited therapeutic options. Plasminogen activator inhibitor (PAI-1) (39), glial fibrillary acidic protein (40), low molecular weight caldesman (41), cathepsin D (42) and others were suggested to represent potential plasma markers for brain tumour diagnosis and surveillance. With respect to the importance of vascular growth factors in tumour biology with potential implications on new therapeutic options, these data on plasma levels of angiopoietic factors might form the basis to extend the diagnostic armamentarium in neuro-oncology. In this context, the recently reported success of VEGF-targeted therapies in GBM treatment seems of specific interest (43). Although no data are available for the potential application of this treatment modality for brain metastasis, the high plasma levels of angiopoietic factors found in our patients with multiple brain metastases suggests the applicability of VEGF blockade-based therapeutic regimes for this selected patient group. The success of therapeutic strategies involving the inhibition of growth factor pathways strongly depends on the identification of susceptible patients. Therefore, the identification of plasma-detectable vascular growth factors in the course of brain tumours might be not only of diagnostic, but also of prognostic and therapeutic relevance.

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