Abstract. Vascular endothelial growth factor (VEGF) is a homodimeric, disulfide-linked glycoprotein which exhibits endothelial cell-specific mitogenic properties. VEGF is also a potent inducer of vascular permeability. There is considerable experimental evidence that VEGF isoforms are strongly involved in provoking neoangiogenesis of neoplastic cells and, consequently, the growth and progression of primary neoplasms (i.e., astrocytic gliomas), including the formation of an invasive and metastatic immunophenotype (IP). During this immunohistochemical study, the presence and tissue localization of VEGF121 was observed in anaplastic, high-grade astrocytomas (AAs) and in glioblastoma multiforme (GBMs) employing the specific monoclonal antibody against it. A sensitive, four-step, alkaline phosphatase-conjugated antigen detection technique was used. The immunoreactivity demonstrated a cytoplasmic, cell surface and extracellular matrix localization pattern in more than 90% of the tumor cells, with high intensity immunoreactivity (+++, +A, B) in every high-grade astrocytic glioma tissue. VEGF121 expression was identified mostly within the cytoplasm of tumor cells, suggesting an embryonic, undifferentiated and more malignant cellular IP of high-grade gliomas. Tumor-related neo-angiogenesis and endothelial cell proliferation were also present. The great majority of high-grade astrocytic gliomas are incurable with the three classic therapeutic modalities. In the future, the development of targeted anti-neoplastic treatment strategies, adapted to individual patients, will require molecular identification of the different classes of neoplasm (including subtypes of astrocytomas) according to their stages, biology, prognosis and therapeutic options.

Malignant childhood astrocytomas (ASTRs) are neuroectodermal tumors appearing within the neuro-glial or macroglial central nervous system (CNS) (1). Gliomas can grow anywhere in the CNS. In children, they usually occur in the brain stem, the cerebrum, or the cerebellum, with the most common brain tumors developing from glial cell precursors (astrocytes, oligodendrocytes and ependymocytes). High-grade glial tumors, including glioblastomas (GBMs), are characterized by hypercellularity, pleomorphism, numerous mitoses, various degrees of necrosis, and multiple endothelial cell proliferations related to tumor neo-angiogenesis (2-4). Astrocytomas account for about 68% of the primary brain tumors occurring in children <20 years old and for over 50% of all intracranial tumors (2-7).

GBM is a grade IV astrocytoma according to the World Health Organization (WHO) classification. It is a highly malignant and aggressive neoplasm, regarded as the prototype of neoplastic tissue capable of inducing neo-angiogenesis (8-10). The vascular phenotype of this tumor is a hallmark for histopathological diagnosis (11). Furthermore, glial tumors are characterized by a strong tendency for local invasiveness, but a relatively low distant metastatic potential. The majority of malignant glial tumors are incurable with the classic therapeutic modalities, including the combination of surgical resection, radiotherapy and chemotherapy, the survival rate not having changed substantially in the last 50 years. The prognosis of high-grade gliomas also remains unpredictable because the histological features alone provide an imperfect assessment and prognostication of the biological behavior of a given lesion.

Vascular endothelial growth factor (VEGF) is a disulfide-linked homodimeric glycoprotein of about 40 kDa that promotes fluid and protein leakage from blood vessels,
being a cytokine which potently enhances microvascular permeability and a selective mitogen for endothelial cells (12, 13). It has been shown to be a potent mediator of brain tumor angiogenesis, vascular permeability and glioma growth (14). Of the four known isoforms, the smaller two, VEGF₁₂₁ and VEGF₁₆₅, are secreted proteins acting as diffusible agents, whereas the larger two, VEGF₁₈₉ and VEGF₂₀₆, remain cell-associated (15).

Implantation and growth of the placenta requires extensive angiogenesis to establish the vascular structures involved in exchange (16). Failure to establish an adequate blood supply to the fetus may have serious clinical consequences such as intrauterine growth retardation. VEGF has significant angiogenic properties. There are four species of mRNA encoding VEGF in both the first trimester and term placenta. In situ hybridization was used to localize the sites of expression of VEGF mRNA in such tissues. VEGF expression was seen in the villous trophoblast in the first trimester and in the extravillous trophoblast at term, and in both fetal macrophages within the villi and maternal macrophages in the decidua. The glandular epithelium in maternal decidua also expressed VEGF mRNA. The strongest site of expression was in maternal macrophages adjacent to Nitabuch's stria, a zone of necrosis at the site of implantation. This complex pattern of expression suggests that VEGF is involved in angiogenesis on both maternal and fetal sides of the placenta and that macrophages are the primary source of VEGF. However, VEGF may also play a role in the term placenta, when extensive angiogenesis has diminished, possibly regulating vascular permeability.

VEGF-B is structurally closely related to VEGF and binds one of its receptors, VEGFR-1 (17). In situ hybridization and immunohistochemistry were employed to localize VEGF-B mRNA and protein in embryonic mouse tissues. In 8.5 to 17.5 day embryos, VEGF-B was most prominently expressed in the developing myocardium, but not in the cardiac cushion tissue. Strong expression in the heart persisted at later developmental stages, while weaker signals were obtained from several other tissues, including developing muscle, bone, pancreas, adrenal gland, and from the smooth muscle cell layer of several larger vessels, but not from endothelial cells. VEGF-B probably acts in a paracrine fashion, as its receptor is almost exclusively present in endothelial cells. It may have a role in vascularization of the heart, skeletal muscles and developing bones, and in paracrine interactions between endothelial and surrounding muscle cells.

The specific aims of this study were: i) to identify the grade of overexpression of VEGF₁₂₁ in neoplastically-transformed cells; ii) to demonstrate the expression pattern of VEGF₁₂₁ in these malignant neuroectodermal tumors; and iii) to identify the intensity of endothelial cell proliferation and tumor-related neo-angiogenesis within high-grade ASTRs.

Materials and Methods

Tissues and tissue handling. Formalin-fixed, paraffin wax-embedded tissue sections of human primary childhood anaplastic ASTRs and GBMs (DAKO Corporation, Carpinteria, CA, USA) were employed. The diagnoses of the specific subtypes were in accordance with the WHO guidelines for the classification of gliomas (18-22).

Technical details of the immunohistochemical techniques used in this study have already been elaborated upon by other investigators (23-30) and in the studies published by our group (31-39). However, the methodology was modified in this study.

Monoclonal antibody. The anti-VEGF₁₂₁ mouse monoclonal antibody (Cat. # MS-350PO; Labvision, Fremont, CA, USA) was used. Recombinant full-length human VEGF₁₂₁ protein was employed as the immunogen. Isootype: IgG₁; Light chain: κ; Clone designation: JH121 (antibody 3). The MoAB was purified from ascites fluid by Protein A chromatography.

Staining of formalin-fixed and paraffin wax-embedded tissue sections required an antigen retrieval technique, employing preliminary boiling in 1 mM EDTA buffer, pH 8.0 (NeoMarkers, Fremont, CA, USA) for 10 to 20 minutes followed by cooling at room temperature for 20 minutes. A detailed description of the antigen retrieval technique employed is available for review (40-44).

Immunohistochemical controls. In order to ensure the specificity of the anti-VEGF₁₂₁ antibody used in this study, the immunoreactivity of several normal human control tissues was tested using a checkerboard multi-tissue block (DAKO: catalog # T1065) (45,46). Several postnatal human thymic specimens were used as negative and positive tissue controls, while a number of neoplastically-transformed tissues, such as malignant melanoma and lung cancer tissues, acted as the positive tissue controls. Additional controls for all tissues and the monoclonal antibody (MoAB) included omission of the primary MoAB, utilization of only the enzymatic developer solution to detect the presence of endogenous alkaline phosphatase (AP) activity, and the use of MOPC 21 mouse myeloma IgG₁ (ICN) as a replacement for the primary MoAB to determine non-specific myeloma protein binding to the antigen epitopes of the screened tissues.

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Immuno-alkaline phosphatase antigen detection technique. An immuno-alkaline phosphatase cytochemical method (23-30) was modified for antigen detection in formalin-fixed, paraffin wax-embedded anaplastic astrocytoma (AA) and glioblastoma (GBM) tissues. The technique is a highly sensitive, indirect, four- to six-step immunocytochemical method, which combines the biotin-streptavidin-based ABC-method with enzyme-linked AP immunohistochemistry, previously described in detail by us (31-39).

Briefly, following deparaffinization in three changes of Xylene substitute (Shandon-Lipshaw, Pittsburgh, PA, USA) for 20 to 30 minutes, rehydration was carried out employing descending dilutions of alcohol (100% to 50%) to TBS. An initial blocking step using 1% glacial acetic acid mixed with the working buffer for 10 minutes was necessary to eliminate the endogenous AP activity from the tissues. The use of levamisole solution was described in earlier observations. As was explained in our earlier publications (31-39), glacial acetic acid (GAA) inhibition was preferred because of the possible presence of levamisole-resistant AP iso-enzyme (25).
Results

Immunohistochemical evaluation. Qualitative and quantitative evaluation of the percentage of antigen-positive cells and the intensity of the immunostaining were conducted using a light microscope (Olympus, Japan). One hundred to 200 cells from each of five to eight distinct areas in non-necrotic, ASTR, GBM and peripheral thymus tissues were counted with artifacts being avoided. The scales used for evaluation were:

Quantitative evaluation. (++++) over 90% of the total cell number are positive; (+++) 50% to 90% of the total cell number are positive; (+) 10% to 50% of the total cell number are positive; (±) 1% to 10% of the total cell number are positive; (−) under 1% of the total cell number are positive; (−) negative.

Qualitative evaluation. (A) very intense red staining; (B) strong red staining; (C) light red staining; (D) negative staining.

Discussion

VEGF is one of the most important mediators of tumor-associated neo-angiogenesis. VEGF mRNA levels (TGF-α, TGF-β, bFGF, VEGF) were screened in human gliomas and meningiomas employing Northern blot analysis to examine their correlation with neoplastic transformation-related angiogenesis (47). The number of blood capillaries was counted after employing von Willebrand factor immunocytochemistry. The authors normalized the growth factor mRNA levels versus the glyceraldehyde phosphate dehydrogenase mRNA level. In the 17 gliomas and 16 meningiomas, only the mRNA level of VEGF correlated significantly with vascularity (r=0.499; p<0.05 in gliomas; r=0.779; p<0.001 in meningiomas). These results clearly suggest an important regulatory role for VEGF in tumor related angiogenesis. A number of in situ hybridization and Northern blot observations identified strong expression of VEGF mRNA in glioblastomas, meningiomas and hemangioblastomas. It has also been suggested that VEGF may be considered the most important stimulatory factor of endothelial cell proliferation in CNS malignancies.

VEGF demonstrated a temporal and spatial expression pattern during neural ontogenesis that is compatible with an inducer function for blood vessel growth from the perineural vascular plexus (48-51). There is considerable experimental evidence that VEGF is strongly involved in neoplasm-provoked neo-angiogenesis and, consequently, the growth and progression of primary neoplasms (i.e., gliomas), including the formation of an invasive and metastatic IP (14, 48-51). The de novo expression of VEGF on endothelial cells depends on activated oncogenes and inactivated tumor-suppressor genes, as well as additional complex regulatory mechanisms involving several other factors (e.g., growth factors, hypoxia and neoplastic promoters).

Basic fibroblast growth factor (bFGF) and VEGF are two growth factors which have been established as potent angiogenic factors and endothelial cell mitogens in human ARS during neoplasm-related angiogenesis (NRA) (52-54). The growth factor levels and staining patterns of tumor cells were evaluated immunocytochemically in seven cases of WHO grade II astrocytoma (55). Four (group A) were diagnosed as expressing anaplastic progression at their second operation while three (group B) were not. The proliferation index was measured by immunostaining with an anti-Ki-67 MoAB (MIB1). Immunostaining for bFGF
Figure 1. Childhood anaplastic astrocytoma (AA). A great majority of the neoplastically-transformed astrocytes express VEGF_{121} in their cytoplasm, cell surface and extracellular matrix. Routine, 10% buffered formalin fixation. Paraffin wax embedding. Alkaline phosphatase conjugated streptavidin-biotin antigen detection technique. Magnification: 200x.

Figure 2. Childhood glioblastoma multiforme (GBM). VEGF_{121} is present in most of the tumor cells with the same localization pattern as evident in Figure 1. Routine, 10% buffered formalin fixation. Paraffin wax embedding. Alkaline phosphatase conjugated streptavidin-biotin antigen detection technique. Magnification: 200x.
was localized to both the nucleus and cytoplasm of ASTR cells, whereas VEGF reactivity was mainly confined to the cytoplasm. The data of this study strongly supports a significant regulatory role for bFGF and VEGF in the development of a more aggressive astrocytoma IP and behavior. In SCID (severe combined immunodeficiency) mice, experimental inhibition of VEGF alone has been found sufficient to prevent the growth of primary neoplasms and their dissemination in vivo. The inhibitory effect on metastasis formation appeared to be distinct from that on primary tumor progression (56).

Machein et al. studied whether the expression of VEGF is correlated with in vivo measurements of the capillary permeability and vascular volume of primary human brain tumors (57). During the observations, 13 brain tumor samples (seven GBMs, one AA, two low-grade ASTRs, one pilocytic ASTR and three primary cerebral lymphomas) were stereotactically obtained from 14 patients. A semi-quantitative polymerase chain reaction was used to quantify the relative expression of VEGF messenger RNA in the tumors. VEGF protein was also demonstrated in tissue sections by immunohistochemical techniques. A two-compartment dynamic computed tomographic method was employed to quantitatively measure the aforementioned parameters in the regions from which the biopsies had been obtained. In glial tumors, there was significant correlation of the VEGF mRNA levels with capillary permeability (p<0.05) and vascular volume (p<0.01). Although all primary cerebral lymphomas showed considerable increases in capillary permeability and vascular volume, the VEGF expression was only slightly up-regulated in these neoplasms. These experimental findings are consistent with the hypothesis that VEGF may be responsible for endothelial cell proliferation and vascular permeability in glial tumors. This relationship has implications for clinical applications, such as assessment of the delivery of water-soluble drugs and treatment of edema and anti-angiogenesis-directed biological therapy based on the inhibition of the function of VEGF.

The clinical manifestations of childhood pilocytic astrocytoma (PA) and AA differ markedly, especially relevant to the time it takes for tumor progression and prognosis. Because of the aggressive course and poor survival rate of AA, one would expect it to be associated with a high angiogenic index, although clinical neuropathologists often find higher microvessel density counts in PA than in AA. Differences in the type or density of the microvasculature between these two neoplasms were examined in a recent study (58). To differentiate established mature vessels from immature growing ones, the authors employed antibodies to Factor VIII (FVIII) to stain endothelial cells (ECs) of blood vessels and alpha-smooth muscle actin (alpha-SMA) antibodies to stain vessels supported by adventitia. The results demonstrated that large, mature, alpha-SMA-positive vessels predominated in PA, and small, immature, alpha-SMA-negative vessels in AA. The vessel maturation index was 54.5% for PA, and 6.1% for AA. Immunoactivity with VEGF and anti-flt-1/VEGFR-1 antibodies showed distinct tissue patterns. VEGF immunoactivity occurred mainly in the processes of the tumor astrocytes in PA; the opposite was observed in AA.flt-1/VEGFR-1 was detected in the tumor cells of AA, but not in those of PA. The results suggested that the predominance of small, alpha-SMA-negative vessels in AA represents immature, unstable microvasculature with a potentially greater susceptibility to anti-angiogenic therapy. The expression of both flt-1 and VEGF by AA tumor cells also suggests a possible autocrine growth-promoting function for VEGF, in addition to its role as paracrine pro-angiogenic growth factor for activated ECs, thus making anti-angiogenesis an attractive therapeutic possibility in the treatment of AA. It has also been suggested that the isolation of highly pure ECs derived from glioma tissues would be more appropriate than ECs from non-transformed sources for studies of tumor angiogenesis and for the testing of potential anti-neoplastic, anti-angiogenic therapeutic targets (59).

VEGF is the major endothelial mitogen in CNS neoplasms and is expressed in about 64 to 95% of GBMs. Neoplastically-transformed astrocytes are the main source of VEGF in GBMs, whereas VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. Infiltrating tumor cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). Recent studies have identified that VEGF expression and bioavailability can be modulated by MMPs. One study employed quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expressions of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and five normal brains (60). The expression of these MMPs was markedly increased in most GBMs with excellent correlation between the mRNA and protein levels. Activated forms of MMP-2 and MMP-9 were present in 8/18 and 7/18 of GBMs, respectively. The majority of GBMs (17/20) also expressed high levels of VEGF, as previously reported, with strong correlation between the VEGF and MT1-MMP gene expression levels, and double immunostaining showed that VEGF and MT1-MMP peptides co-localize in tumor and endothelial cells. These experimental results suggest that the interplay between MMPs and VEGF previously described in carcinogenesis experiments may also be operative in human GBMs. Because of its dual ability to activate MMP-2 and to up-regulate VEGF, MT1-MMP might be of central
importance in the growth of GBMs and represent an interesting target for anti-neoplastic treatments.

Oncostatin-M (OSM), a hematopoietic cytokine, and VEGF, a quintessential angiogenic signal, are co-expressed in development, cancer and inflammation. A recent article reported that in vitro OSM treatment of human astroglioma cell lines increased the VEGF levels by approximately threefold (61). Interleukin-1beta (IL-1beta), in combination with OSM, induced up to seven-fold higher VEGF expression, without significantly inducing VEGF on its own. Specifically examining the OSM contribution to VEGF expression, neutralizing antibodies to OSM receptor subunits gp130 and OSMRbeta, but not LIFRbeta, inhibited OSM induction of VEGF, indicating that the OSM-specific receptor OSMRbeta/gp130 transduces the OSM signal for VEGF synthesis. OSM induction of VEGF promoter activity maps to the (-1171, -786) region of the VEGF promoter, which contains a STAT-3-binding site. STAT-3 is essential for this response, since overexpression of a dominant-negative STAT-3 blocks OSM induction of VEGF promoter activity, as well as endogenous VEGF expression. It is significant that OSM is expressed in biopsies of GBM, a particularly malignant form of glial brain tumor. This novel mechanism of VEGF regulation in astroglioma cells may be active in pathophysiological states where both OSM and IL-1beta are present.

The data about the correlation between angiogenic patterns and their prognostic impact in human GBM is minimal. Such data is relevant for translation of anti-neoplastic, anti-angiogenic therapies into clinical applications (62). Employing immunohistochemistry for CD34, the vascular patterns in 114 primary glioblastomas were assessed. These patterns comprised bizarre unevenly distributed glomeruloid/garland-like/clustered vascular formations and evenly distributed delicate capillary-like microvessels ("classic" vascular pattern). The combination of few bizarre vascular formations and a prominent classic vascular pattern (n=29) was an independent factor for longer survival ($p=0.006$, Cox regression), as well as a high postoperative Karnofsky performance status ($p=0.005$). In patients with a prominent classic vascular pattern as compared to all other patients, there was no difference of MIB1 labeling index whereas microvesSEL density and apoptotic index (TUNEL) were significantly higher ($p<0.05$). In addition, the diffuse expression of hypoxia-inducible factor (HIF)-1alpha and strong expression of VEGF were more common ($p<0.05$, Chi-square test). FISH revealed loss of chromosomes 1p and 19q only in one out of seven long-time survivors with the classic pattern. Based on their immunocytochemical results, the authors concluded that the vascular patterns in primary glioblastoma influence the clinical outcome and are associated with variable expression of angiogenic proteins. To our best knowledge, this report is the first to distinguish angiogenic subtypes of human glioblastoma, which may prove relevant for a more accurate classification and future biological, anti-angiogenic, anti-neoplastic therapy approaches.

There has also been a recent report indicating that the expression of VEGF receptor in astrocytic gliomas correlated well with tumor malignancy, even better than actual VEGF content (63). It has also been found that irradiation enhances VEGF secretion in glioma cell lines, possibly representing one factor in establishing the radioresistance of GBM (64).

The inhibition of angiogenesis as a form of anti-neoplastic therapy has been extensively studied (65, 66). A number of anti-angiogenic agents have been discovered and observed in vitro and in vivo, including IL-12, combretastatin A-4, AGM-1470 (TNP-470), anti-endoglin antibody TEC-11, endostatin, angiotatin, suramin, edelfosine, 2-methoxyestradiol, taxol, and thalidomide. The driving influence on angiogenesis of activating and inactivating mutations in oncogenes (ras) and tumor suppressor genes (p53), respectively, was also determined and presents further possibilities for gene therapy-based therapeutic protocols. The comparative aspects of neoplastic transformation in the brain have been explored and may allow for the better observation of novel therapies of xenotransplanted malignancies in animals, which may relate to humans. It still remains clear that, in all neoplastic diseases, a combination of classic therapeutic modalities with adjuvant biological therapies, which target different molecular biological pathways of the malignant neoplastic growth and dissemination, should be used, but only an "individualized cocktail" will ensure the best possible results.

References


