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Expression of Endothelial Cell-Specific Receptor Tyrosine Kinases and Growth Factors in Human Brain Tumors

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Key growth factor-receptor interactions involved in angiogenesis are possible targets for therapy of CNS tumors. Vascular endothelial growth factor (VEGF) is a highly specific endothelial cell mitogen that has been shown to stimulate angiogenesis, a requirement for solid tumor growth. The expression of VEGF, the closely related placental growth factor (PlGF), the newly cloned endothelial high affinity VEGF receptors KDR and FLT1, and the endothelial orphan receptors FLT4 and Tie were analyzed by* in situ *hybridization in normal human brain tissue and in the following CNS tumors: gliomas, grades II, III, IV; meningiomas, grades I and II; and melanoma metastases to the cerebrum. VEGF mRNA was up-regulated in the majority of low grade tumors studied and was highly expressed in cells of malignant gliomas. Significantly elevated levels of Tie, KDR, and FLT1 mRNAs, but not FLT4 mRNA, were observed in malignant tumor endothelia, as well as in endothelia of tissues directly adjacent to the tumor margin. In comparison, there was little or no receptor expression in normal brain vasculature. Our results are consistent with the hypothesis that these endothelial receptors are induced during tumor progression and may play a role in tumor angiogenesis. (Am J Pathol 1995, 146:368–378)

Angiogenesis, or the formation of new blood vessels from vascular endothelium, is important in embryogenesis, ovulation, and wound healing, and in pathological processes such as neovascular glaucoma, arthritis, diabetic retinopathy, and neoplasia.[1] Vascular proliferation is a requirement for solid tumor growth,[2,3] and blood vessel counts have been found to correlate significantly with metastases and prognosis in breast cancer,[4] non-small cell lung cancer,[5] and melanoma.[6] Endothelial proliferation almost always accompanies malignant intracranial neoplasms, particularly glioblastoma multiforme (GBM), but may also occur to a lesser extent in histologically low grade brain tumors. Vascular proliferation and necrosis are among the factors that distinguish high grade from low grade brain neoplasms and generally indicate a poor prognosis. It is therefore feasible that a greater understanding of the mechanism of angiogenesis may lead to improved characterization, imaging, and therapy of brain neoplasms.

Various growth factors have been shown to stimulate angiogenesis *in vitro* and *in vivo*. These include transforming growth factor-α,[7] acidic and basic fibroblast growth factors (FGF) a and b,[8] and vascular endothelial growth factor (VEGF) or vascular permeability factor (VPF).[9–12] VEGF is a glycosylated, dimeric, secreted angiogenic growth factor that is structurally related to platelet-derived growth factor (PDGF) and is highly specific for endothelial cells. Previous studies have shown that the mRNAs of VEGF and its receptors KDR and FLT1 are up-regulated in kidney and bladder carcinomas[13] and in some primary brain tumors of humans.[14–16] Placental growth factor (PlGF) is a recently identified VEGF-related angiogenic factor[17] whose receptor has not yet been characterized. At least four mRNA transcripts of VEGF[18] and two of PlGF[19] encode growth factor isoforms differing, for example, in their affinity for heparin. The formation of new blood vessels in tumors may be partially mediated by a range of VEGF/PlGF-endothelial cell receptor tyrosine kinase interactions. Clarification of these specific ligand-receptor interac-

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tions involved in angiogenesis may lead to the design of therapies based on the modulation of angiogenic responses induced by growth factor binding.

The two transmembrane endothelial cell-specific tyrosine kinase receptors that have been cloned and identified as high affinity receptors for VEGF, KDR and FLT1, are closely related to the class III receptor tyrosine kinases and are characterized by seven immunoglobulin-like loops in their extracellular domain.[20–24] The structurally similar *fms*-like tyrosine kinase 4 or FLT4[25–27] comprises a third member of this receptor subfamily. A recent comparative study suggests that these three receptors have distinct, overlapping expression patterns in human fetal endothelial cells and function as regulators of vasculogenesis and angiogenesis during development.[28]

Two novel endothelial cell-specific orphan receptors, Tie and Tek, have recently been identified. Tie, a tyrosine kinase with immunoglobulin and epidermal growth factor homology domains,[29] is expressed in angioblasts as well as in mature endothelial cells during mouse angiogenesis and in adult mouse during wound healing and hormonally induced corpus luteum formation in the ovaries, suggesting a possible role in physiological neovascularization.[30,31] In addition, Tie may be involved in angiogenesis during tumor progression, as enhanced expression of the receptor mRNA has been observed in the endothelia of metastatic melanomas (A. Kaipainen et al., in press). The closely related Tek (tunica interna endothelial cell kinase) is also expressed in developing and mature endothelial cells.[32,33]

In this study we have analyzed by *in situ* hybridization the mRNA distribution and level of expression of the four endothelial receptor tyrosine kinases Tie, KDR, FLT1, FLT4, and two endothelial growth factors VEGF and PlGF, in the most frequent human primary brain neoplasms, gliomas and meningiomas, as well as in metastases to the cerebrum. In addition, we have assessed the expression of the Tie protein by immunohistochemistry. Our results show increased levels of Tie, KDR, and FLT1 mRNAs, as well as Tie protein, in tumor vasculature and in adjacent brain endothelia in comparison to little or no expression in normal brain.

Materials and Methods

Tissue Specimens

Fresh samples of previously untreated cerebral gliomas and intracranial meningiomas were obtained during open surgery at the Department of Neurosurgery, Helsinki University Central Hospital. All tumor patients were receiving corticosteroids. A sample of non-neoplastic control brain tissue was obtained during surgery for intractable epilepsy. Several of the tumor samples included adjacent brain tissue. All samples were frozen in liquid nitrogen as soon as possible after surgical removal and stored at −70 C. Histopathological diagnoses were based on hematoxylin and eosin- stained cryostat sections as well as on routinely stained paraffin sections of adjacent formaldehyde-fixed tumor specimens. A complete list of the samples is shown in Table 1.

Table 1. *Human Tissue Specimens*

No.	Tissue Samples	Age (yr)	Sex
1	Control brain	66	M
2	Control brain	11	F
3	Grade II oligoastrocytoma	34	F
4	Grade II oligoastrocytoma	66	M
5	Grade III astrocytoma	36	M
6	GBM	53	M
7	GBM	63	M
8	GBM	56	M
9	Tissue adjacent to GBM	69	F
10	Grade I meningioma	52	M
11	Grade II meningioma	35	F
12	Grade II meningioma	55	M
13	Melanoma metastasis (mm)	54	M
14	Tissue adjacent to mm	67	M
15	Grade III glioma	44	M
16	GBM	65	M

Preparation of Tissue Sections

Sterilized slides were dipped in 2% 3-aminopropyltriethoxysilane (TESPA) in acetone to ensure tissue adherence. 5-μ cryostat sections were cut at

Table 2. *Summary of Probe Preparation*

Probe*	Plasmid	Endonuclease	Polymerase	Nucleotides
FLT4 s	pGEM 3Z f(+)	***Sph*I**	T7	1–595
FLT4 as	pGEM 3Z f(+)	***Eco*RI**	SP6	1–595
FLT1 s	pGEM 3Z f(+)	***Sac*I**	T7	706–2310
FLT1 as	pGEM 3Z f(+)	***Ava*I**	SP6	706–2310
KDR s	pBS KII +/−	***Hind*III**	T7	6–715
KDR as	pBS KII +/−	***Eco*RI**	T3	6–715
Tie s	pGEM 7Z f(+)	***Pvu*II**	SP6	1–2190
Tie as	pGEM 7Z f(+)	***Sma*I**	T7	1–2190
VEGF s	pGEM 3Z f(+)	***Eco*RI**	T7	57–639
VEGF as	pGEM 3Z f(+)	***Eco*RI**	T7	57–639
PlGF s	pGEM 3Z f(+)	***Eco*RI**	T7	304–944
PlGF as	pGEM 3Z f(+)	***Eco*RI**	SP6	304–944
vWF s	pGEM 3Z f(+)	***Eco*RI**	SP6	1–2334
vWF as	pGEM 3Z f(+)	***Hind*III**	T7	1–2334

*s, sense; as, antisense.

–20 C onto the pretreated slides, fixed in 4% paraformaldehyde, and stored at –70 C.

Preparation of Probes

Each human tyrosine kinase receptor or ligand cDNA sequence was subcloned into an appropriate transcription vector with RNA polymerase promoters on either side of the insert. The plasmids were linearized by restriction endonuclease digestion and purified. Sense and antisense radiolabeled RNA probes were obtained via the incorporation of [^{35}S]UTP on addition of specific polymerases (Promega Riboprobe Gemini II Core System). A summary of the specifications of probe preparation is shown in Table 2. Synthesis of radioactive RNA was followed by treatment with DNAse I (ribonuclease-free) and partial alkaline hydrolysis of RNA to obtain fragments of 200 to 400 nucleotides in length. The RNA strands were separated from unincorporated nucleotides by fractionation on a Sephadex G-50 column and ethanol-precipitated.

In Situ *Hybridization*

The technique used was based on that described by Wilkinson et al[34,35] with the following modifications. Post-hybridization washing of sections was carried out at low stringency (2X SSC, 20 mmol/L dithiothreitol) for 1 hour at 50 C and at high stringency (1X SSC, 30 mmol/L dithiothreitol, 50% formamide) for 30 minutes at 65 C. Autoradiography was carried out using NTB2 Kodak emulsion, and the film-coated slides were stored with desiccant at 4 C. Exposure time was determined empirically for each probe and varied from 2 to 8 weeks, after which the slides were developed (Kodak D19 developer), fixed, counterstained with hematoxylin, and mounted in Permount.

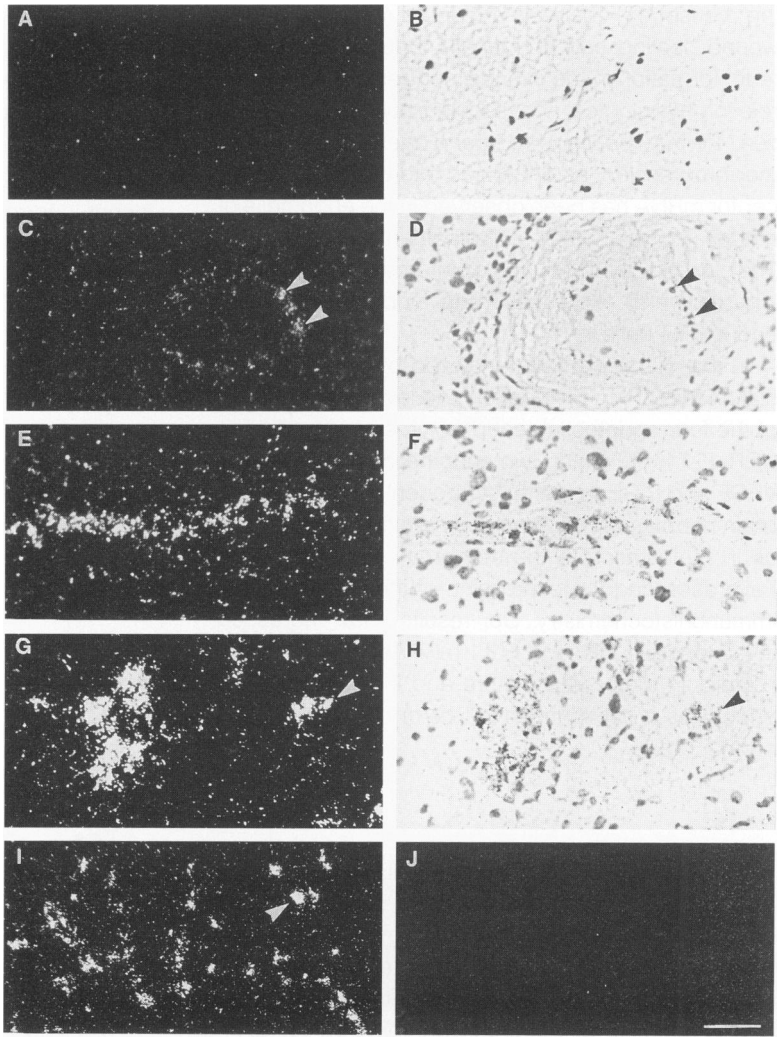

Figure 1. *A comparison of Tie expression in gliomas. Dark field* (left) *and bright field* (right) *micrographs of cryostat sections hybridized with [^{35}S]-labeled Tie antisense probe, autoradiographed and counterstained with hematoxylin. Tie mRNA is not detectable in the area of control cortical brain vasculature studied* (**A, B**)*, but is up-regulated in a few endothelial cells in a grade II astrocytoma* (**arrows, C, D**)*. Abundant Tie mRNA expression can be observed in GBM vessels* (**E, F**)*, as well as in endothelia in areas of infiltrating GBM* (**arrows, G, H**)*. Panel* I *illustrates the expression pattern of Tie mRNA in GBM at a lower magnification (scale bar,* **I, J** *= 0.1 mm), showing uniform decoration of capillaries* (large arrow) *and small vessels* (small arrow)*. The corresponding hybridization with Tie sense probe is shown* (**J**)*. Scale bar,* **A–H** *= 0.05 mm.*

Control experiments were carried out using corresponding sense probes to demonstrate signal specificity, and control hybridization signals did not significantly differ from the background.

Immunohistochemistry

5-μ cryostat sections from the samples used for *in situ* hybridization analysis, as well as two additional malignant glioma samples, were cut onto TESPA slides as described previously. The sections were vacuum-dried at 37 C overnight and stained immunohistochemically using mouse monoclonal antibodies against human Tie (a cocktail of monoclonal antibodies against Tie extracellular domain; a kind gift from Dr. Juha Partanen) and rabbit antibodies (Dakopatts) against human von Willebrand factor (vWF) as an endothelial cell-specific marker.[36]

Staining was carried out using the Vectastain ABC Elite biotin-avidin system for mouse IgG (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity in the dried, unfixed tissue sections was blocked by treatment with 0.5% H_2O_2 in methanol for 30 minutes. After phosphate-buffered saline washes, the sections were incubated with diluted Vectastain blocking serum for 20 minutes and then with undiluted Tie antibody (1:1) overnight at 4 C or von Willebrand factor antibody (1:50) for 1 hour at room temperature. Biotinylated secondary antibody was added for 45 minutes, followed by the Vectastain ABC reagent for 30 minutes. The reaction was visualized with 0.2 mg/ml 3-amino-9-ethylcarbazole (AEC), 0.03% H_2O_2, 14 mmol/L acetic acid and 33 mmol/L sodium acetate. Normal serum and antigen-blocked primary antibody were used as controls. Sections were lightly counterstained in hematoxylin and mounted in Aquamount.

Results

Expression of Endothelial Cell Receptor Tyrosine Kinases in Glial Tumors

Normal brain cortical tissue analyzed by *in situ* hybridization did not show any specific hybridization signal for Tie mRNA (Figure 1, A and B). In contrast, expression was increased in some endothelia of low grade astrocytomas (C and D) and significantly up-regulated in the vasculature of high grade gliomas, particularly GBM (E and F). Strong hybridization signals were observed in capillaries and small vessels in GBM, and also in some endothelial cells of larger, more mature vessels in several of the glioma samples studied (example shown in C and D). A similar hybridization pattern and level of expression was noted in endothelial cells in tissues directly adjacent to the tumor (G–I). Adjacent sections hybridized with the Tie sense probe did not show any specific signal (J).

Little or no KDR or FLT1 mRNA expression was observed in the vasculature of control human brain cortex (Table 3). A few endothelia in low grade astrocytomas expressed KDR (Figure 2, A and B) and to a greater extent, FLT1 (Table 3). Both receptors were significantly up-regulated in GBM vasculature, both in the area of the infiltrating tumor margin (C and D) and within the tumor (E and F). Endothelial cells lining a variety of vessel types expressed high levels of FLT1 transcripts (G and H). In contrast, no FLT4 mRNA was detected in normal brain cortex or in any of the brain tumors tested including GBM (I; see also Table 3). Positive control hybridizations with the FLT4 probe showed strong expression in fetal lung tissue (data not shown), in accordance with previous studies using the same probes and technique, which showed FLT4 mRNA expression in this tissue.[28]

Table 3. In Situ *Hybridization of Growth Factor and Receptor Tyrosine Kinase mRNAs*

No.	Tissue	GF		RTK				
		VEGF	PIGF	KDR	FLT1	FLT4	Tie	vWF
1	Control brain	–	–	–	–	–	–	–
2	Control brain	+	–	–	+	–	–	+
3	Grade II oligoastrocytoma	+	–	–	+	–	+	–
4	Grade II oligoastrocytoma	+	–	–	++	–	–	+
5	Grade III astrocytoma	+	–	++	++	–	++	++
6	GBM	*	*	*	+++	–	*	*
7	GBM	+++	–	++	+++	–	+++	++
8	GBM	+++	–	++	+++	–	++	++
9	Tissue next to GBM	+++	–	+	++	–	++	++
10	Grade I meningioma	–	–	–	–	–	–	+
11	Grade I meningioma	++	–	++	++	–	++	+
12	Grade II meningioma	++	–	++	++	–	+	++
13	Melanoma metastasis	++	–	++	+++	–	+++	++
14	Tissue next to metastasis	++	–	+	+	–	+++	++

GF, growth factor; RTK, receptor tyrosine kinase; –, signal indistinguishable from background; +, low level of expression; ++, positive hybridization signal; +++, high levels of expression; *, not studied.

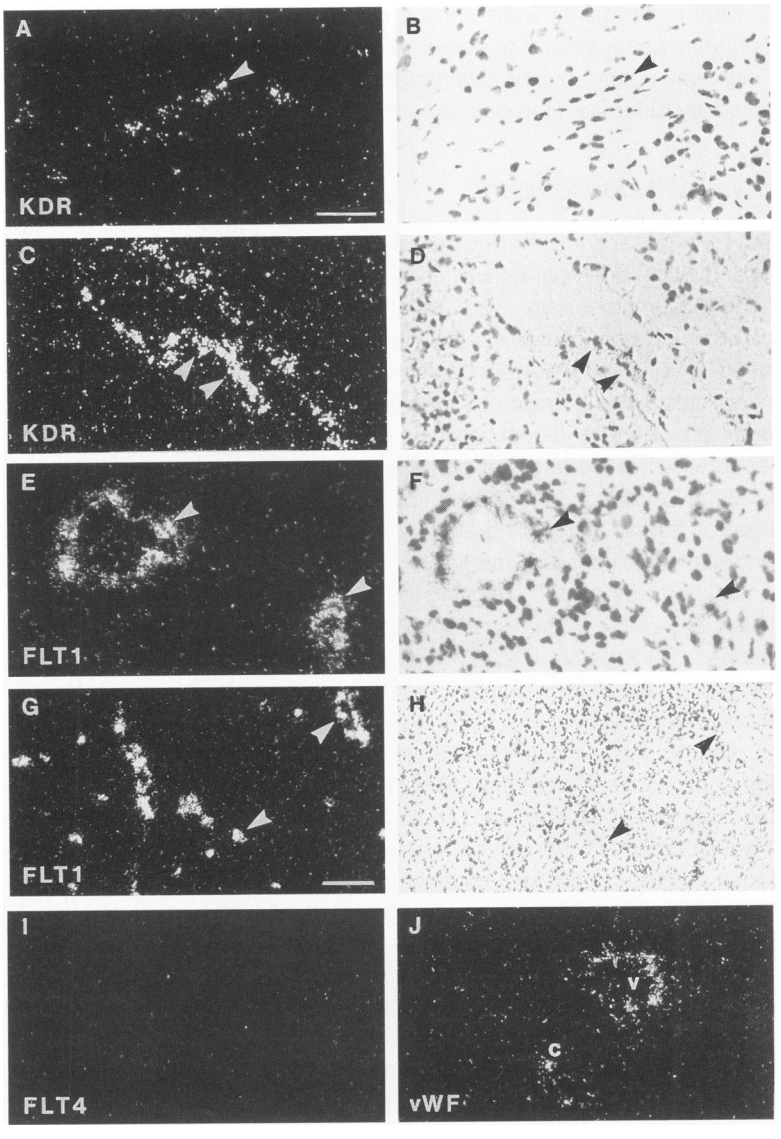

Figure 2. *Expression of KDR, FLT1, and FLT4 in gliomas. KDR is weakly expressed in a few endothelial cells of grade II astrocytoma vessels* (arrow, A, B) *and particularly highly expressed in endothelial cells lining vessels around areas of GBM infiltration* (arrows, C, D). *Equally high levels of FLT1 mRNA can be observed in a sample from GBM* (arrows, E, F). G *and* H *illustrate the pattern of FLT1 expression in GBM at lower magnification (scale bar,* G, H = *0.1 mm), with strong hybridization signals in small vessels and capillary sprouts* (arrows). *By contrast, there is no detectable FLT4 mRNA in the same sample* (I). *As a positive control, sections from GBM were also hybridized with vWF antisense probe, specific for blood vessel and capillary endothelia (v and c respectively,* J). *Scale bar,* A–F, I, J = *0.05 mm.*

VEGF and PlGF Expression in Glial Tumors

Very low levels of VEGF mRNA were detected in a control cortical brain specimen (Figure 3A). Most of the tumors analyzed showed intense hybridization signals in tumor cells but not endothelial cells (C to F). The highest grain densities were observed in clusters of tumor cells, particularly in areas adjacent to necrotic foci (n in panel D) as well as in areas of tumor infiltration into surrounding tissue (E and F). In comparison, no PlGF mRNA was detected in any of the samples analyzed (data not shown). PlGF was, however, detected in placenta, hybridized as a positive control, as it was previously reported to be highly expressed in this tissue.[19]

Endothelial Growth Factor and Receptor mRNAs in Meningiomas

In two of three meningiomas, VEGF mRNA was upregulated in clusters of tumor cells, but overall expression levels were lower than in GBM (Figure 4, A and B). Two of three low grade meningiomas analyzed expressed relatively low levels of Tie mRNA (G and H). Tie signal was generally observed in a few endothelial cells in capillaries and small vessels and, to a lesser extent, in larger vessels. Two meningiomas, grades I and II, expressed KDR (C and D) and FLT1 (E and F) mRNA, and one grade I tumor was negative for both. Low levels of vWF mRNA were detected in endothelial cells in control brain, low grade oligoastrocytoma and meningioma, but the vascula-

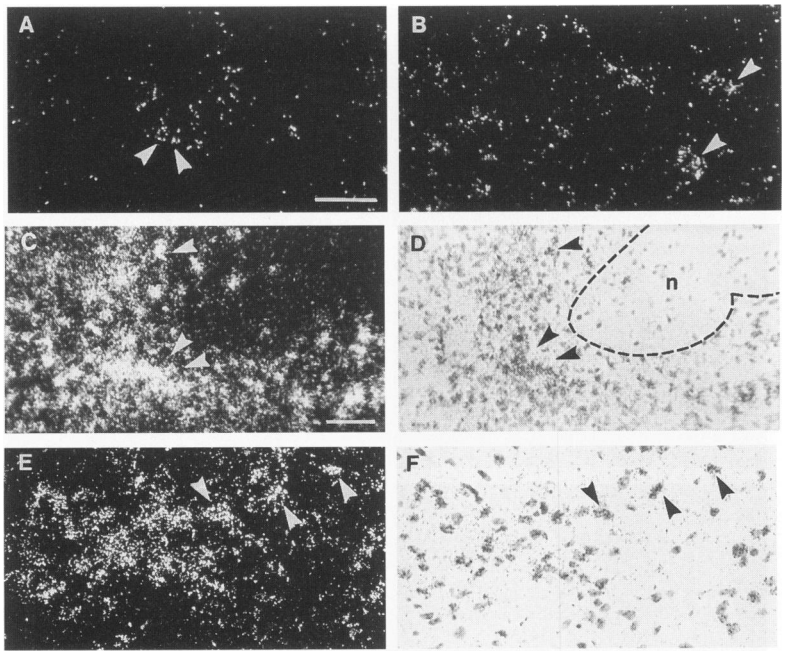

Figure 3. *VEGF expression in gliomas and normal brain tissue. Very low levels of VEGF mRNA can be observed in control brain* (**arrows, A**), *whereas slightly higher signals can be detected in some tumor cells in a grade II astrocytoma* (**arrows, B**). *Clusters of glioblastoma cells express very high levels of the transcript* (**arrows, C, D**) *particularly adjacent to areas of necrosis* (*n in panel* **D**, *necrotic margin indicated by an interrupted line*) *as well as along the invasive tumor margin* (**arrows, E, F**). *Scale bars,* **A, B, E, F** = *0.05 mm;* **C, D** = *0.1 mm.*

ture of malignant glioma showed a higher signal intensity (Figure 2J; Table 3).

Analysis of Cerebral Melanoma Metastases

VEGF transcripts were also observed in groups of metastatic melanoma cells, notably, in islands of tumor cells in close proximity to necrotic regions (Figure 5, A and B). Similar foci of increased VEGF mRNA expression occurred along the tumor margin infiltrating adjacent brain tissue, where there were no apparent neighboring areas of necrosis (Table 3). Samples hybridized with VEGF sense probes showed no signal above background. The VEGF-related PlGF was not detected in the samples analyzed.

Endothelia in melanoma metastases and in reactive tissue bordering the tumor showed equally strong hybridization signals for KDR and FLT1 (C–H). The two receptors were noted in a variety of vessel types in all the tumors studied (Figures 1I; 2, G and H; 4, I and J; 5, E and F) but were not expressed in all vessels in adjacent sections that were positive for vWF mRNA (positive control hybridization)). Although the KDR and FLT1 mRNAs appeared to be coexpressed to some extent, the expression patterns were not completely overlapping. Sections hybridized with the corresponding sense probes were devoid of any specific signal (data not shown).

The data for Tie mRNA expression in melanoma metastases to the cerebrum are included in another study (A. Kaipainen, T. Vlaykova, E. Hatva, T. Böhling, A. Jekunen, S. Pyrhönen, and K. Alitalo, in press) and is therefore not presented here.

Immunohistochemical Detection of the Tie Protein

Blood vessels within normal cortical tissue were negative for Tie protein (Figure 6A), whereas strong staining was observed in glioblastoma vasculature (B) and in the endothelia of tissue adjacent to GBM (inset, B). Tie protein was also detected in endothelial cells lining vessels in melanoma metastases (C) as well as bordering the tumor (data not shown). No specific staining was observed when the sections were incubated with antigen-blocked antibody or normal serum instead of the Tie antibodies. To further assess the extent and specificity of Tie staining, adjacent sections were stained for factor VIII (D). A summary of the Tie staining results is shown in Table 4.

Discussion

Angiogenesis is a crucial but so far poorly characterized aspect of brain tumor biology. Detailed knowledge on angiogenic factors and receptors in healthy and neoplastic CNS tissues could lead to

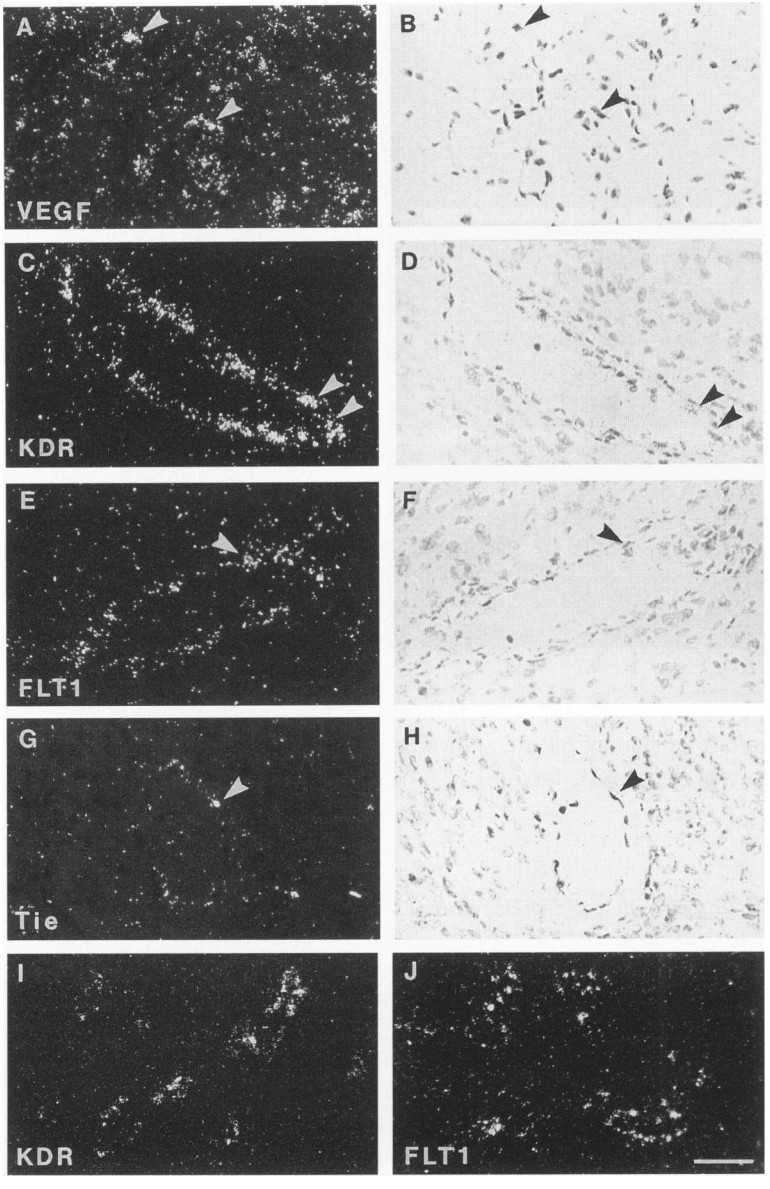

Figure 4. *Expression of VEGF and endothelial cell receptors in meningioma. VEGF mRNA is expressed by grade I meningioma tumor cells* (arrows, A, B). *KDR* (C, D) *and FLT1* (E, F) *mRNAs are up-regulated and partially coexpressed in grade II meningioma vasculature* (*arrows indicate endothelial cells showing strong expression*). *Increased levels of Tie mRNA can be observed in a few meningioma endothelial cells* (arrows, G, H). I *and* J *illustrate the similar intensities and patterns of expression of KDR and FLT1 mRNAs, respectively. Scale bar,* A–H = *0.05 mm;* I, J = *0.1 mm.*

improved imaging and therapy of the CNS tumors. Several studies have suggested a significant role for VEGF in tumor angiogenesis.[14–16,37] In particular, antibodies against VEGF have been shown to reduce tumor vascularity and inhibit tumor growth in nude mice.[37] We were therefore interested in analyzing the expression patterns of endothelial cell specific growth factors and receptor tyrosine kinases, including VEGF, PlGF, KDR, FLT1, and FLT4 in the most commonly occurring human brain tumors.

The probes used for *in situ* hybridization analysis were designed to minimize cross-reactivity and were generated from the extracellular domains of the receptors, where nucleic acid sequence identity between the probes is low, approximately 30%. In addition, these probes were previously tested by Northern blotting and did not cross-react[26,28]

By *in situ* hybridization analysis, VEGF mRNA was found to be expressed at relatively low levels in normal human brain, up-regulated in low grade glioma and meningioma cells, and highly expressed in GBM cells, which is in accordance with previous reports and consistent with its suggested role as an *in vivo* tumor angiogenesis factor.[12,15,16] Our observation that normal cortical brain cells express low levels of VEGF suggests that, in addition to its function as an endothelial cell mitogen,[12] this

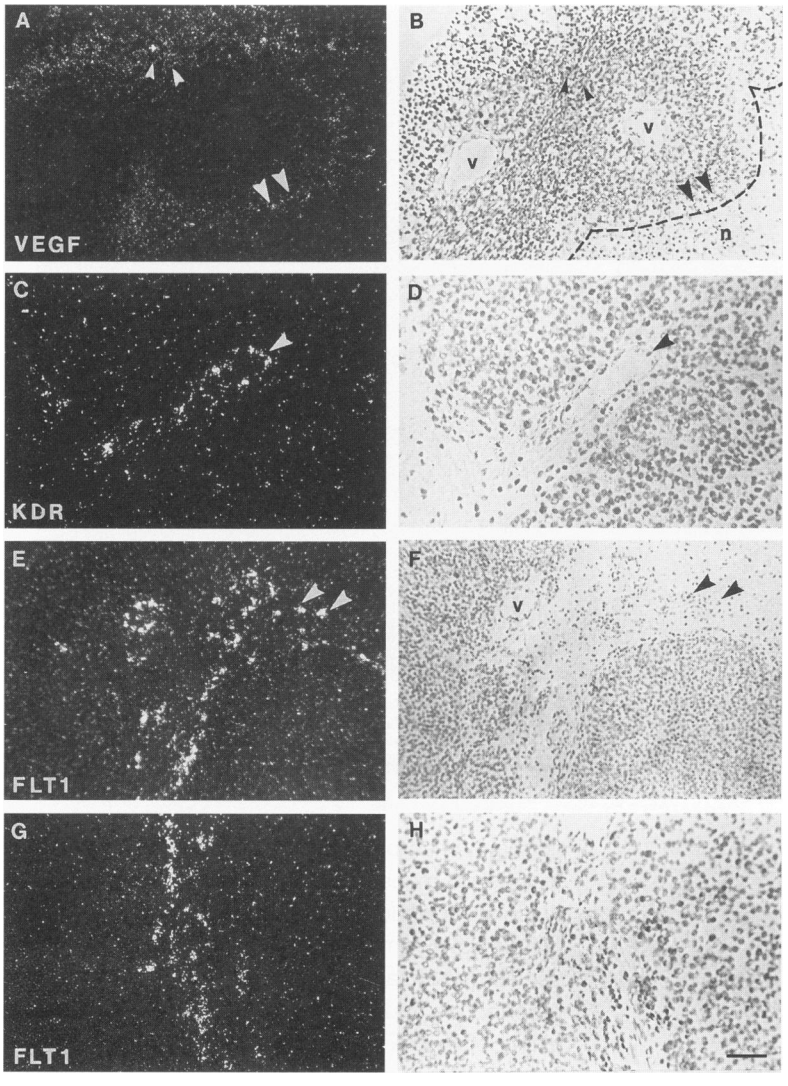

Figure 5. *Analysis of cerebral melanoma metastases. Increased levels of VEGF mRNA can be observed in melanoma metastases* (**arrows, A, B**), *particularly in groups of cells* (**large arrows**) *directly adjacent to areas of necrosis* (*n, necrotic margin indicated by an interrupted line*) *and furthest from tumor vasculature* (*v*). *High levels of KDR* (**arrows, C, D**) *and FLT1* (**E–H**) *mRNAs are partially coexpressed in vessels* (*v*) *and capillaries* (**arrows, E, F**) *of tissue bordering vital tumor, as well as within the tumor* (**G, H**). *Scale bar,* **A, B, E, F** = *0.1 mm;* **C, D, G, H** = *0.05 mm.*

growth factor may play a role in endothelial cell maintenance. In our present study, the tumors with the highest VEGF mRNA content also expressed high levels of vWF in their endothelia. VEGF is also associated with enhanced microvascular permeability,[11] and increasing VEGF expression may be partially responsible for edema accompanying tumor progression. This is compatible with our finding and that of Berkman et al[16] that VEGF is significantly up-regulated in glioblastomas, meningiomas, and metastases to the cerebrum, tumors associated with peritumoral brain edema.

The observed VEGF mRNA expression in these tumors was not uniform. The highest levels of expression occurred in tumor cells immediately adjacent to necrotic foci, which agrees with recent studies showing enhanced VEGF expression in specific subpopulations of glioblastoma cells bordering areas of necrosis, known as palisading cells.[14,15] These results suggest that the growth factor may be induced in the tumors under conditions of hypoxia, as has been shown in cell culture.[14] The abundant expression of VEGF in other, more vascular, areas of the tumors analyzed (with no apparent adjacent necrosis), as well as in areas of tumor infiltration into bordering brain tissue, suggests that, in addition to hypoxia, there may be other factors that result in VEGF up-regulation *in vivo.*

We also hybridized normal and neoplastic brain with PlGF riboprobes and did not detect specific signal in the tissues tested. Previous analysis of PlGF expression in several cell lines and tissues, normal and neoplastic, showed mRNA expression in placenta and in human colon and mammary carcinomas.[19] However, PlGF is expressed in normal human thyroid, but not in thyroid tumors, unlike VEGF, whose

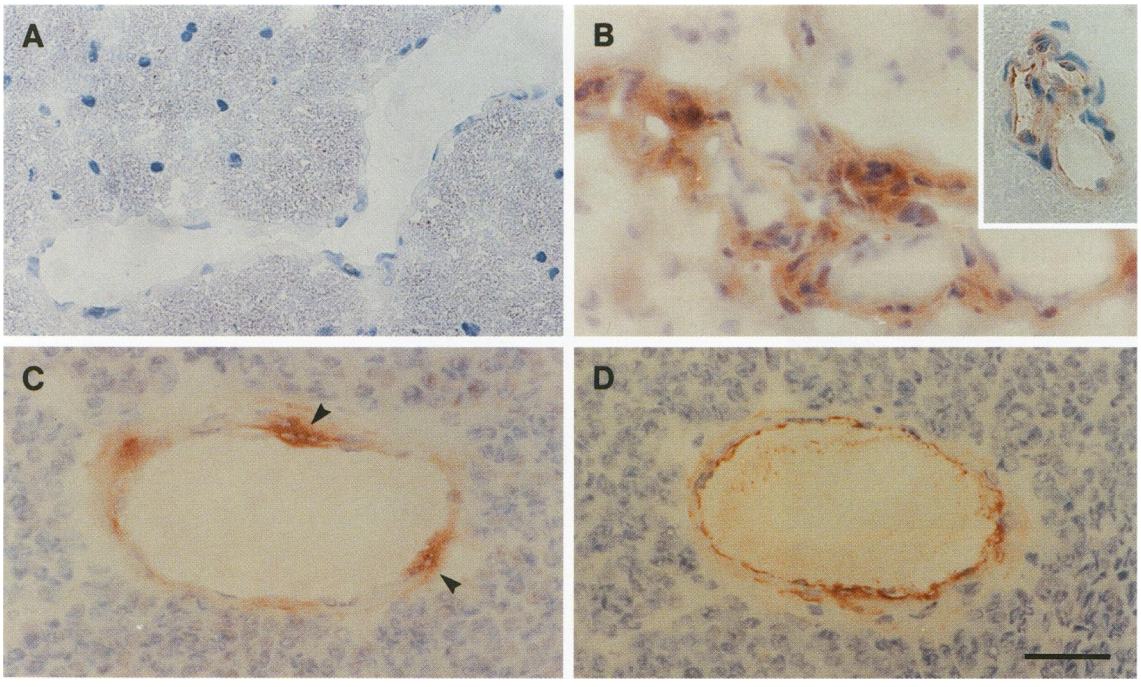

Figure 6. *Immunohistochemical staining of Tie in normal brain, GBM, and melanoma metastasis. No Tie expression is seen in control cortical brain vasculature* (**A**). *By contrast, Tie is up-regulated in GBM vasculature* (**B**) *and in endothelia of tissue bordering GBM* (**inset, B**). **C** *and* **D** *show the same vessel in a section of melanoma metastasis stained for Tie* (**left**) *and for factor VIII* (**right**). *Abundant Tie protein is detected in several endothelial cells* (**arrows, C**). *The sections were first stained immunohistochemically and then lightly counterstained with hematoxylin. Scale bar = 0.05 mm.*

Table 4. *Tie Immunohistochemical Staining*

No.	Tissue	Tie ab
2	Normal brain*	–
15	Grade III glioma	++
7	GBM*	+++
16	GBM	+++
11	Grade I meningioma*	++
13	Melanoma metastases*	++

*Samples also used for *in situ* hybridization analysis.

expression is up-regulated in thyroid tumors (M. Graziella Persico, personal communication).

A recent study of the FLT/KDR family of receptors in human fetal tissues[28] demonstrated different receptor expression patterns for FLT4 and FLT1/KDR within the developing fetal vasculature, and identifies KDR and FLT1 as early markers for endothelial cell progenitors.[38] These two receptors are also known to be expressed in tumor vasculature.[13,15,16]

In this study, *in situ* hybridization analysis of KDR and FLT1 mRNA expression showed receptor up-regulation in the endothelia of low grade gliomas and meningiomas and to a greater extent in the highly vascular GBM and melanoma metastases, whereas little or no expression was observed in normal brain vasculature. The lack of expression of receptor mRNA in normal human brain tissue is consistent with earlier studies on mouse tissues, demonstrating KDR expression in endothelial cells throughout embryonic mouse development, but not in adult brain.[38] The observed hybridization pattern for FLT1 is similar to that reported previously for gliomas.[15] Both intensity and extent of mRNA expression were enhanced with increasing tumor grade, and the observed expression patterns of the related receptors KDR and FLT1 were similar, indicating that they may be partially co-expressed. Both receptors were noted in proliferating tumor microvasculature, small vessels and, to a lesser extent, larger vessel endothelia, which agrees with previous studies on the FLT1 receptor.[13,15]

A comparison of ligand and receptor expression in the tumors analyzed showed clear correlation between VEGF and KDR/FLT1 expression such that KDR and FLT1 transcripts were located in vessels near tumor cells expressing VEGF mRNA. These findings suggest that both VEGF and its receptors are induced during tumor development and support the hypothesis that VEGF is secreted by tumor cells and binds to its receptors located on endothelial cells.

FLT4 mRNA was not observed in normal human brain tissue or in any of the tumors analyzed by *in*

situ hybridization. Studies have shown that the receptor is expressed in placenta[17,19] and in certain differentiating endothelial cells of human fetal tissues.[28] A recent analysis of FLT4 expression in mouse embryos and adult tissues suggests that FLT4 is specifically expressed in normal and pathological lymphatic vessels and in some high endothelial venules (A. Kaipainen et al, submitted for publication). The lack of FLT4 mRNA in brain tumor vasculature would agree with such a restricted pattern of expression.

The endothelial receptor tyrosine kinase Tie,[29] with an unknown ligand and function, is characterized by a unique multidomain extracellular region, including three cell adhesion-associated domains, and together with the related Tek receptor,[32] it comprises a new subfamily of endothelial cell-specific receptors. Tie has been identified as an early endothelial cell marker in vasculogenesis and has been shown to be induced during neovascularization[30]. To assess its possible role in tumor progression and angiogenesis, Tie mRNA expression was also analyzed. Our results showed little or no Tie mRNA expression in normal human cortical vasculature, which is consistent with the finding that Tie is downregulated in mouse adult tissues, including normal brain.[30] The most malignant tumors studied, GBM and melanoma metastases, expressed the highest levels of Tie mRNA in their endothelia, indicating that Tie may be induced in endothelial cells during malignant tumor progression. In addition, equally strong hybridization signals were noted in the microvasculature of areas of infiltrating GBM and in reactive tissue directly adjacent to vital tumor areas. This pattern was also observed for the Tie protein, detected by immunohistochemical staining of serial sections from the same samples used in *in situ* hybridization analysis, with strong staining of small vessels and capillaries. The distribution and degree of Tie protein expression determined by immunohistochemical staining was consistent with the patterns and intensity of Tie mRNA hybridization signals observed via *in situ* analysis.

In summary, our results demonstrate up-regulation of VEGF mRNA in tumor cells, and Tie, KDR, and FLT1 mRNAs in tumor vasculature, correlating with increased tumor aggressiveness. These results provide evidence that VEGF and the three receptor tyrosine kinases are induced during tumor development. Further studies should clarify the mechanisms of enhanced expression of these mRNAs and the specific functions of their protein products.

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