Early Induction of Angiogenetic Signals in Gliomas of GFAP-v-src Transgenic Mice

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Angiogenesis is a prerequisite for solid tumor growth. Glioblastoma multiforme, the most common malignant brain tumor, is characterized by extensive vascular proliferation. We previously showed that transgenic mice expressing a GFAP-v-src fusion gene in astrocytes develop low-grade astrocytomas that progressively evolve into hypervascularized glioblastomas. Here, we examined whether tumor progression triggers angiogenetic signals. We found abundant transcription of vascular endothelial growth factor (VEGF) in neoplastic astrocytes at surprisingly early stages of tumorigenesis. VEGF and v-src expression patterns were not identical, suggesting that VEGF activation was not only dependent on v-src. Late-stage gliomas showed perinecrotic VEGF up-regulation similarly to human glioblastoma. Expression patterns of the endothelial angiogenic receptors *flt-1*, *flk-1*, *tie-1*, and *tie-2* were similar to those described in human gliomas, but *flt-1* was expressed also in neoplastic astrocytes, suggesting an autocrine role in tumor growth. In crossbreeding experiments, hemizygous ablation of the tumor suppressor genes *Rb* and *p53* had no significant effect on the expression of VEGF, *flt-1*, *flk-1*, *tie-1*, and *tie-2*. Therefore, expression of angiogenic signals is an early event during progression of GFAP-v-src tumors and precedes hypervascularization. Given the close similarities in the progression pattern between GFAP-v-src and human gliomas, the present results suggest that these mice may provide a useful tool for antiangiogenic therapy research. (Am J Pathol 1999, 154:581-590)

Angiogenesis is defined as the sprouting of capillaries from existing blood vessels. With the exception of the female reproductive cycles, angiogenesis takes place in adult organisms mainly in pathological situations such as wound healing and tumor growth.¹ It has been suggested that tumor progression and metastasis require persistent new blood vessel growth and that manipulating the "angiogenic switch" is an important feature that a tumor must

acquire for successful growth.² Some important evidence for this hypothesis was derived from transgenic mouse models of cancer. For example, mice expressing the SV 40 large T antigen in pancreatic β cells exhibit a stage-dependent onset of pathological angiogenesis during tumor development.^{3,4} Although all β cells express the transgene, only 50% of the islets switch to a hyperproliferative stage (CIS). Angiogenesis is turned on in a small fraction of hyperplastic islets and facilitates progression to large solid tumor formation.⁵ Similar observations were made in various other transgenic models for cancer.⁶⁻⁸

Vessel density in invasive human cancers is clearly a significant prognostic indicator, in both breast⁹ and prostate¹⁰ cancers. Prognosis deteriorates with increasing density of blood vessels. In human gliomas, the presence of microvascular hyperproliferations is suggested to be the most important single predictor of survival.¹¹ Low-grade gliomas (WHO grade II),¹² which consist of highly differentiated neoplastic astrocytes and sparse blood vessel growth, have a mean survival time of several years, whereas higher-grade gliomas (WHO grade III) with highly proliferative cells have a prognosis of 2 to 5 years.¹¹ This is in stark contrast to glioblastoma multiforme (WHO grade IV), which is defined by the presence of extensive microvascular proliferations and necroses and has a mean survival time of less than 1 year.¹³ Because pathological angiogenesis is such an ominous sign in human gliomas, glioblastoma multiforme may be an ideal candidate for antiangiogenic therapy.¹⁴

A large body of direct evidence incriminates vascular endothelial growth factor (VEGF) as a central mediator of tumor-induced angiogenesis in gliomas *in vivo*. For example, tumor growth is inhibited by neutralizing antibodies against VEGF¹⁵ by retrovirus-mediated gene transfer of a dominant-negative VEGF receptor (*flk-1*) mutant that inhibits its signaling pathway¹⁶ and by expression of antisense VEGF RNA in tumor cells.¹⁷ In human brain tumors,

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VEGF expression significantly correlates with the density of counted vessels, and thus represents a useful marker for glioma angiogenesis.¹⁸

We have generated a transgenic mouse model for astrocytoma by expressing the viral *v-src* tyrosine kinase under control of the glial fibrillary acidic protein (GFAP) regulatory elements in astrocytes.¹⁹ Abnormal astrogliosis and dysplastic changes were observed frequently, and within the first year of life 20% of the mice developed overt astrocytoma in the brain and in the spinal cord (Maddalena A and Aguzzi A, unpublished results). We have defined dysplasia in this context as abnormal accumulation of nondividing astrocytes without frank tumorigenesis. This preneoplastic stadium is obvious in all GFAP-*v-src* mice older than 2 weeks. While early lesions were histologically consistent with low-grade astrocytoma, at later stages most tumors were highly mitotic and frankly malignant. Late-stage tumors showed, in addition to cellular polymorphism and high proliferative activity, numerous highly vascularized areas with cells palisading around necroses, thereby mimicking the morphological characteristics of human glioblastoma multiforme. The stochastic character of tumorigenesis in GFAP-*v-src* mice is compatible with a multistep model carcinogenesis to which additional genetic lesions concur with *v-src*.

The characteristics described above suggest that GFAP-*v-src* transgenic mice may represent a useful model for testing antiangiogenic therapies. We therefore investigated the expression patterns of molecules involved in angiogenesis at various time points during malignant progression in astrocytomas derived from these mice. *In situ* hybridization was used to assess the transcription of VEGF, its receptors *flt-1* and *flk-1*, and the endothelial-specific transmembrane receptor tyrosine kinases *tie-1* and *tie-2* (which act as receptors for angiotensin-1) in early neoplastic lesions as well as in compact, frankly malignant tumors. Because inactivation of cell cycle regulatory genes seem to be an early genetic event in the development and progression of gliomas²⁰ we intercrossed GFAP-*v-src* transgenic mice with mice hemizygous for the tumor suppressor genes *p53* and *Rb* and investigated the angiogenetic parameters described above in double mutant mice. This allowed us to ask whether *p53* and *Rb* may have an effect on the angiogenic switch in our model of gliomagenesis.

Materials and Methods

Animals and Tissue Specimens

Neoplastic and non-neoplastic brain tissues from GFAP-*v-src* transgenic mice, GFAP-*v-src* × *p53*^{+/-21}, and GFAP-*v-src* × *Rb*^{+/-22} double transgenic mice were analyzed.¹⁹ Animals were sacrificed by CO₂ overdose, brains were removed, fixed overnight in 4% (w/v) paraformaldehyde in phosphate-buffered saline, and embedded in paraffin. Two-μm sections were mounted on silanized slides and stained with hematoxylin and eosin (H&E). For each genotype, we chose samples from three low-grade and three high-grade lesions according to the

following features: low-grade lesions had only very few mitotic figures, nuclear atypia, displayed diffuse infiltration of the surrounding brain parenchyme, and could not easily be distinguished from the surrounding tissue thus reflecting characteristics of human low-grade astrocytoma (WHO grade II). In addition, those early lesions, which had a small diameter (<1.5 mm), already showed cellular pleomorphism and small foci of endothelial proliferation. Malignant high-grade lesions consisted of compact tumor masses (diameter >2 mm) with higher mitotic activity, nuclear atypia, and cellular pleomorphism according to human anaplastic astrocytoma (WHO grade III). Some of them additionally had areas with extensive vascular proliferations and/or necroses, thus exhibiting characteristics of human glioblastoma multiforme (WHO grade IV).²³ High-grade lesions consistently showed higher cellular density than low-grade lesions.

In Situ Hybridization

Sense and antisense RNA probes were transcribed *in vitro* with T3 and T7 RNA polymerase from linearized pBluescript vectors carrying cDNAs for *v-src* (700 bp), GFAP²⁴ (1200 bp), VEGF²⁵ (600 bp), *flk-1*²⁶ (2600 bp), *flt-1*²⁷ (2000 bp), *tie-1* (600 bp), and *tie-2* (1200 bp)²⁸ in the presence of digoxigenin-11-dUTP (Boehringer Mannheim). As described, 400 to 800 ng of labeled transcripts (0.6 to 2.6 kb) were hybridized to tissue sections at 65°C.²⁹ *tie-2* probes were hybridized at 60°C. Digoxigenin was detected with alkaline phosphatase-labeled anti-digoxigenin Fab fragments and 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim). Coverslips were mounted with glycerol gelatin.

Results

We investigated the transcription pattern of VEGF, *flk-1*, *flt-1*, *tie-1*, and *tie-2* by *in situ* hybridization in 20 different tissue samples. The morphological features of the neoplastic lesions were analyzed and correlated to the *in situ* hybridization analysis (Table 1).

We discovered incipient abnormal endothelial proliferations in all of the low-grade lesions. However, these were very small and were present only in few foci per lesion (Figure 1a). In contrast, most of the high-grade tumors had large areas with extensive microvascular proliferation (Figure 1b). To unequivocally localize neoplastic cells and also to provide an internal quality control for mRNA, we performed additional *in situ* hybridizations for GFAP mRNA on consecutive sections. We found that all neoplastic lesions expressed abundant amounts of GFAP irrespective of their degree of anaplasia (Figure 2, c and d).

VEGF, *flt-1*, and *flk-1* Expression

In all low-grade lesions, we found extensive expression of VEGF in neoplastic astrocytes, whereas neither brain tissue surrounding the neoplasms, nor control tissues from normal mouse brains (data not shown), showed any

Table 1. Histological Characterization of the Tissue Samples Generated in GFAP-v-*src*, GFAP-v-*src* × *p53*^{+/-} and GFAP-v-*src* × *Rb*^{+/-} Transgenic Mice by H&E Staining and GFAP *in Situ* Hybridization

Sample	Malignancy	Genotype	Age (weeks)	Mitotic figures [†]	Growth pattern	Neovascularization [‡]	Necroses [§]	GFAP MRNA
1	—	Wild-type	6	—	—	—	—	+
2	LG	<i>Src</i>	4	—	D	+	—	++
3	LG	<i>Src</i>	4	—	D	+	—	++
4	LG	<i>Src</i>	4	—	D	+	—	++
5	LG-HG	<i>Src</i>	14	+	D-C	+	—	++
6	LG	<i>Src</i> × <i>Rb</i>	20	—	D	+	—	++
7	LG	<i>Src</i> × <i>Rb</i>	20	—	D	+	—	++
8	LG	<i>Src</i> × <i>Rb</i>	4	—	D	+	—	++
9	LG	<i>Src</i> × <i>p53</i>	4	—	D	+	—	++
10	LG	<i>Src</i> × <i>p53</i>	12	++	D	+	—	++
11	LG-HG	<i>Src</i> × <i>p53</i>	4	+	D-C	+	—	++
12	HG	<i>Src</i>	11	+	C	++	—	+++
13	HG	<i>Src</i>	10	+++	C	+++	+++	+++
14*	HG	<i>Src</i>	20	+++	C	+++	++	++
15	HG	<i>Src</i> × <i>Rb</i>	30	++	C	+	+	+++
16	HG	<i>Src</i> × <i>Rb</i>	9	+++	C	++	—	+++
17	HG	<i>Src</i> × <i>Rb</i>	16	+++	C	+++	++	+++
18	HG	<i>Src</i> × <i>p53</i>	36	+	C	+	++	++
19	HG	<i>Src</i> × <i>p53</i>	18	+	C	++	+	++
20	HG	<i>Src</i> × <i>p53</i>	46	+++	C	++	—	+++

src, *Rb*, and *p53* mice had a hemizygous genotype for the respective gene. All samples were from different individual mice.

20 different tissue samples from nonmalignant wild-type brain, low-grade (LG) and high-grade (HG) gliomas were analyzed. One subcutaneous tumor was localized in the neck (*). Neoplastic lesions were either growing as compact masses (C), diffusely infiltrating (D), or presented a combination of both growth patterns (D-C).

[†] —, Normal mitotic activity; +, slightly elevated mitotic activity; ++, moderate elevated mitotic activity; +++, numerous mitotic figures.

[‡] +, very few and small foci of neovascularization; ++, several larger foci of neovascularization; +++, areas with extensive microvascular proliferation.

[§] —, no necrotic areas; +, 1 to 4 necrotic areas; ++, 5 to 10 necrotic areas; +++, over 11 necrotic areas (diameter >0.25 mm). For GFAP mRNA evaluation see Table 2.

detectable VEGF mRNA (Figure 2e). In some areas of early neoplastic lesions, the expression pattern of VEGF and v-*src* were almost identical (Figure 3, a and b), whereas in others, VEGF and v-*src* expression appeared to be independent from each other. We found both areas in which VEGF was transcribed in the absence of v-*src* (Figure 3, c and d), and vice versa (Figure 3, e and f).

In high-grade neoplasms, extensive up-regulation of VEGF was evident in tumor cells palisading around necroses (Figure 2f) at areas with low vascular density and also at the periphery of each tumor (data not shown). In

contrast to VEGF, v-*src* transcription showed a homogeneous expression pattern throughout the tumor without any specific relationship to its topography (Figure 3, g and h).

We found that *flt-1* (the receptor of VEGF, also named VEGFR-1) was expressed in a dot-like pattern in normal endothelial cells of mouse brains, especially in cells lining small vessels and capillaries (Figure 4a). The same pattern was seen in non-neoplastic brain parenchyme of transgenic mice (data not shown). In some low-grade lesions, we found additional up-regulation of *flt-1* in en-

Figure 1. Vascular pattern in low-grade (a) and high-grade (b) gliomas of GFAP-v-*src* transgenic mice. Low-grade lesions were well vascularized (arrows) but showed very little or no endothelial proliferations. In contrast, high-grade lesions exhibited distinct areas with extensive microvascular proliferation (arrows). Original magnification, ×158.

Figure 2. Low-grade (a, c, and e) and high-grade (b, d, and f) neoplastic lesions of GFAP-v-src transgenic mice: H&E stains (a and b) and *in situ* hybridization for GFAP (c and d) and for VEGF (e and f). Low-grade tumors consisted of small (<1.5 mm) nodular neoplastic lesions that diffusely infiltrated the surrounding brain parenchyma (a, region enclosed by dots). Neoplastic astrocytes showed strong GFAP mRNA expression when compared with the surrounding tissue (c, arrows). In the cerebral cortex adjacent to the tumor (c; Cx), only a few reactive astrocytes were GFAP-positive, whereas neurons and other neuroectodermal cells failed to express GFAP (thus confirming the specificity of the GFAP *in situ* hybridization reaction). High-grade lesions were large tumors, some of them showed necrotic areas (Ne) surrounded by palisading cells (arrowheads). *In situ* hybridization for VEGF on consecutive sections (e and f) showed extensive transcription of the VEGF gene in clusters of neoplastic astrocytes of low-grade lesions (e, arrows). In contrast, neither brain tissue surrounding the neoplasms (peripheral areas of e), nor normal mouse brain control tissues (not shown) showed VEGF mRNA. High-grade tumors expressed the highest levels of VEGF mRNA in palisading cells surrounding necrotic foci (f, arrowheads), in areas of low vascular density, and at the periphery of the tumor (not shown). Original magnification, $\times 75$.

endothelial cells of newly formed, sprouting capillaries (Figure 4e) with no significant relationship to the areas of VEGF expression. Most of the highly malignant tumors showed strong staining for *flt-1*, especially in small vessels and capillaries and also at the periphery of the

tumor, whereas larger vessels were only weakly stained (Figure 4i). In one sample of a glioblastoma-like tumor there was additional expression of *flt-1*, *flk-1*, *tie-1*, and *tie-2* in endothelial cells in the non-neoplastic tissue adjacent to the tumor (not shown).

Figure 3. Comparative *in situ* hybridization analysis of the expression patterns of *v-src* (a, c, e, and g) and of VEGF (b, d, f, and h) on consecutive sections. In low-grade lesions, we found certain areas with superimposable spatial expression of *v-src* and VEGF (a and b, arrows). However, other areas displayed expression of only VEGF or only *v-src* (c-f, arrows). In high-grade tumors, *v-src* transcription showed a homogeneous expression pattern throughout the tumor without any specific relationship to its histoarchitecture (g). In contrast, VEGF up-regulation was most prominent in astrocytes palisading around necrotic areas (h, arrows; see Figure 2), indicating that the regulatory mechanisms of VEGF transcription active in human gliomas were conserved in GFAP-*v-src* tumors. Original magnification, $\times 75$.

Figure 4. *In situ* hybridization with antisense probes for the VEGF receptors *flt-1* and *flk-1*, the orphan/angiopoietin receptors *tie-1* and *tie-2* in normal mouse brain (a-d), and low-grade (e-h) and high-grade (i-l) neoplastic lesions. In normal mouse brains, *flt-1* was expressed in a punctuate pattern exclusively endothelial cells (see arrows in Figure 3a), whereas no *flk-1*, *tie-1*, or *tie-2* mRNA could be detected (b-d, arrows). A similar pattern of expression was detected in non-neoplastic brain parenchyme of GFAP-v-*src* transgenic mice (not shown). A number of low-grade lesions expressed sizeable amounts of *flk-1* and, to a lesser extent, of *tie-1* and *tie-2*. In addition, we observed further up-regulation of *flt-1* in endothelial cells of newly formed, sprouting vessels (e; see Figure 5). Moreover, we found *flt-1* mRNA also in neoplastic astrocytes of most low-grade lesions (e, arrowheads; see Figure 5). In high-grade tumors, most small vessels and capillaries showed strong staining for *flt-1* and *flk-1* and to a lesser extent for *tie-1* and *tie-2* (i-l, arrows), whereas larger vessels were only weakly stained (see asterisks in Figure 3, i, j, and k). Original magnification, $\times 158$.

Surprisingly, we found expression of *flt-1* also in neoplastic astrocytes in a proportion of low-grade lesions (Figure 5, a and b). This finding was consistently reproduced in six of nine tumors analyzed.

In contrast to *flt-1*, we found no or only very little expression of *flk-1* (also named VEGFR-2) in normal brain tissue as well as in non-neoplastic tissue of transgenic mice (Figure 4b). Some low-grade lesions clearly ex-

Figure 5. Details of the tumor shown in Figure 4e illustrating *flt-1* mRNA expression in endothelial cells of sprouting vessels (arrows) as well as, to a lesser extent, in neoplastic astrocytes of low-grade lesions (arrowheads). Original magnification, $\times 500$.

pressed *flk-1* on endothelial cells of small and larger vessels but, in contrast to *flt-1*, failed to express on anaplastic astrocytes (Figure 4f). In highly malignant lesions, the expression pattern of *flk-1* in endothelial cells was similar to *flt-1* but with even higher affinity to small vessels (Figure 4j). There was no obvious topographical relationship of the presence of *flk-1* mRNA to expression of VEGF (not shown).

tie-1 and tie-2 Expression

tie-1 and *tie-2*, both receptor tyrosine kinases known to be involved in embryonal angiogenesis²⁸ showed no expression in adult brain tissue (Figure 4, c and d). Also, only few low-grade lesions exhibited expression of *tie-1* or *tie-2* on endothelial cells (Figure 4, g and h). We found strong expression of both receptor mRNAs only in glioblastoma multiforme-like tumors (Figure 4, k and l). As in the case of *flt-1* and *flk-1*, endothelial cells of small vessels displayed a stronger staining for *tie-1* and *tie-2* than the larger ones. In general, we detected a stronger signal in endothelial cells for *tie-1* than for *tie-2*.

Effect of p53^{+/-} and Rb^{+/-} on the Expression Pattern of Angiogenic Molecules

We were interested in potential effects of loss of the tumor suppressor genes, *Rb* and *p53*, on angiogenesis. We found that the neoplastic lesions arising in GFAP-v-*src* $\times$ *p53*^{+/-} and in GFAP-v-*src* $\times$ *Rb*^{+/-} double transgenic mice were morphological identical to those of GFAP-v-*src* mice (Maddalena A, Hainfellner J, and Aguzzi A, unpublished observations). *In situ* hybridization analysis of these neoplastic lesions revealed no significant alteration in the expression pattern of VEGF, *flt-1*, *flk-1*, *tie-1*, and *tie-2* (Table 2). Neither the localization nor the quantity of mRNA during malignant progression differed from those of GFAP-v-*src* single transgenic mice.

Discussion

Pathological angiogenesis is a crucial but poorly understood aspect of astrocytoma biology. Detailed knowledge of the interactions between angiogenic factors and their receptors, and particularly of their timing, during malignant progression may lead to improved imaging and therapy of human gliomas. Indeed, several studies have suggested a significant role for VEGF in tumor angiogenesis.^{15,17,30}

Before the various forms of antiangiogenic therapy can be attempted in humans, it is most desirable to assess their potential in a reliable animal model. The GFAP-v-*src* transgenic mice provide such a model because tumorigenesis in these mice recapitulates the sequence of events found in human "career gliomas", ie, the progression of small pre-neoplastic foci to low-grade, slowly growing neoplastic lesions and finally to higher grade, rapidly growing glioblastoma-like tumors.³¹

We have therefore analyzed the expression patterns of VEGF and its receptor tyrosine kinases *flt-1* and *flk-1* as well as the *tie-1* and *tie-2* receptor molecules in early neoplastic lesions and late end-stage gliomas in this transgenic mouse model for glioma. Our results strongly suggest that the angiogenic switch, defined as the abrupt up-regulation of molecules (growth factors and their receptors) capable of inducing abnormal vessel growth, is a very early event during malignant progression in astrocytoma produced by GFAP-v-*src* transgenic mice. In all early neoplastic lesions we found clusters of dysplastic astrocytes expressing VEGF mRNA.

In an earlier study, we had speculated that VEGF expression may be directly induced by v-*src* at this stage,¹⁹ based on previous reports showing transactivation of the VEGF promoter by v-*src* in a dose-dependent manner in U87 glioblastoma cells.³² However, although the expression pattern of VEGF and v-*src* mRNA was superimposable in some areas of low-grade lesions, other areas showed no correlation between the expression of these

Table 2. Distribution of the Expression of VEGF, *flt-1*, *flk-1*, *tie-1*, and *tie-2* mRNA in Normal Brain, Low-Grade Lesions and High-Grade Tumors of GFAP-v-*src* Transgenic Mice, and in GFAP-v-*src* × *p53*^{+/-} and GFAP-v-*src* × *Rb*^{+/-} Double Transgenic Mice

Sample	VEGF	<i>flt-1</i>	<i>flk-1</i>	<i>tie-1</i>	<i>tie-2</i>
1	—	+	—	—	—
2	++	++*	+	+	+
3	++	++*	+	—	—
4	++	+	+	—	—
5	++	++*	+	+	—
6	+	n.d.	—	n.d.	n.d.
7	++	+	++	—	—
8	++	+	—	—	—
9	n.d.	++	+	n.d.	+
10	++	+	+	—	+
11	++	+	+	—	—
12	++	+++	++	+	—
13	+++	+++	+++	++	+
14	++	+++	+++	++	++
15	+++	++	n.d.	+	—
16	++	n.d.	++	++	+
17	+++	+	+	+	+
18	++	+++	++	+	—
19	+++	++	+++	—	+
20	++	+	+	+	+

v-*src* and VEGF were expressed in neoplastic astrocytes, whereas *flt-1*, *flk-1*, *tie-1*, and *tie-2* were expressed only in endothelial cells. Surprisingly, we found that some low-grade gliomas expressed *flt-1* also in neoplastic astrocytes (*).

Semiquantitative evaluation of expression: —, no expression detectable; +, weak expression, at the threshold of detectability; ++, clearly detectable expression; +++, strong expression; n.d., not determined.

two genes. This may indicate that other mechanisms, in addition to direct transcriptional induction of VEGF by v-*src*, are likely to be involved in early VEGF expression. This phenomenon was even more evident in high-grade gliomas, in which VEGF mRNA was strongly up-regulated in tumor cells directly adjacent to necrotic foci, in the periphery of the tumors, and in areas of low vessel density. In contrast, v-*src* showed a uniform expression pattern throughout the tumors.

These findings are in agreement with previous reports showing enhanced VEGF mRNA transcription in pseudopalisading cells of human gliomas, ie, in those tumor cells that line areas of necrosis, as well as in areas of tumor infiltration into surrounding tissue.^{14,33,34} Because VEGF levels are dramatically increased within a few hours of exposing different cell cultures to hypoxia,³⁵ it has been proposed that VEGF induction is driven by insufficient perfusion. Hypoxia-induced transcriptional activation is mediated by direct binding of hypoxia-inducible factor, to regulatory *cis*-acting enhancer elements located in the 5'-flanking region of the VEGF gene^{36,37} and by increased RNA stability.³⁸ In addition to hypoxia, there may be other factors inducing VEGF up-regulation in high malignant tumors: for example, oncogenic Ras and hypoxia have been shown to synergistically up-regulate VEGF via hypoxia-inducible factor-1.³⁹ Other mechanisms of VEGF induction via glucose deprivation⁴⁰ and mutations in the VHL tumor suppressor gene⁴¹ have been proposed. Because they are easily amenable to genetic and other manipulations, we hope that the GFAP-v-*src* transgenic mice will be of help in clarifying the role of these pathways *in vivo*.

Both VEGF receptor tyrosine kinases, *flt-1* and *flk-1*, were expressed on endothelial cells in most of the low-grade lesions. In general *flt-1* mRNA, which was found

also on normal brain vasculature, was expressed to a greater extent in low-grade tumors than *flk-1*, again in strict analogy to the situation in human gliomas.^{33,34} In highly malignant tumors, strong hybridization signals were observed in capillaries and small tumor vessels, and in one sample also in endothelial cells of tissues directly adjacent to the tumor. These findings suggest that VEGF acts specifically on those vessels that are primed to grow into the tumor from peripheral, unaffected tissue and are in agreement with observations made in human gliomas.³⁴

The factors that induce the expression of VEGF receptors in tumor endothelium are as yet unknown. It has been suggested that VEGF itself may induce expression of its receptors by means of a positive feed-back mechanism.⁴² However, we found no significant correlation between areas of VEGF induction and the expression pattern of *flt-1* and of *flk-1*, suggesting that other factors may be involved in *flt-1/flk-1* induction. According to experiments with cultured cells, *flt-1* is up-regulated by hypoxia in endothelial cells, and up-regulation is not prevented by addition of neutralizing antibodies against VEGF.⁴³

Apart from endothelial cells, we found *flt-1* mRNA in neoplastic astrocytes, which, to the best of our knowledge, was not described before. Although in normal healthy organisms in which expression of *flt-1 in vivo* appears to be restricted to endothelial cells, there are reports demonstrating *flt-1* expression in stromal cells of human hemangioblastoma⁴⁴ and in monocytes.⁴⁵ One could speculate that *flt-1* is also up-regulated by hypoxia in dysplastic astrocytes and that stimulation by VEGF leads to tissue factor production and chemotaxis like in monocytes and endothelial cells. Tissue factor itself can also regulate VEGF expression and seems to be impor-

tant for tumor angiogenesis as tumors expressing tissue factor antisense mRNA exhibit reduced angiogenesis.⁴⁶

The *tie* receptors comprise a novel family of endothelial-specific transmembrane receptor tyrosine kinases.⁴⁷ *tie-1* and *tie-2* are expressed in early stage of angiogenesis of the mouse, but the onset of their expression occurs somewhat later than that of *flt-1* and *flk-1*. *tie-1*, whose ligand still is unknown, is required for the structural integrity of the endothelium,⁴⁸ whereas *tie-2* and the recently identified ligands angiopoietin-1 and angiopoietin-2 are involved in vascular remodeling.^{49,50} We found very little *tie-1* and *tie-2* mRNA in a few low-grade lesions, suggesting that the *tie* receptors may play a minor role in early angiogenesis. In high-grade tumors, a larger amount of *tie-1* and *tie-2* mRNA was identified, and again their expression was particularly pronounced in small vessels. A similar pattern of *tie-1* expression was reported in human gliomas,³⁴ whereas *tie-2* expression was not detectable in transplanted astrocytoma cell lines.³⁰

Alterations of the retinoblastoma (*Rb*) and *p53* tumor suppressor genes have been reported in human gliomas.²⁰ It was proposed that progression of astrocytoma to glioblastoma involves clonal expansion of tumor cells carrying mutations of the *p53* tumor suppressor gene.⁵¹ Moreover, mutant *p53* up-regulates VEGF in NIH 3T3 cells⁵² and wild-type *p53* protein enhances angiogenesis inhibitors, eg, TSP-1.^{53,54} However, we did not find any significant changes in the transcription patterns of VEGF and any of the angiogenic receptor molecules in GFAP-*v-src* × *p53*^{+/-} and in GFAP-*v-src* × *Rb*^{+/-} double transgenic mice.

In summary, our results indicate that the angiogenic switch takes place at an early stage during the malignant progression of astrocytomas observed in GFAP-*v-src* transgenic mice. In addition, the expression pattern of VEGF, *flt-1*, *flk-1*, *tie-1*, and *tie-2* is similar to what has been described in human gliomas. Therefore, we have reasons to hope that GFAP-*v-src* transgenic mice will provide a useful tool for deepening our insight into the molecular pathophysiology of glioma angiogenesis and will perhaps even claim a use in antiangiogenic therapy research.

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