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R S Morrison

F Yamaguchi

H Saya

J M Bruner

Alan M. Yahanda MD, FACS

See next page for additional authors

Authors

R S Morrison; F Yamaguchi; H Saya; J M Bruner; Alan M. Yahanda MD, FACS; L A Donehower; and M Berger

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Basic fibroblast growth factor and fibroblast growth factor receptor I are implicated in the growth of human astrocytomas

Richard S. Morrison,[1,2] Fumio Yamaguchi,[1] Hideyuki Saya,[2,3] Janet M. Bruner,[4] Alan M. Yahanda,[5] Lawrence A. Donehower[6] and Mitchel Berger[7]

Departments of [1] Neurosurgery, [2] Tumor Biology, [3] NeuroOncology, [5] Surgical Oncology and [4] Neuropathology; The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA; [6] Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030, USA; [7] Department of Neurosurgery, University of Washington, Seattle, WA 98195, USA

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Summary

Malignant astrocytomas are highly invasive, vascular neoplasms that comprise the majority of nervous system tumors in humans. A strong association has previously been made between malignancy in human astrocytic tumors and increased expression of certain fibroblast growth factor (FGF) family members, including basic and acidic FGF. The influence of endogenous basic FGF on glioblastoma cell growth *in vitro* was evaluated using basic FGF-specific antisense oligonucleotides. These studies indicated that human glioblastoma cell growth *in vitro,* can be inhibited by suppressing basic FGF expression. Human astrocytomas also exhibited changes in FGF receptor (FGFR) expression during the course of their progression from a benign to a malignant phenotype. FGFR2 (bek) expression was abundant in normal white matter and in all low grade astrocytomas, but was not observed in glioblastomas. Conversely, FGFR1 (flg) expression was absent or barely detectable in normal white matter, but was significantly elevated in glioblastomas. Glioblastomas also expressed an alternatively spliced form of FGFR1 containing two immunoglobulin-like disulfide loops (FGFR1β), whereas normal human adult and fetal brain expressed a form of the receptor containing three immunoglobulin-like disulfide loops (FGFR1α). Intermediate grades of astrocytic tumors exhibited a gradual loss of FGFR2 and a shift in expression from FGFR1α to FGFR1β as they progressed from a benigh to a malignant phenotype. The underlying cytogenetic changes that contribute to these alterations are not entirely understood, but abnormalities in the p53 tumor suppressor gene may influence expression of bFGF as well as the FGFR. These results suggest that alterations in FGFR signal transduction pathways may play a critical role in the malignant progression of astrocytic tumors.

Introduction

A major goal in the field of neuro-oncology is to identify cytogenetic changes in astrocytomas and to correlate these changes with specific biochemical alterations that enhance cellular proliferation, invasiveness and vascularity. We have evaluated the contribution made by specific fibroblast growth factor (FGF) family members and their receptors to malignant progression in human astrocytic tumors.

Basic fibroblast growth factor (bFGF) is a multifunctional protein recognized primarily for its mitogenic and angiogenic properties. On the basis of cell culture studies bFGF has been shown to be mitogenic for a wide range of cell types derived from mesoderm and neuroectoderm. In addition to the

many *in vitro* studies performed with bFGF, it is also active in numerous *in vivo* models of angiogenesis and wound healing [1, 2]. bFGF has been identified in many normal and malignant tissues [3, 4], and at several developmental time points [3, 5, 6], implying that it may play a role in normal tissue function, embryonic development, and neoplastic progression. bFGF and related members of the FGF family have been implicated in the autocrine regulation of human tumor growth based partly on transfection studies with bFGF expression vectors, which result in amplified autocrine growth in monolayer culture and soft agar [7–9]. In addition, several recently described oncogenes discovered in human tumors encode proteins structurally related to the FGF family growth factors [10–15].

The biological responses of FGFs are mediated through specific, high affinity, transmembrane receptors. Four structurally related genes encoding high affinity receptors have been identified [16–20]. In addition to high-affinity sites, cells exhibit low affinity FGF binding sites [21] which have been characterized as extracellular heparin sulfate proteoglycans [22, 23]. Binding to the low affinity, glycosaminoglycan sites appears to be obligatory for FGF binding to high affinity receptors and biological activity [24–26].

Structural variants of FGFR1 and FGFR2 can be generated by alternative RNA splicing of their RNA transcripts [27–30]. The divergent receptors generated by this process manifest different ligand binding specificities and affinities [31, 32]. One common structural variant involves the second half of the third immunoglobulin-like (Ig) disulfide loop of FGFR1 and FGFR2 which dramatically alters their ligand-binding properties [31]. Another splicing variant results in FGFRs containing either two or three Ig-like domains in the extracellular region [17, 29, 30, 33]. Alternative RNA splicing involving both the first and third Ig-like domain are subject to cell and tissue-specific processing indicative of the changing FGF requirement that occurs during tissue growth and differentiation [17, 29, 31, 34]. Changes in ligand binding affinity and specificity resulting from alternative splicing, are also likely to be important in some types of human cancers that rely on FGF family members to sustain growth and invasiveness.

In the present review, we discuss efforts to inhibit glioma growth using antisense oligonucleotides to suppress bFGF expression. Specific changes in FGFR expression are also described which relate to malignant progression in astrocytic tumors and which may provide novel targets for therapy. Finally, a transgenic mouse model producing inactivation of both copies of the p53 gene (–/–) is described. Astrocytes derived from the p53 –/– mice exhibit several aspects of malignant transformation associated with their malignant human astrocytic counterparts. The conclusion of these studies is that cytogenetic abnormalities in transformed astrocytes ultimately alter growth factor signaling pathways providing them with a selective growth advantage.

bFGF is an astrocyte-derived mitogen

The first reports localizing bFGF to cells in the CNS suggested that bFGF was synthesized by neurons [35, 36]. However, recent immunocytochemical analyses, employing several well defined bFGF-specific antibodies, have demonstrated that bFGF is principally expressed by astrocytes [37–40]. The cellular localization of bFGF as demonstrated by immunocytochemistry correlates well with the biochemical identification of bFGF in highly purified cultures of astrocytes as opposed to neurons [37, 38, 40]. In marked contrast to bFGF, acidic FGF (aFGF) appears to be expressed by neurons [41–45] suggesting these two related factors may have unique actions within the CNS.

The function of astrocyte-derived bFGF is not understood. bFGF has a multitude of actions on cultured CNS cells. These include promoting neuronal survival and neurite outgrowth, stimulating proliferation and differentiation of oligodendrocytes and enhancing the proliferation of type I astrocytes. Additional actions on microglial cells and endothelial cells may also occur following injury to the CNS.

The presence of bFGF in normal astrocytes suggests that bFGF may subserve a maintenance func-

tion in these cells. Malignant transformation of astrocytes appears to result in elevated bFGF expression [46–48] which may enhance their proliferation and invasiveness.

bFGF promotes autocrine growth of astrocytoma cells

Endogenous bFGF expression was suppressed in glioblastoma cells to evaluate its role in promoting abnormal cell growth and invasiveness. This was accomplished using bFGF-specific antisense oligonucleotides [49]. The addition of 50 μM bFGF-specific antisense oligonucleotide to the human glioblastoma cell line SNB-19 resulted in an 80% inhibition in cell growth. This effect was saturable and specific. Antisense oligonucleotides directed to two different sites of bFGF mRNA were effective in suppressing SNB-19 growth, whereas sense strand oligonucleotide was ineffective. A subsequent report using a separate astrocytoma cell line also demonstrated that bFGF suppression by antisense oligonucleotides inhibited the growth of transformed human astrocytes in culture [50]. Interestingly, antisense oligonucleotide directed against the B-chain of human PDGF did not effect SNB-19 cell growth when tested at concentrations equivalent to those used for bFGF [49]. Thus, the growth inhibitory actions of bFGF antisense oligonucleotides appeared to be specific and due to the formation of specific hybrids between the oligonucleotides and their respective mRNA.

The lack of growth inhibition observed with bFGF-specific sense oligonucleotide and the PDGF-B chain-specific antisense oligonucleotide suggested that the inhibitory effect of bFGF-specific antisense oligonucleotides was related to alterations in bFGF expression. This possibility was evaluated by measuring bFGF protein in SNB-19 cells using a slot-blot immuno-detection technique. bFGF protein was quantitated by slot-blot analysis against a standard human-recombinant bFGF curve. SNB-19 cells grown in serum free medium until approximately 75% confluent exhibited 5.56 ng bFGF/μg protein. The sense strand primer, which did not effect SNB-19 cell growth, correspondingly had no effect on SNB-19 bFGF content (6.44 ng bFGF/μg protein). In marked contrast, an antisense primer complementary to the initiation codon (AS-1, 35 μM) significantly reduced bFGF expression in SNB-19 cells (1.87 ng bFGF/μg protein). In this particular experiment the 67% reduction in bFGF content was parelleled by a 55% reduction in cell number, implying that inhibition of SNB-19 cell growth was directly related to the loss of bFGF.

The relevance of bFGF expression to the growth of human astrocytoma cells was addressed further by investigating the actions of bFGF antisense primers on the growth of non-transformed human astrocytes. Non-transformed human astrocytes were prepared from surgically resected temporal lobe biopsies. Cells prepared in this manner were identified as astrocytes based upon their expression of the astrocyte intermediate filament protein, glial fibrillary acidic protein (GFAP). bFGF-specific antisense primers failed to alter the growth of non-transformed human astrocytes. However, a recent report demonstrated that the growth of non-transformed rat astrocytes was sensitive to bFGF-specific antisense oligonucleotides [51]. The discrepancy between these results may be related to species differences since we have observed that non-transformed rat astrocytes contain significantly greater amounts of bFGF protein than non-transformed human astrocytes. Therefore, the observation that bFGF-specific antisense primers do not effect the growth of non-transformed human astrocytes suggests that bFGF pathways are activated in astrocytoma cells following neoplastic transformation.

bFGF activity has also been suppressed using neutralizing monoclonal antibodies [52, 53] and an inhibitor of the agonist action of bFGF [53]. The application of neutralizing antibodies or inositolhexakisphosphate ($InsP_6$), a newly identified antagonist of FGF binding and activity [Morrison *et al.*, submitted, 54], suppressed SNB-19 cell growth in soft agar culture. A 70% reduction in the number of surviving colonies was observed with 100 μM $InsP_6$. Inhibition of clonogenic growth by $InsP_6$ was dose-dependent and saturable at 200 μM. $InsP_6$ also reduced the size and altered the morphology of individual colonies in a manner analogous to treatment

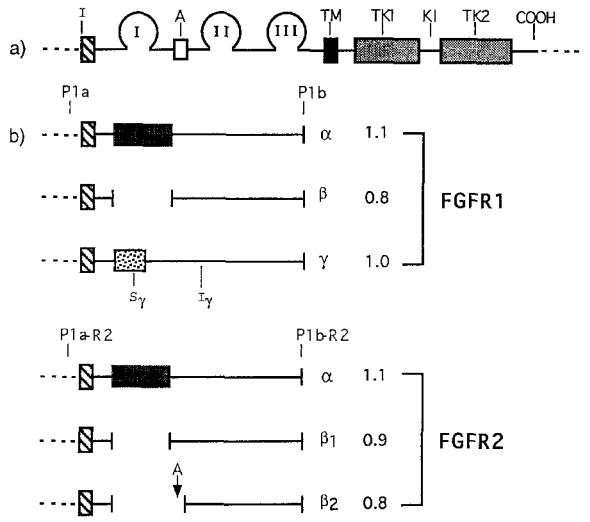

Fig. 1. Schematic representation of the FGFR and the location of primers used for PCR analysis. a) Stucture of the FGFR including the non-translated regions (----), initiation start site (I), immunoglobulin-like disulfide loops I–III, acidic box domain (A), transmembrane domain (TM), tyrosine kinase consensus sequences 1 and 2 (TK1, TK2), kinase insert region (KI) and the carboxy terminal (COOH). b) Primer location and expected sizes of PCR amplification products for FGFR1 and FGFR2 analysis. Structural variations in the NH_2 terminus of FGFR1 were evaluated using PCR primers Pla and Plb. The PCR reaction yielded 1.1-kb (3 Ig-loops), 1.0-kb (2 Ig-loops) and 0.8-kb (2 Ig-loops) fragments encoding the α γ, and β motifs, respectively. The gamma transcript (γ), contains a unique 144 bp insertion in place of the 267 bp α exon. There is an in frame stop codon (Sγ) present down stream of the translation initiation site and an alternate candidate translation initiation site (Iγ) that purportedly encodes and intracellular form of FGFR1 containing two IgG-like disulfide loops [30]. Primers Pla-R2 and Plb-R2 resulted in the detection of three structural variants of FGFR2. The PCR reaction yielded 1.1-kb (3 Ig-loops), 0.9-kb (2 Ig-loops) and 0.8-kb (2 Ig-loops lacking the acidic box) fragments encoding the α, β_1, and β_2 motifs, respectively.

with antisense oligonucleotides. However, $InsP_6$ had no effect on the proliferation of non-transformed human fetal astrocytes or a low-level bFGF expressing glioma cell line suggesting that growth inhibition was not the result of non-specific cytotoxicity [Morrison *et al.*, submitted]. These results correlate well with earlier studies demonstrating that exogenous treatment with bFGF significantly increased the number and size of SNB-19 colonies growing in soft agar [55]. The results of studies using neutralizing antibodies and $InsP_6$ imply that bFGF release or secretion may be related to an autocrine pathway promoting astrocytoma growth. Furthermore, these studies collectively imply that bFGF expression potentiates phenotypic malignancy in transformed human astrocytes.

Malignant astrocytes express an alternatively spliced form of FGFR1 mRNA

We have evaluated benign and malignant human astrocytomas for alterations in FGF receptor expression [74]. Astrocytomas of varying degrees of malignancy were evaluated for alterations in FGFR expression that might signify progressive changes in malignancy. FGFR1 oligonucleotide primers were used to amplify cDNAs which correspond to an NH_2-terminal motif containing three (α) or two (β) immunoglobulin (IgG)-like disulfide loops (Fig. 1). A third alternatively spliced transcript γ, purportedly encodes an intracellular form of FGFR1 containing two IgG-like disulfide loops [30]. PCR amplification of cDNA prepared from frozen sections of histologically defined glioblastomas demonstrated the presence of α, β, and γ transcripts. Glioblastomas preferentially expressed the β-form of FGFR1 (Fig. 2). In marked contrast, frozen sections of adjacent normal brain expressed significantly less FGFR1 mRNA demonstrating that FGFR1 mRNA is overexpressed in malignant astrocytomas. In addition, normal brain immediately adjacent to the tumor preferentially expressed the α transcript. It was shown that the paucity of FGFR1 amplification product in normal brain was not related to the absence or quality of mRNA by amplifying the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH).

FGFR1 transcripts were examined in astrocytomas of varying degrees of malignancy to determine if the shift from FGFR1α to FGFR1β was associated with a particular stage of astrocyte transformation. The relationship between tumor phenotype and the ratio of β/α transcripts was established by measuring the signal intensity of individual transcripts following PCR Southern blot analysis. As seen in Table 1, the β/α ratio increased significantly as astrocytic tumor grade varied from low-grade (LGA) to high-grade (GBM). Anaplastic astrocytomas (AA)

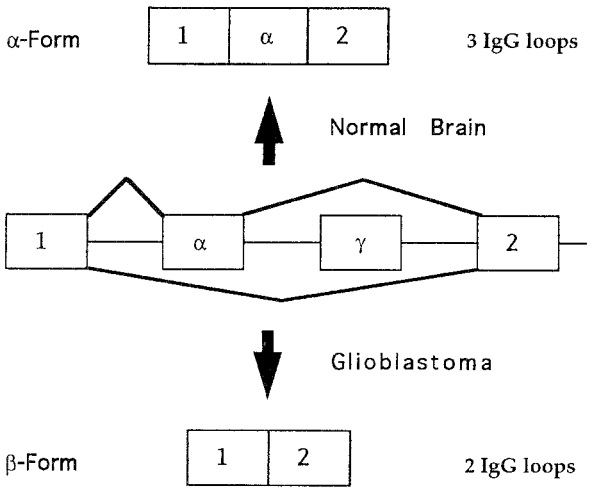

Fig. 2. Alternative mRNA splicing of FGFR1 transcripts in normal brain and glioblastoma. This schematic summarizes the changes that are detected in FGFR1 during the malignant progression of astrocytic tumors. After FGFR1 expression is activated in transformed astroctyes (see Fig. 2) there is a progressive shift in the mRNA splicing pattern of FGFR1 transcripts that parallels malignant progression. Normal adult and fetal brain include α-exon which encodes the first immunoglobulin-like (IgG) disulfide loop in the extracellular domain of the receptor [30]. Thus, normal brain expresses almost exclusively a form of the receptor containing three IgG-like loops (3 IgG loops, α-form). In contrast, glioblastomas exclude the α-exon thereby producing FGFR1 transcripts that join exons 1 to 2. This produces an mRNA transcript that includes only two IgG-like loops in the extracellular domain of the receptor (2 IgG loops, β-form). As shown in Table 1, the relative abundance of the β-form of FGFR1 is selectively favored during the course of malignant progression. The regulation of exon usage in this receptor is not understood, but the β-form of the receptor has recently been reported to exhibit a ten fold higher affinity for basic and acidic FGF than the α-form [65].

displayed two receptor phenotypes, one similar to glioblastomas, the other similar to normal brain. The average β/α ratio for GBMs and the higher subset of AAs was significantly different from LGAs and normal brain ($P < 0.005$). There was no difference in the β/α ratio between normal adult brain, LGAs and the lower subset of AAs ($P > 0.002$). The β/α ratio for normal fetal brain was significantly different from that for adult brain ($P < 0.005$). Interestingly, the ratio for medulloblastoma, the most common malignant central nervous system tumor of childhood, closely resembled fetal brain, expressing almost exclusively FGFR1α.

Table 1. Relationship between tumor phenotype and the ratio of FGFR1β/FGFR1α

Tumor	Sample # (n)	FGFR1β/α (X ± s.e.m.)
Glioblastoma Multiforme	11	2.54 ± 0.32**
Anaplastic Astrocytoma-H	3	1.70 ± 0.12**
Anaplastic Astrocytoma-L	3	0.60 ± 0.12
Low Grade Astrocytoma	5	0.74 ± 0.0
Normal Adult Brain	10	0.53 ± 0.04
Normal Fetal Brain	2	0.16 ± 0.03*
Medulloblastoma	10	0.16 ± 0.04

The relative intensity of FGFR1β and FGFR1α transcripts were determined from PCR-Southern blots as described [74]. Anaplastic astrocytomas (AA) displayed two distinct sets of β/α ratios. The set displaying an average value similar to the glioblastomas was designated the high group (H), while the set displaying an average ratio similar to low-grade astrocytomas and normal adult brain was designated the low group (L). Values represent the mean of three separate determinations. ** The average β/α ratio for GBMs and the higher subset of AAs was significantly different from LGAs and normal brain ($P < 0.005$). There was no difference in the β/α ratio between normal adult brain, LGAs and the lower subset of AAs ($P > 0.02$). * The β/α ratio for normal fetal brain was significantly different from that for adult brain ($P < 0.005$).

FGFR1 and FGFR2 exhibit reciprocal expression in normal and transformed human brain tissue

FGFR2 expression was also evaluated in human astrocytomas and normal brain. FGFR2 exhibits alternative RNA splicing in a manner analogous to FGFR1 (Fig. 1) [56]. Surprisingly, the distribution of FGFR2 transcripts in astrocytomas and normal brain was opposite to that observed for FGFR1 expression as shown schematically in Fig. 3. Whereas, glioblastomas expressed elevated levels of FGFR1, low to non-detectable levels of FGFR2 were observed using the same cDNA samples. Adjacent normal brain obtained from the same tumor specimen expressed low levels of FGFR1, but abundant levels of FGFR2. Low-grade astrocytomas (LGA) also expressed abundant levels of FGFR2, similar to normal brain. Anaplastic astrocytomas expressed variable but higher levels of FGFR2 mRNA than those observed in glioblastomas. The concomitant loss of FGFR2 and gain of FGFR1 expression demonstrated in glioblastoma samples was also observed in three human gliomblastoma cell

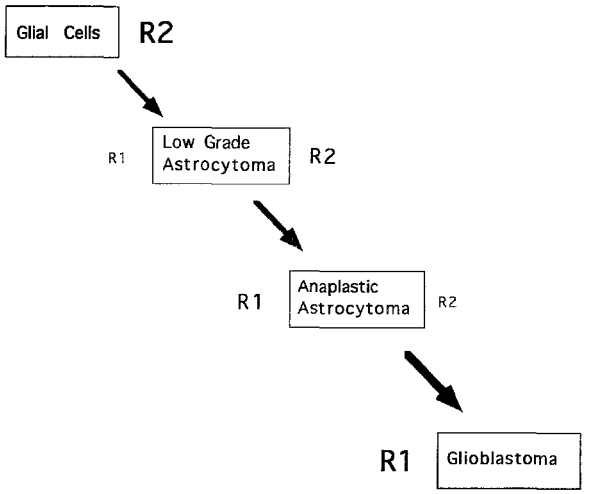

Fig. 3. Differential expression of FGFR1 and FGFR2 in astrocytomas and normal human brain. This schematic summarizes the changes that are detected in FGFR1 and FGFR2 during the malignant progression of astrocytic tumors. FGFR2 is the predominant mRNA detected in normal white matter glial cells while FGFR1 is poorly expressed or not detectable. In addition, FGFR1 immunoreactivity is not detected in normal white matter astrocytes. Cultured rat astrocytes express almost exclusively FGFR2 consistent with its abundant expression in white matter samples. However, malignant astrocytomas express significantly elevated levels of FGFR1 and by the time they progress to glioblastoma FGFR2 expression is low to non-detectable.

lines in culture, suggesting that the loss and gain of FGFR2 and FGFR1, respectively, is associated with malignant progression in astrocytomas from low-grade to high-grade tumors.

Our studies have demonstrated that malignant astrocytomas exhibit several significant alterations in FGFR expression. Analysis of normal white matter specimens, normal brain adjacent to the malignant tumor and low-grade astrocytomas suggest that FGFR2 is the principal FGF receptor in astrocytes as recently proposed by Lai and Lemke [57] and Peters *et al.* [58]. In contrast, FGFR1 appears to be poorly expressed in normal astrocytes, but is abundantly expressed in neurons as shown by recent *in situ* hybridization studies [58–61]. Therefore, a major finding of this work is the observation that astrocytes fail to express FGFR2 when transformed to high-grade malignancy. The mechanism underlying the loss of FGFR2 expression in glioblastomas is not known. FGFR2 has been localized to the long arm of chromosome 10 (q. 26) [62]. Approximately 80% of all glioblastomas exhibit a loss of heterozygosity for 10q [63]. The loss of chromosome 10, which is one of the most frequent genetic abnormalities occurring in glioblastomas, has not been associated with lower-grade astrocytic tumors [64]. While most glioblastomas lose an entire copy of chromosome 10, a subgroup has been shown to exhibit partial loss of chromosome 10. The common region of chromosome 10 deletion extends from 10q24 to 10q26, a region encompassing FGFR2. Although it is not known if FGFR2 is an imprinted gene, it is conceivable that one copy of FGFR2 is lost during the astrocyte transformation process leaving behind a copy that is transcriptionally inactive. Alternatively, both copies of the FGFR2 gene may be present in glioblastomas, but silent following translocation to another chromosome.

In addition to exhibiting a loss of FGFR2 expression, glioblastomas appear to overexpress an alternatively spliced form of the FGFR1 gene [74]. When present in normal brain, the α-form of FGFR1 seems to predominate over the β-form. In this regard the brain is unique from other tissues which express nearly equal levels of the α and β forms of FGFR1 [56]. When the FGFR1 gene is activated or upregulated in glioblastomas there is a significant shift in the splicing pattern of the gene. The β-form represents the predominant transcript in glioblastomas, whereas the α-form is predominant in low-grade astrocytic tumors. The shift from α to β correlates with the grade of malignancy in astrocytomas and may eventually provide a useful prognostic indicator for astrocyte-derived tumors.

The presence of FGFR1 in low-grade (LGA) astrocytomas relative to the lack of FGFR1 in normal white matter suggests that increased FGFR1 expression may be an early event in the genesis of astrocytomas. However, the change in alternative splicing is clearly a late event in the development of astrocytic tumors and may be coupled to the loss of FGFR2 or other genes on chromosome 10.

The functional consequence of a shift in alternative RNA splicing from the α-form (three IgG-like disulfide loops) to the β (two IgG-like disulfide loops) of the FGFR is not understood. The first IgG-like disulfide loop encoded by the α exon is not essential for binding FGF [28]. The FGF binding

domain has recently been localized to the second and third lgG-like disulfide loops [28, 30]. Early transfection studies using vectors encoding either the α form or β form of the receptor indicated that both forms bound acidic or basic FGF equally well. However, using insect cells which do not express endogenous FGFR, FGFR1β has recently been shown to exhibit a 10 fold greater affinity for acidic and basic FGF than FGFR1α [32]. If this is true, then glioma cells expressing FGFR1β would be more responsive to FGF than non-transformed cells or low-grade astrocytoma cells expressing primarily FGFR1α. These types of structural differences may impart a growth advantage to glioma cells expressing FGFR1β.

Inactivation of the p53 gene dramatically increases astrocyte proliferation

Human astrocytomas are associated with several cytogenetic changes affecting loci located on chromosomes 9, 10, and 17 [65]. Among the consistent changes exhibited by astrocytomas is a loss of chromosome 17p. This is commonly correlated with a loss or mutation in the remaining wild type (Wt) p53 allele [66]. Although the exact percentage of astrocytomas expressing p53 abnormalities varies from study to study, alterations in the p53 gene are common in astrocyte-derived tumors. In fact, the histological progression of human astrocytomas from low-grade to high-grade tumors has been associated with clonal expansion of cells that had previously acquired a mutation in the p53 gene [67, 68]. Cells expressing the p53 mutation were presumably able to expand because they acquired a selective growth advantage over normal cells. Furthermore, induced expression of Wt p53 suppressed glioma cell growth [69] implying that the p53 gene product may directly or indirectly regulate the expression and activity of growth-promoting genes. Thus, while p53 abnormalities may not be observed in all glial tumors they are clearly related to malignant transformation in a subset of these tumors.

The mechanism by which p53 genetic lesions promote transformation has not been elucidated. The loss of p53 has been associated with increased genetic instability and a tendency toward increased aneuploidy [70, 72]. Genetic alterations subsequent to the loss of p53 may result in the activation of growth promoting genes or loss of additional growth suppressive genes. Consistent with this idea, we have recently observed that Wt p53 transfection of SNB-19 glioblastoma cells resulted in a 7 fold reduction in bFGF protein levels [Morrison *et al.*, in preparation]. This was correlated with significant growth suppression of SNB-19 cells in monolayer culture.

In order to further evaluate the relationship between p53 gene expression and astrocyte transformation we have begun to examine astrocyte cultures derived from mouse brains containing two wild-type copies (+/+) or two inactivated copies of the p53 gene (–/–) [73]. Initial results with this model suggest that the loss of p53 confers a remarkable growth advantage on astrocytes. Astrocytes with two inactivated copies of the p53 gene (–/–) are immortalized and ultimately reach saturation densities 30 times greater than +/+ astrocytes [Yahanda *et al.*, in preparation]. The –/– astrocytes also express significant alterations in FGFR expression characteristic of malignant astrocytomas cells.

In summary, malignant transformation of astrocytes appears to be associated with increased expression of bFGF, loss of FGFR2 and overexpression of an alternatively spliced form of FGFR1. The reciprocal gain and loss of FGFR1 and FGFR2 respectively, are closely associated with malignant progression in astrocyte-derived tumors and many eventually serve as useful diagnostic and prognostic indicators. A further understanding of the mechanisms that regulate bFGF and FGFR expression in malignant astrocytomas may shed light on the transformation process in astrocytes.

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Address for offprints: R.S. Morrison, Department of Neurosurgery (Box 064), U.T.M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA