REVIEW ARTICLE

Ras Activation in Astrocytomas and Neurofibromas

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ABSTRACT: Oncogenic mutations resulting in activated Ras Guanosine Triphosphate (GTP) are prevalent in 30% of all human cancers, but not primary nervous system tumors. Several growth factors/receptors are implicated in the pathogenesis of malignant astrocytomas including epidermal growth factor (EGFR) and platelet derived growth factor (PDGF-R) receptors, plus the highly potent and specific angiogenic vascular endothelial growth factor (VEGF). A significant proportion of these tumors also express a truncated EGFR, which is constitutively activated. Our work demonstrates that the mitogenic signals from both the normal PDGF-R and EGFR and the truncated EGFR activate Ras. Inhibition of Ras by genetic or pharmacological strategies leads to decreased astrocytoma tumorgenic growth *in vitro* and decreased expression of VEGF. This suggests that these agents may be potentially important as novel anti-proliferative and anti-angiogenic therapies for human malignant astrocytomas.

In contrast to astrocytomas, where increased levels of activated Ras GTP results from transmitted signals from activated growth factor receptors, the loss of neurofibromin is postulated to lead to functional up-regulation of the Ras pathway in neurofibromatosis-1(NF-1). We have demonstrated that NF-1 neurofibromas and neurogenic sarcomas, compared to non-NF-1 Schwannomas, have markedly elevated levels of activated Ras GTP. Increased Ras GTP was associated with increased tumor vascularity in the NF-1 neurogenic sarcomas, perhaps related to increased VEGF secretion. The role of Ras inhibitors as potential therapy in this tumor is also under study.

RÉSUMÉ: Activation de Ras dans les astrocytomes et les neurofibromes (médaille du Collège Royal en Chirurgie, 1997). Les mutations d'oncogènes provoquant une activation de Ras-GTP ont une prévalence de 30% dans tous les cancers humains, mais non dans les tumeurs primitives du système nerveux. Plusieurs facteurs de croissance / récepteurs sont impliqués dans la pathogenèse des astrocytomes malins dont le récepteur du facteur de croissance épidermique (EGF-R) et celui du facteur de croissance plaquettaire (PDGF-R), ainsi que le facteur de croissance endothélial vasculaire (VEGF), un facteur de croissance très puissant et hautement spécifique. Une grande proportion des astrocytomes malins expriment un EGF-R tronqué qui est activé constitutivement. Nos travaux démontrent que les signaux mitogènes du PDGF-R normal et du EGFR normal et du EGFR tronqué activent Ras. L'inhibition de Ras par des stratégies génétiques ou pharmacologiques provoque la diminution de la croissance tumorale astrocytaire *in vitro* et diminue l'expression du VEGF. Ceci indique que ces agents pourraient être importants comme traitements antiprolifératifs et antiangiogéniques dans les astrocytomes malins humains. Contrairement aux astrocytomes où des niveaux augmentés de Ras.GTP activé résultent de signaux transmis provenant de récepteurs de facteurs de croissance activés, on pense que la perte de la neurofibromine amène une régulation fonctionnelle à la hausse de la voie Ras dans la neurofibromatose I (NFI). Nous avons démontré que les neurofibromes de la NFI et les sarcomes neurogéniques non-NFI ont des niveaux très élevés de Ras.GTP activé comparés aux Schwannomes. Un Ras.GTP augmenté était associé à une vascularité tumorale augmentée dans les sarcomes neurogéniques de la NFI, possiblement en relation avec une augmentation de la sécrétion du VEGF. Le rôle des inhibiteurs de Ras en tant que thérapie dans cette tumeur est aussi à l'étude présentement.

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The Clinical Problem

Astrocytomas: Astrocytomas are the most prevalent primary intracranial neoplasm, accounting for about 4-5% of all cancer related deaths (approx. 20,000 deaths/year in North America).[1] Astrocytomas are classified by the World Health Organization[2,3] into four increasing grades of malignancy; Grade 1: pilocytic astrocytomas found in children which usually have an indolent course, Grade 2: astrocytoma (low grade), Grade 3: anaplastic astrocytoma (AA) and Grade 4: glioblastoma multiforme (GBM). This histo-pathological classification is somewhat arbritary, as low grade adult astrocytomas (Grade 1, 2) will inevitably progress to a more malignant grade (Grade 3, 4). At the present time, there is no antigenic or molecular marker which distinguishes an astrocytoma cell from a normal astrocyte

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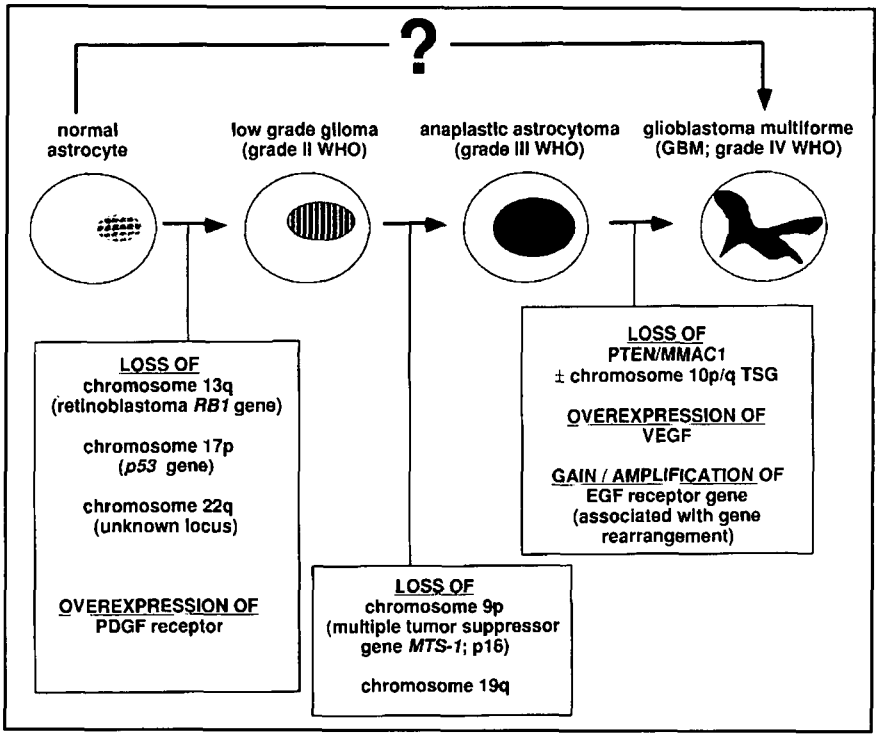

Figure 1: *The molecular pathogenesis of human astrocytomas is depicted, with their origin from normal astrocytes. The progression from a grade III (anaplastic astrocytoma) to a highly-malignant glioblastoma multiforme is characterized by loss of PTEN/MMAC and other putative tumor suppressor genes on chromosome 10 and amplification of the EGF-R gene. The question mark (?) refers to those patients who present at initio with glioblastoma multiforme; whether these tumors progress through each step of this progression (either very rapidly or in a subclinical state) or skip various stages is not currently known.*

or between a low and higher grade tumor cell. This often leads to misdiagnosis especially when small specimens are obtained, such as by stereotactic biopsies. Acknowledging the potential difficulties in classification, the W.H.O. grading is nevertheless an important survival prognosticator.

Anaplastic astrocytomas and GBMs are by far the most common type of astrocytomas seen in adults, with fifty percent of the patients dying at 18 (AA) and 12 (GBM) months post-diagnosis.[3] This morbid statistic includes patients who have received radiation therapy, which shifts the survival curve favorably only by a few months. Aggressive gross neurosurgical removal, variations in dose and delivery of radiotherapy, chemotherapy, and other adjuvant therapy has had little impact on survival over the last thirty years in management of malignant astrocytomas.

Neurofibromatosis-1: Neurofibromatosis-1(NF-1) and 2, collectively known as von-Recklinghausen's Syndrome[4] due to their partial clinical overlap, are two distinct diseases based on loss of function of two different genes. The hallmark of NF-1 is the presence of multiple benign peripheral (cutaneous) neurofibromas consisting of a mixture of Schwann cells, fibroblasts, and mast cells.[5] Other clinical features include café au lait (CAL) spots, freckling of the axilla, groin and other intertriginous areas and pigmented hamartomas of melanocytic origin in the iris known as Lisch nodules. While these are the most commonly noted signs in NF-1, the disease is characterized by a variable number of diverse pathologies throughout the body, including cutaneous, osseous, hematological, developmental, and nervous system abnormalities.[6] Despite recent advances in our understanding of the molecular basis of NF-1, these clinical criteria continue to be the most reliable means for making the diagnosis.[7]

The two major life-threatening complications of NF-1 are hypertension and a significantly higher rate of malignancy.[8,9] In a 12 year Swedish study, 22 of 70 NF-1 adults being followed died (10-hypertension and 12-malignancies), a rate four times that of the general population. Malignancies noted at a higher frequency in NF-1 patients include pheochromocytomas, astrocytomas (most notably optic gliomas), chronic myeloid leukemias of childhood, and malignant peripheral nerve sheath tumors which arise following malignant transformation of non-cutaneous, more deeply-located plexiform neurofibromas.[10,11] Despite this, the diagnosis of NF-1 is usually not life threatening, with the majority of the patients surviving well into their adulthood, with a mean survival age of 61.2 years in the Swedish study.[8]

Review of Current Molecular Understanding:

Astrocytomas: As schematized in Figure1, a molecular pathogenic description of the progression of astrocytomas is slowly evolving and involves both tumor suppressor genes (TSG) and oncogenes. Most patients present with a malignant astrocytoma (AA, GBM), suggesting that a normal astrocyte can be directly transformed to a malignant astrocyte. Another plausible explanation is that in these patients the lower grades of astrocytomas were not detected as they were subclinical. Karyotypic analysis of malignant astrocytomas demonstrate that most tumor cells are diploid (2N), although they have gross chromosomal abnormalities including total or partial deletions, duplications,

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translocations and amplifications.[12] Mutations have been identified in the p53 tumor suppressor gene on chromosome 17p in low grade astrocytomas, suggesting that this may be an early pathogenic event.[13] Furthermore, there is clonal expansion of the p53 mutated cells (perhaps due to selective growth advantage) as the tumor progress to a more malignant grade.[14] Loss of entire or parts of chromosome 10 is exclusively found in GBMs[15-17] and not in lower grade astrocytomas. Recently a dual specific phosphatase, termed PTEN/MMAC has been identified as a TSG on chromosome 10 associated with GBM's and several other tumors including breast and prostate cancers.[18-20] Other yet to be identified TSG(s) on chromosome 10 lost in GBMs are probably present. The multiple tumor suppressor (MTS1) gene, encoding for the cell cycle inhibitor p16, may also be relevant in progression to a malignant astrocytoma.[21,22] Loss of heterozygosity on 19q may be related to progression of astrocytomas to a higher grade.[23]

Epidermal Growth Factor Receptor (EGFR): The proto-oncogene c-erbB which encodes for the EGFR is located on chromosome 7 and amplified in about 50% of GBMs and found at much lower levels in low grade astrocytomas.[24,25] Established human astrocytoma cultures do not retain the DNA amplification, though they still may overexpress EGFR at the protein level. In those GBMs with amplified EGFR, 25-40% express both normal (170kDa) and a truncated EGFR (140^{EGFR}) which is unable to bind EGF or TGF-α, due to a 801 base pair deletion (exons 2-7; residue 6-273) in its N-terminal extracellular domain.[26-28] There is recent evidence that the 140^{EGFR} is constitutively activated and enhances *in-vivo* growth of transfected malignant astrocytoma cells in animal models.[29] The signal transduction mechanism(s) that the 140^{EGFR} utilizes are unknown but may include activation of the Ras pathway.

Platelet Derived Growth Factor Receptor (PDGF-R): The majority of established malignant astrocytoma cell lines overexpress a combination of PDGF ligand and receptor genes which could, in principle, form an autocrine/paracrine loop.[30] Amplification or rearrangements of the PDGF subunits (A, B) or PDGF-R (α,β) genes are not found in the majority of astrocytoma specimens.[31,32] However, overexpression of the PDGF-αR as determined by western analysis was demonstrated in 24% of the GBMs examined. Expression of both PDGF-A & B subunits are increased in malignant astrocytomas (AA, GBMs), compared to lower grade astrocytomas. PDGF-αR was overexpressed in all astrocytoma grades compared to non-neoplastic glia.[33,34] This suggests that PDGF-αR expression, which is capable of binding both PDGF subunits, may be an early event in transformation of a normal astrocyte to an astrocytoma cell, and that subsequent progression to a malignant astrocytoma cell may depend on increased expression of PDGF ligands and stimulation of the astrocytoma cell by autocrine/paracrine mechanisms. In another study, we documented that the increased expression of PDGF and PDGF-αR in sporadic malignant astrocytomas, were also prevalent in malignant astrocytomas associated with patients with germline p53 mutations or the Li-Fraumeni syndrome.[35] Expression of PDGF and PDGF-Rs is postulated to be functionally relevant in the growth of astrocytomas. To test this hypothesis we created PDGF dominant-negative mutants by site-directed mutagenesis of the mouse PDGF-A cDNA.[36,37] We found that growth of human established astrocytoma cells was decreased both *in-vitro* and *in-vivo*, by expression of the PDGF dominant-negative mutants blocking activation of the overexpressed PDGF-Rs.

Vascular Endothelial Growth Factor (VEGF): Angiogenesis is dynamically regulated with both positive and negative endogenous factors, with VEGF being the most potent and specific endothelial cell mitogen.[38-45] VEGF was initially isolated as a 40-46kDa protein from tumor fluid, which was 10,000-50,000 times as potent as histamine in increasing vascular permeability.[46-48] Subsequently, a highly potent and specific endothelial mitogen was identified from bovine pituitary follicular stellate cells,[49] which was found to be identical to the previously isolated permeability factor.[50] VEGF is a dimeric growth factor and is highly secreted due to a N-terminal signal peptide.[50] Four isoforms of VEGF (121, 165, 189 and 205 amino acids) have been identified as a result of alternate splicing, with $VEGF^{165}$ being predominant.[51,52] Hypoxia is a strong transcriptional stimulant of VEGF, as are other mitogenic growth factors.[53-57] Recent evidence suggests that both mitogenic signals and angiogenic signals (via induction of VEGF) share a common link by activation of the Ras signaling pathway,[58,59] and hence inhibition of Ras activity may lead to control of both tumor cell and tumor angiogenic growth.

The two primary biological functions of VEGF (endothelial cell mitogen and endothelial cell permeability) are mediated through two high affinity protein receptor tyrosine kinases, Flt-1 and Flk-1 (human counterpart of the latter known as KDR).[60-65] Expression of these receptors only on vascular endothelial cells accounts for the main biological function of VEGF, as it can induce the entire sequence of angiogenesis,[49,50,66] and makes VEGF the most potent of all known angiogenic factors, in contradistinction to other non-specific angiogenic growth factors. In addition to acting as a potent endothelial cell mitogen, VEGF also induces the endothelial cells to express various factors such as proteases, collagenases, urokinase and tissue plasminogen activators, which are all involved in turning on the "angiogenic switch" thereby promoting angiogenesis and metastasis.[67,68]

In human astrocytomas, one of the main pathological criteria for grading the degree of malignancy and hence the prognosis is tumor vascularity.[2,3,69] Whether tumor angiogenesis is similarly correlated to malignant potential of peripheral nerve tumors in both NF-1 and non NF-1 patients is presently unknown, although some evidence exists that plexiform neurofibromas and neurogenic sarcomas can be highly angiogenic.[70,71] In contrast to low grade astrocytomas, malignant astrocytoma cells express increased levels of VEGF, especially around the hypoxic peri-necrotic zones, with increased expression of Flk-1/KDR in the hyperproliferative vascular endothelium.[50,54,72-75] In addition to astrocytomas, we have demonstrated a correlationship between tumor vascularity, peri-tumoral edema and VEGF expression in meningiomas,[76] the second most common adult CNS tumor. Anti-angiogenic therapeutic strategies including VEGF neutralizing antibodies,[74,77-79] antisense constructs,[80] inhibitory mutants against the ligand or receptor (Flk-1/KDR dominant negative mutant)[81] have demonstrated some efficacy in animal models of astrocytomas. Pharmacologic therapies such as tyrophostins and other small molecules directed at blocking Flk1/KDR receptor activation[82] are under current study. In the future signaling pathways such as Ras activation

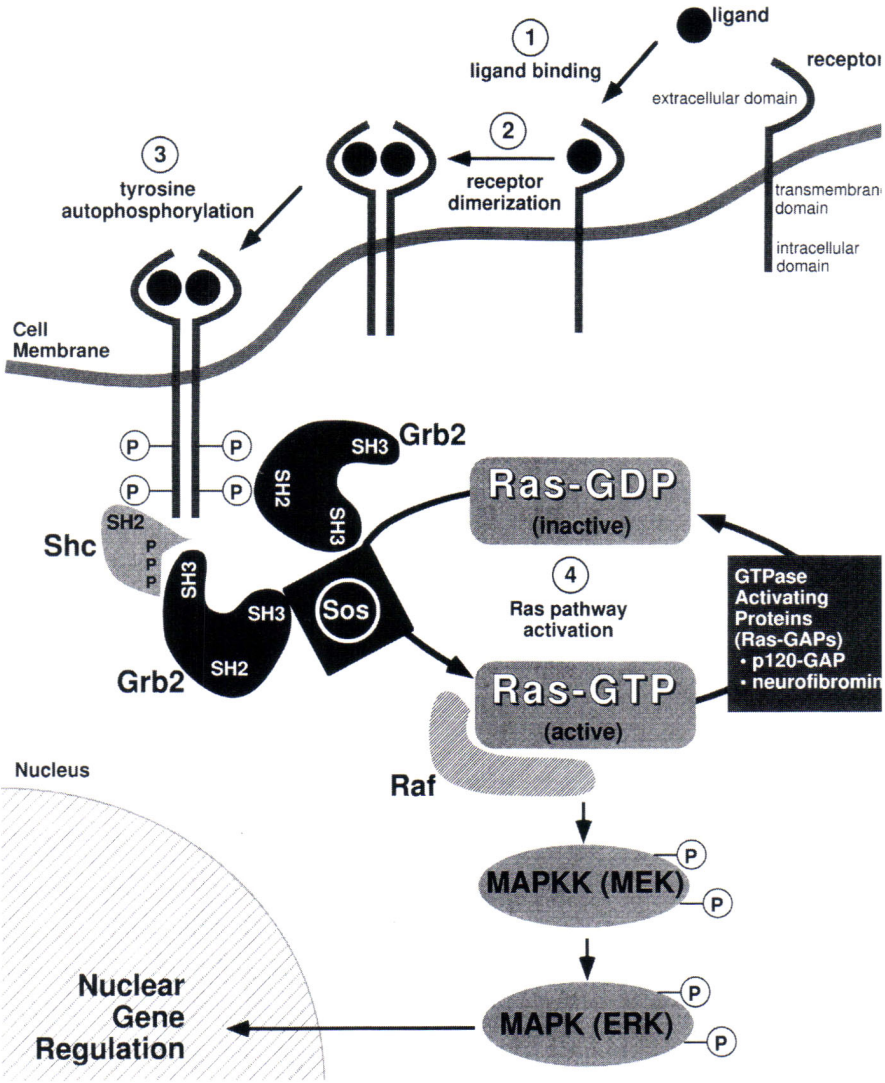

Figure 2: *Schematic representation of the Ras-Raf-MAPK mitogenic signaling pathway. This cascade is activated in normal cells when ligand (a growth factor such as platelet-derived growth factor) binds its cognate surface receptor* (STEP 1). *Ligand:receptor interaction results in receptor dimerization* (STEP 2), *resulting in transautophosphorylation of tyrosine residues on the intracellular domain of the receptor* (STEP 3). *This allows signaling molecules with SH2 domains (Shc, Grb2 or the Shc:Grb2 complex) to interact with the phosphotyrosine residues, bringing the nucleotide exchange factor Sos in proximity to the cell surface, where it exchanges GDP with GTP, activating Ras. Raf interacts with activated Ras•GTP, phosphorylating MAPKK, which subsequently phosphorylates MAPK. MAPK translocates to the nucleus where it participates with other molecules in activating the transcription of the transcription factors fos and jun, resulting in the increased transcription of genes involved in cell division and other functions* (STEP 4).

which up-regulate VEGF expression, and those pathways involved in transmitting signals from activated VEGF receptors in endothelial cells may be of therapeutic value.

Neurofibromatosis-1: The *NF1* gene, located on the pericentromeric region of chromosome 17, is extremely large,[83] with a variety of mutations identified in NF-1 patients.[84,85] However, no specific mutational hotspots or significant genotype-phenotype correlationship, where one can predict the clinical presentation based on the location and type of mutation, have been identified.[84] The lack of mutational hot spots, the large size of the *NF1* gene, high spontaneous mutation rate and lack of genotype-phenotype correlationship, have all contributed to the hurdles which still make routine genetic screening of NF-1 patients impractical. The NIH clinical diagnostic criteria,[86] remains the best method of detecting new patients with NF-1.

Homology screening of neurofibromin, the protein product of the *NF1* gene, offered clues as to its function. A small region in the central portion of neurofibromin demonstrated 30% homology with the mammalian p120-GAP, and with the *Saccharomyces cerevisiae* genes *Ira1* and *Ira2* (inhibitory regulators of the *Ras*-cAMP pathway in yeast).[87,88] This region has been named the *G*AP-*R*elated *D*omain (GRD), and has identified neurofibromin as a member of the Ras-GAP family, of which there are currently four mammalian members, all of which are the key negative regulators of the signal transduction protein Ras.[10,88-94] In NF-1 peripheral nerve tumors it is hypothesized that decreased levels of neurofibromin leads to increased Ras.GTP, with subsequent aberrant mitogenic signals leading to tumor formation. In support of this hypothesis, neurofibrosarcoma cell lines established from NF-1 patients not only lacked neurofibromin

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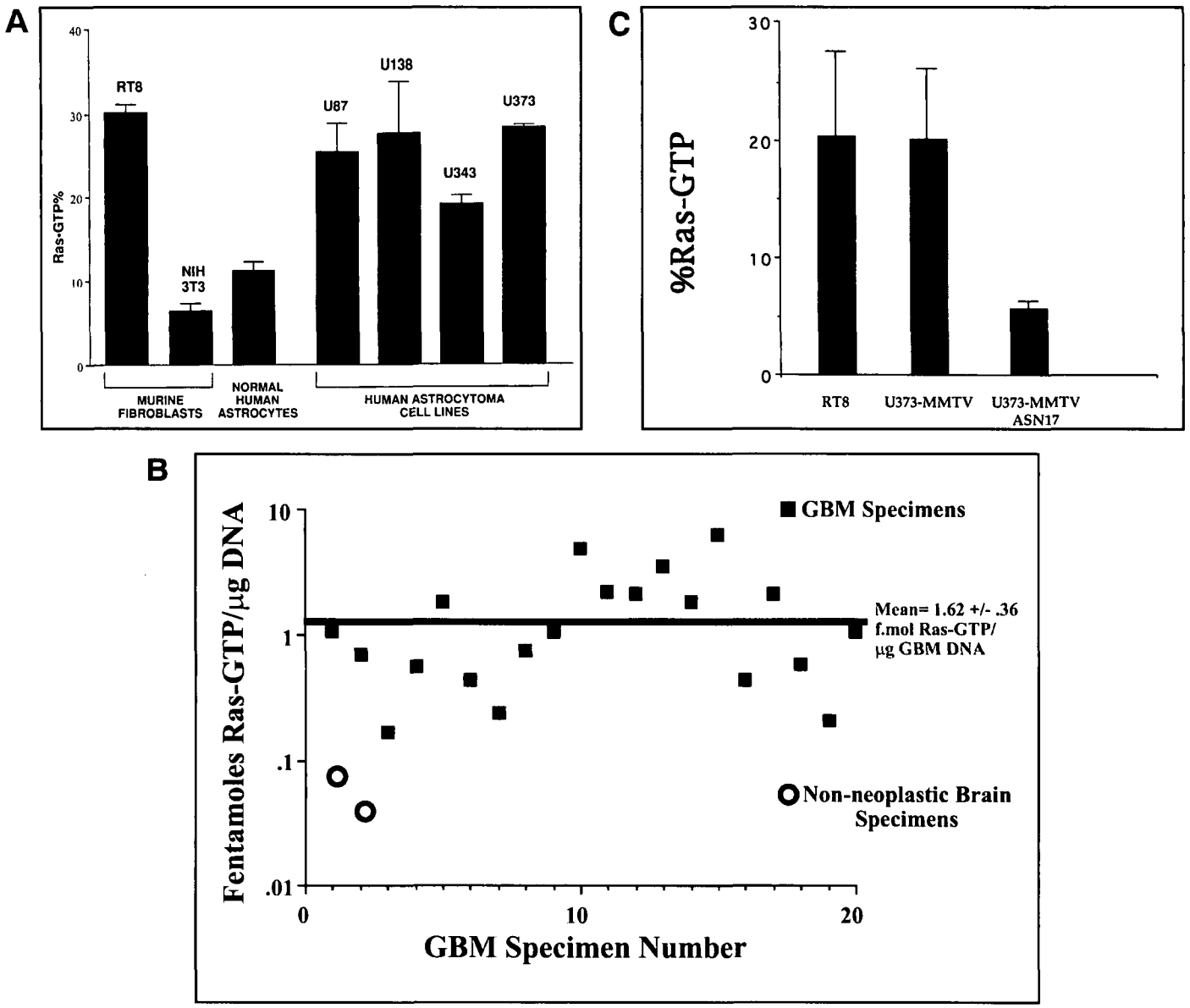

Figure 3A: *Levels of activated Ras.GTP in parental human malignant astrocytoma cell lines (U373, U343, U138, U87) and in control mouse fibroblast cells (NIH-3T3) and in mouse fibroblast cells transformed with* v-Ha-Ras *(RT8). Levels of activated Ras.GTP were determined using the* ^{32}*P-Ras loading assay, and are expressed as a percentage of total cellular %Ras (Ras.GTP/Ras.GDP+Ras.GTP). The human malignant astrocytoma cell lines have levels of activated Ras.GTP which are similar to the transformed RT8 cells and much higher than non-transformed 3T3 cells, though they do not harbor oncogenic mutations of Ras.* ***3B:*** *Femtomoles Ras-GTP normalized to µG DNA extracted from 20 human malignant astrocytoma (GBM) specimens measured by the enzymatic assay. The amount of Ras.GTP, hence Ras activity, is markedly elevated in the GBM specimens, compared to the two normal brain specimens.* ***3C:*** *The mean +/- SEM of four* ^{32}*P-Ras loading assays demonstrates that the U373 human malignant astrocytoma cell line stably transfected with the MMTV-Asn17 Ras inhibitory mutant has decreased Ras.GTP levels compared to MMTV transfected U373 cells.* v-Ha-ras *transformed RT8 fibroblasts serve as positive controls.*

expression but have elevated levels of activated Ras.GTP.[95,96] Furthermore, the elevated levels of Ras.GTP directly contributed to mitogenesis, since cellular proliferation could be effectively blocked by microinjecting neutralizing Ras antibody into these cells,[95,96] or by inhibiting Ras activation by farnesyl transferase inhibitors (FTI).[97] We asked whether aberrant activation of the Ras signaling pathway was also present in peripheral nerve tumor specimens, by adapting an enzymatic assay which allows for quantitative determination of Ras.GTP and Ras.GDP in tissues.[10,98]

Ras-mediated Signal Transduction:

The three human *Ras* genes which code for four 21kDa proteins (Ha,N,K4A,K4B) belong to the small G protein family, and comprise important intracellular signal transduction molecules. Oncogenic activating mutations in Ras (residues 12,13,61), prevent conversion of activated GTP bound Ras to the basal inactive GDP bound Ras by *G*TP'ase *A*ctivating *P*roteins (i.e., $_{p120}$GAP & neurofibromin). The importance of Ras in cellular control is exemplified by the fact that 30% of human cancers, but not those primarily from the nervous system, have

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oncogenic activating mutations.[92,99,100] Activation of Ras is pivotal in transmitting proliferative, differentiating or transforming signals from a variety of activated growth factor receptor tyrosine kinases (RTKs) such as EGFR and PDGF-R.[101-105] These responses are blocked by micro-injection of neutralizing Ras antibody (Y13-259) into the growth factor stimulated cells, or by over expression of the Ha-Ras-Asn17 dominant inhibitory mutant. This Ha-Ras-Asn17 mutant is unable to effectively bind Mg^{+2}, thereby decreasing its affinity to both GDP and GTP but increasing its affinity towards nucleotide exchange factors (see below), which are required for the activation of Ras. By depleting the availability of these nucleotide exchange factors, normal endogenous Ras cannot be activated to Ras.GTP effectively blocking this signaling pathway, resulting in the dominant inhibitory function of the mutant.[106-112]

Activation by receptors: The discovery of protein modules, such as SH2 (src homology-2), SH3 (src homology-3), PTB (phosphotyrosine binding domain) and PH (pleckstrin homology) domains, have led to elucidation of many of the protein-protein interactions involved in signal transduction, including the upstream regulators and downstream substrates of the Ras pathway, Figure 2.[113-118] Ras activation requires post-translational addition of hydrophobic isoprenyl groups to its C-terminal containing CAAX box, mediated by farnesyl transferase, allowing Ras to bind to the inner cell membrane.[119-121] Exchange of GDP for GTP can then occur by nucleotide exchange factors, such as mSos (*mammalian homologue of the son of sevenless gene product identified as a Ras activator in D. melanogaster.*)[122] mSos is thought to be always bound to Grb-2, which is an adaptor protein containing a SH2 and two SH3 domains, by its proline rich regions interacting with SH3 binding sites on Grb-2. This Grb-2:Sos complex is brought to the cell membrane near Ras.GDP by binding of the Grb-2 SH2 domain to specific phosphorylated tyrosine(**Y**) residues on activated RTKs either directly or indirectly. Specificity for SH2 domains is conferred by the 3-4 amino acids C-terminal to the phosphotyrosine binding site (**Y**XXX);[118] Indirectly, the Grb-2:Sos complex can bind activated RTKs through another SH2 and PTB containing adapter protein called Shc, which has transforming properties itself, Figure 2.[123-127]

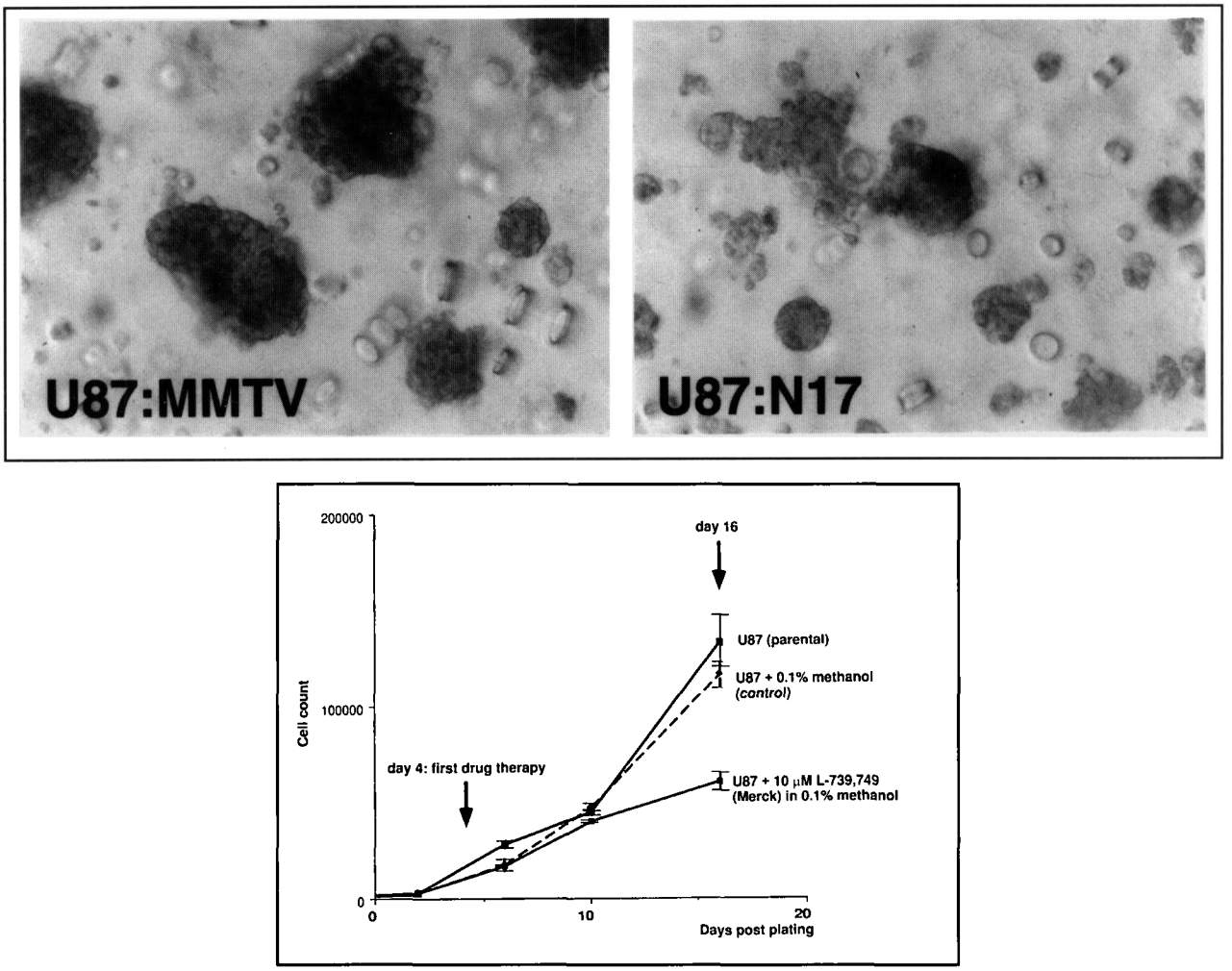

Figure 4A: *Anchorage-independent growth inhibition of the U87 astrocytoma cells in a soft agar methylcellulose assay, due to decreased Ras activity by the Ha-Ras-Asn17 dominant negative mutant (40X).* ***4B:*** *Mean +/- SEM of U87 parental cells, U87 cells treated with vector alone (0.1% methanol), and U87 cells treated with 10 μM L-739,749 (methanol concentration 0.1%). All cells were maintained in 10% DMEM + 10% calf serum. 4000 cells were plated on day 0, and drug therapy began on day 4. Drug and medium were replenished every 4 days. Cell counts on day 16 demonstrate a significant proliferation inhibition ($p < .05$:paired t-tests) of L-739,749 on U87 cells.*

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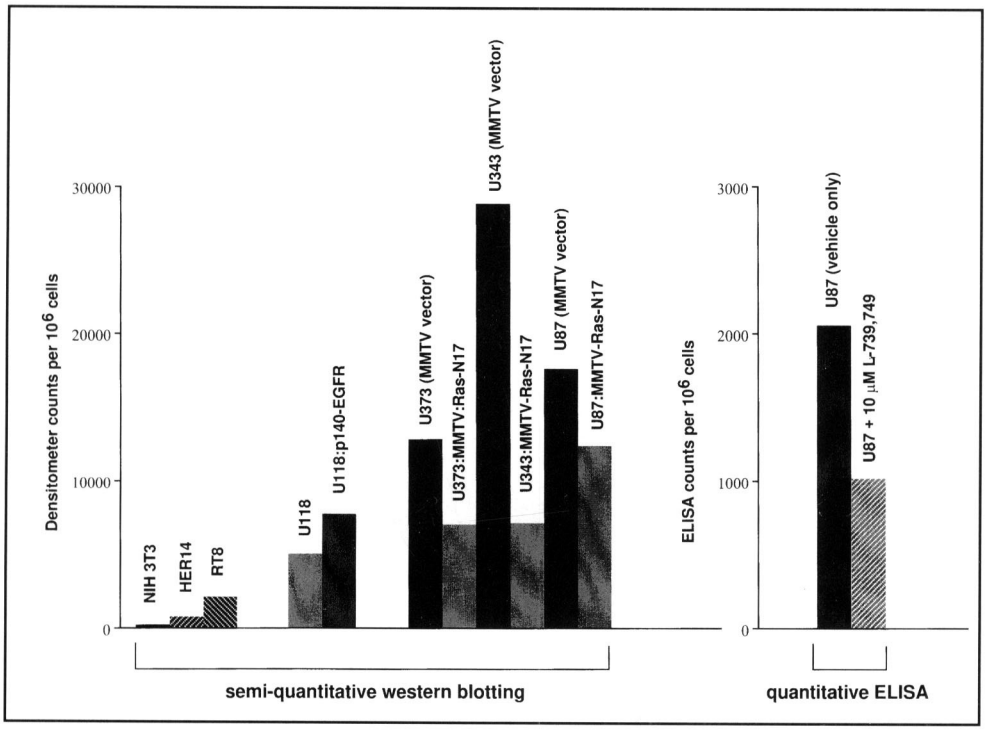

Figure 5: Secretion of VEGF by human malignant astrocytomas, and inter-relationship with Ras activity. Western immunoblot analysis of conditioned media of the cell lines with polyclonal anti-VEGF that recognizes all four isoforms, with protein levels normalized to cell numbers. U87 secrete large amounts of VEGF which is decreased by inhibiting the Ras pathway with the Ha-Ras-Asn17 mutant. Similar decrease in VEGF secretion compared to vehicle controls, is observed by treating the U87 and to a lesser degree the U373 cells with the FTI, L739,749, to pharmacologically inhibit the Ras pathway. Of ongoing interest is the increased VEGF secretion due to expression of the mutant $p140^{EGFR}$ (U118 vs. U118:EGFRv111 lanes). The mutant $p140^{EGFR}$ expressed by a large percentage of GBMs, has increased basal Ras activity due to constitutive activation, and provides in-vitro *and* in-vivo *growth advantage, the latter perhaps related to increased VEGF secretion.*

Once mSos exchanges Ras bound GDP for GTP, activated Ras.GTP can associate with several downstream effectors. These effectors include neurofibromin (a major negative regulator of Ras activity as described below), but in particular the main mitogenic cascade through Raf, MAPKK (mitogen-activated protein kinase kinase, also known as MEK or ERK kinase), and MAPK (mitogen-activated protein kinase, also known as extracellular signal related kinase or ERK) (Figure 2).

Inactivation: Inactivation of Ras.GTP to Ras.GDP requires binding of the family of enzymes called GAPs (*G*TP'ase *A*ctivating *P*rotein). The two main mammalian Ras-GAPs are $_{p120}$GAP and neurofibromin (the gene product of NF-1).[88-91,94,128-133] Decreased levels of these Ras-GAPs could in theory lead to elevated levels of active Ras.GTP, the presence of which has been documented in neurofibrosarcoma cells lacking neurofibromin.[95,96] Using an enzymatic assay that we have developed, that allows for the first time measurement of levels of GDP and GTP bound to Ras in tissues quantitatively, we have demonstrated that levels of Ras.GTP are increased in NF-1 peripheral nerve tumors compared to other non NF-1 tumors.[10] Which Ras-GAP is predominantly utilized by a particular cell type in regulation of Ras is an area of ongoing research. Recent evidence suggests that neurofibromin (ubiquitously expressed) may be the most important Ras-GAP in basal regulation of Ras.GTP levels, while $_{p120}$GAP (containing an SH2 domain capable of interacting with RTKs) may play a dominant role when Ras activation is stimulated by activated RTKs (personal communication -Tony Pawson).

Like other tumor suppressor genes, mutations in the NF-1 gene exist in many sporadic tumors including astrocytomas.[134] We have examined low and high grade astrocytoma specimens for neurofibromin expression using RT-PCR and Western immunoblot analysis.[135] Contrary to what one may predict, we have found that neurofibromin levels are elevated in the more malignant astrocytomas. We hypothesized that this may reflect increased Ras.GTP levels in these malignant astrocytomas as suggested by our current work, with neurofibromin levels being increased secondarily by the tumor cells in an attempt to decrease Ras.GTP levels. Elevated levels of neurofibromin (mRNA and protein) in both Ras transformed fibroblasts compared to their normal counterparts, and human malignant astrocytoma cells (high levels of Ras.GTP) compared to those transfected with the Ha-Ras-Asn17 inhibitory mutant (low levels of Ras.GTP), are in agreement with our hypothesis.[135]

Methodology

Inhibition of Ras signaling leads to decreased proliferation and VEGF secretion of human astrocytoma cell lines: Four established human malignant astrocytoma cell lines were transfected with the Ha-Ras-Asn17 dominant inhibitory mutant and also treated with the farnesyl transferase inhibitor (FTI) L-739,749 (Merck Research Laboratories) to inhibit the Ras signaling pathway. The cell lines were characterized for expression and tyrosine phosphorylation of PDGF and EGF receptors,

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	Ras-GTP femtomoles/ μg Tumor DNA	%Ras-GTP/ (Ras-GTP + Ras-GDP)
non-NFI Schwannomas	3.5 +/-0.6	1.3 +/0.3
NF1 Neurofibromas	13.3 +/-.2.6	6.0 +/2.5
NF1 Neurogenic Sarcomas	46.7 +/-7.0	15.4 +/-5.2

Figure 6: Ras activity of peripheral nerve tumors expressed as a percentage of total Ras in the activated Ras.GTP form, or quantitatively as femtomoles of Ras.GTP/μg of tissue DNA analyzed by the enzymatic assay. Compared to non NF-1 Schwannomas, the amount of activated Ras.GTP in NF-1 neurofibromas and NF-1 neurogenic sarcomas were approximately elevated 6X and 15X respectively.

Shc and Grb2 under serum starved and ligand stimulated conditions with Western immunoblot analysis. Ras activity in the cells was measured with the ^{32}P-Ras loading assay, Figure 3A, 3C and in 20 flash frozen human GBM specimens and two normal human brain specimens using the enzymatic assay described in prior publications.[10,98,136] To determine if inhibition of Ras activation resulted in decreased MAPKinase activity, the major mitogenic signal downstream of activated Ras, the mobility shift assay, an activation-specific MAPKinase antibody (New England Biolabs, U.S.A.), and the myelin basic protein kinase assays were used (data not shown). Colony formation and anchorage dependent and independent proliferation assays, as demonstrated with the U87 cell line, were undertaken on the stably derived clones to determine the effects of inhibiting the Ras signaling pathway in human astrocytoma cells, Figure 4A, 4B. VEGF secretion was evaluated by Western-immunoblot analysis (Figure 5) and ELISA (data not shown) assays, on the conditioned media obtained from the parental astrocytoma cell lines or those with inhibition of Ras activity.

Ras-GTP levels and VEGF expression are elevated in malignant NF-1 neurogenic sarcomas: Ras.GTP levels were measured in the flash frozen specimens of both astrocytomas (Figure 3B) and peripheral nerve tumors (Figure 6), using a novel non-radioactive enzymatic assay for quantitative measurement of Ras.GTP.[10,98,136] Expression of NF-1 mRNA (data not shown) was detected using RT-PCR from total RNA obtained from the NF-1 neurogenic sarcoma, NF-1 neurofibromas and non NF-1 schwannomas specimens using primers and techniques as described in a prior publication.[10] Western-immunoblotblot analysis (Figure 7) and immunohistochemistry (data not shown) with an affinity purified antibody made against a trpE:-human neurofibromin fusion protein injected into rabbits[130](generously donated by Dr. N. Ratner, Univ. of Cincinnati), was undertaken on the specimens. Embryonic mouse fibroblasts with homozygous knockout of both *NF1* alleles (*NF1*:-/-; gift of Dr. Tyler Jacks, M.I.T., Boston, Massachusetts), and normal mice (*NF1*:+/+) were used as negative and positive controls, respectively, in the Western blot analysis. VEGF mRNA expression in the NF-1 neurogenic sarcoma cell lines was quantified by Northern blot analysis, Figure 8. Expression of the 4.2Kb VEGF mRNA was determined by hybridization

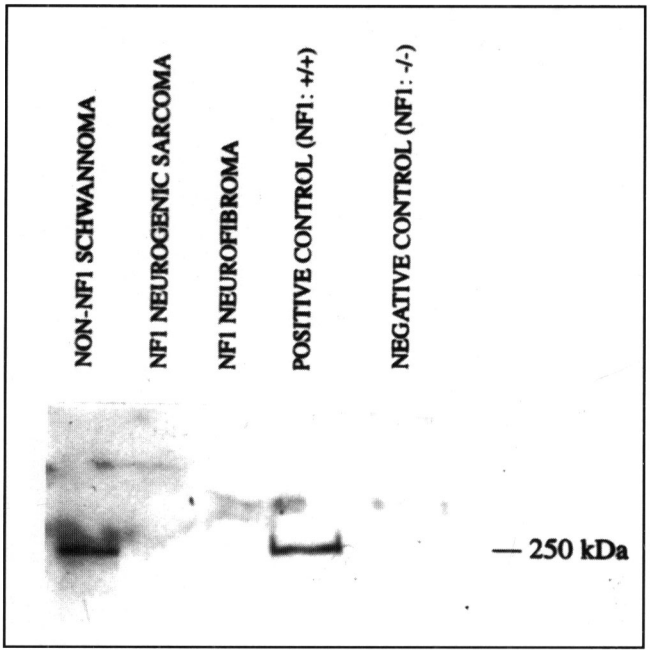

Figure 7: Western immunoblot analysis of tumor lysates for neurofibromin with a polyclonal antibody. Positive and negative controls were NF-1(+/+) and NF-1(-/-) mouse fibroblasts respectively. The non NF-1 Schwannoma expressed abundant neurofibromin, while none were detected in either the NF-1 neurofibroma or neurogenic sarcoma.

of the Northern blot, with a ^{32}P radiolabelled VEGF cDNA probe which contains 204 base pairs of the human VEGF sequence, common for all four isoforms (gift from Dr. B. Berse).

Results

Levels of Ras.GTP are elevated in human malignant astrocytoma cell lines and operative specimens which overexpress PDGFRs and EGFRs: Of the four established human malignant astrocytoma cell lines, PDGF-αR was expressed only by the U373 cells, while the other three expressed variable but increased amounts of PDGF-βR compared to NIH/3T3 cells (data not shown). All the cell lines expressed large amounts of EGFR. Upon activation by exogenous PDGF or EGF the corresponding receptors expressed by the astrocytoma cell lines bound to Shc and Grb2, which are signaling proteins involved in activation of Ras (data not shown).

^{32}P-Ras loading experiments demonstrated that in *v-Ha-ras* transformed fibroblasts (RT8 cells) approximately 30% of the total Ras was in the Ras.GTP bound state, compared to 5% for non-transformed NIH/3T3 cells, Figure 3A. Although human malignant astrocytomas do not harbor oncogenic Ras mutations,[99] 20-30% of the total Ras was in the GTP bound state in each of the four astrocytoma cell lines. Thus the level of Ras.GTP in human malignant astrocytoma cell lines is similar to that found in Ras transformed RT8 cells. Using an enzymatic assay (Figure 3B), 20 human GBMs and 2 non-neoplastic brain specimens (head injury) were evaluated for Ras activity. The mean amount of Ras.GTP was 1.62 +/- .36 f.moles/μg of GBM DNA, compared to .08 and .04 f.moles/μg of non-neoplastic brain DNA. When Ras activity was expressed as percent of total

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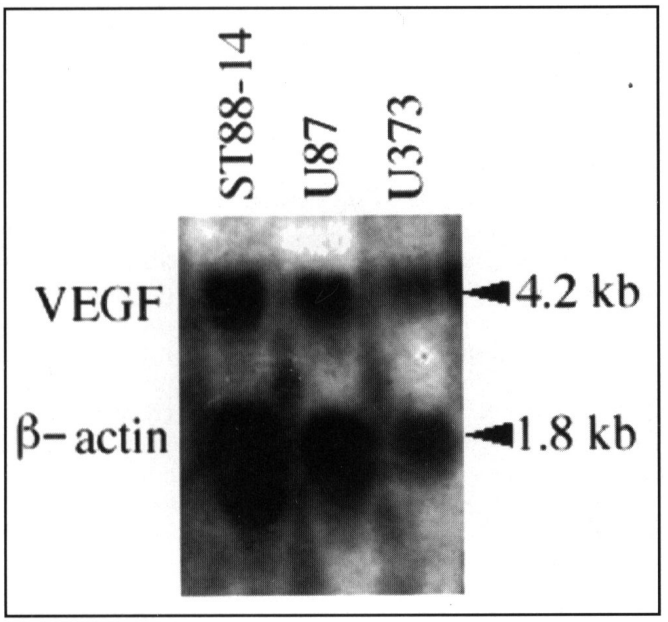

Figure 8: Northern blot analysis of human NF-1 neurogenic sarcoma cell line (ST88-14) and human astrocytoma cell lines for VEGF mRNA expression. The NF-1 neurogenic sarcoma cell line, whose Ras activity has been documented to be elevated by other labs, expresses comparable levels of VEGF mRNA to the astrocytoma cells, whose increased Ras activity and inter-relationship to VEGF secretion is documented (see Figures 5, 11, 12). Effect of inhibiting Ras activity on cellular proliferation and VEGF secretion by the NF-1 neurogenic sarcoma cells are ongoing.

Ras normalized to mg tumor protein (%Ras.GTP/Ras.GDP + Ras.GTP), the value for the GBM specimens were similarly elevated at 53.5 +/- 5%, compared to .5 and 2.3% in the non-neoplastic human brain samples (data not shown). In comparison, values for Ras.GTP in *v-Ha-ras* transformed RT8 and non-transformed NIH/3T3 fibroblasts varied between .8-1.0 and below detection level to .03 Ras.GTP f.moles/μg of DNA respectively (data not shown).

Effect of blocking Ras activation on *in-vitro* proliferation of human malignant astrocytoma cell lines: To inhibit Ras activation, astrocytoma cells were transfected with the Ha-Ras-Asn17 dominant inhibitory mutant. ^{32}P-Ras loading assays were used to measure levels of Ras.GTP in the U373 clones transfected with Ha-Ras-Asn17 construct, Figure 3C. The mean percentage of total Ras in the Ras.GTP bound form in the U373 cells transfected with the empty MMTV vector was about 20%, measured in four separate experiments. This value was similar to the *v-ras* transformed RT8 fibroblasts and parental U373 cells in Figure 3A. In comparison, expression of Ha-Ras-Asn17 significantly decreased levels of activated Ras.GTP in the U373 cells to a mean value of about 10%. Activation of MAP kinase was decreased by expression of Ha-Ras-N17 as detected by the mobility shift, an activation specific antibody, and the myelin basic protein kinase assay (data not shown).

Colony formation and anchorage-dependent proliferation assays, on pooled or stably selected clones of transfected astrocytoma cells, both demonstrated that the proliferation rates of the four astrocytoma cell lines were decreased by blocking Ras activation by the Ha-Ras-Asn17 dominant inhibitory mutant (data not shown). Anchorage independent soft agar assay on U87 human malignant astrocytoma cells transfected with the Ha-Ras-Asn17 inhibitory mutant also demonstrated decreased tumorgenic proliferation, Figure 4A, with the number and average size of the U87 colonies with decreased Ras.GTP levels due to Ha-Ras-Asn17 expression much reduced, compared to MMTV vector only transfected U87 cells.

Treatment with an FTI (12 days of 10μM L-739,749), reduced by 54% and 48% the number of U87 cells treated with L-739,749 compared to parental U87 cells ($p = 0.0108$ by paired t-test) and vehicle-treated cells ($p = 0.0063$ by paired t-test) respectively, Figure 4B. Inhibition of proliferation by FTIs has been noted in a wide panel of human astrocytoma cell lines,[137] within a dose range found to be effective in Ras transformed human cancer cell lines and that well tolerated in animals with minimal toxicity.

VEGF expression and effect of Ras inactivation in human astrocytoma cell lines: The interrelation of the mitogenic Ras-Raf-MAPKinase pathway and VEGF mediated angiogenic pathway as discussed in the introduction, was explored by examining the conditioned media from human astrocytoma cell lines for VEGF secretion. Western immunoblot analysis of the conditioned media demonstrated abundant secretion of all four VEGF isoforms by the parental U118,U87 and U373 astrocytoma cell lines, which were decreased by inhibition of Ras activity by transfection with the Ha-Ras-Asn17 dominant inhibitory mutant or treatment with 10μM L739,749, a FTI, Figure 5. Appropriate controls for the experiments (MMTV vector only, or vehicle (methanol)) are included. Of interest, the constitutively activated and truncated EGFR ($p140^{EGFR}$) secretes higher levels of VEGF when expressed in the U118 astrocytoma cells, compared to U118 parentals. Recent experiments have demonstrated that this mutant receptor, which is expressed in 25-40% of GBMs and confers both *in-vitro* and *in-vivo* growth advantage, further elevates Ras activity (data not shown). This would be in keeping with our hypothesis that activation of the Ras pathway leads not only to mitogenic signals, but also angiogenic signals via increased VEGF expression, both elements vital to tumorgenic growth. The interrelationship of Ras activation and VEGF expression in the astrocytoma cells was further quantified with ELISA assays (data not shown), demonstrating decreased VEGF secretion with Ras inactivation by Ha-Ras-Asn17 or the FTI (L739,749).

Expression of neurofibromin by peripheral nerve tumors and levels of Ras activity: Western immunoblot (Figure 7) and immunohistochemistry (data not shown) were used to analyze expression of neurofibromin in the peripheral nerve specimens. Lysates from the NF-1 (+/+) embryonic fibroblasts (+'ve control) expressed the large approximately 240kDa neurofibromin protein, while the fibroblasts derived from homozygous NF-1 (-/-) deleted embryos (-'ve control) did not. Lysates from a non-NF-1 sporadic Schwannoma expressed abundant neurofibromin, while both NF-1 neurofibromas and NF-1 neurogenic sarcomas did not, consistent with the RT-PCR data (data not shown).

Ras activity was measured using the enzymatic assay described, with levels of Ras activity expressed as a percentage of total Ras (%Ras.GTP/Ras.GDP + Ras.GTP), or quantitatively as femtomoles of Ras.GTP/μg tumor DNA. The latter measurement gave a quantitative value, and normalized against a nuclear derived parameter (μg DNA) which may reflect more accurately

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the number of tumor cells in the samples assayed. This would theoretically account for differences in cellular size and amount of extracellular material between the samples, thereby giving a more accurate measurement of the amount of activated Ras in each of the tumor cells. In five NF-1 neurogenic sarcomas, the average percentage of activated Ras was 15.4 +/- 5.2%, or 46.0 +/- 7.0 fmol/μg DNA, Figure 6. The average activated Ras.GTP levels in the four NF-1 benign neurofibroma specimens was 6.0 +/- 2.5% or 13.3 +/- 2.6 fmol GTP/μg DNA, Figure 6. Levels of activated Ras.GTP in the benign neurofibromas, expressed either as a percentage of total Ras or in absolute amounts per μg DNA were therefore approximately one-third the levels detected in the NF-1 neurogenic sarcomas. In the four non NF-1 benign Schwannomas levels of activated Ras.GTP was 1.3 +/- .3% or 3.5 +/- .6 fmol GTP/μg DNA, Figure 6. These levels of activated Ras.GTP in the benign Schwannomas in non NF-1 patients were approximately 8% and 21% the levels found in the NF-1 neurogenic sarcomas and neurofibromas, respectively.

VEGF expression and angiogenesis in NF-1 neurogenic sarcomas: Our recent experiments have started to explore the inter-relationship between Ras activation, VEGF expression and tumor angiogenesis based on the rationale discussed in the introduction in peripheral nerve tumors. Although not as progressed as our work with astrocytomas presented above, some preliminary data do support our hypothesis that increased Ras activity in these tumors would be accompanied by a more angiogenic tumor and increased VEGF expression. In Figure 8, Northern blot analysis demonstrates abundant VEGF mRNA expression in a human NF-1 neurogenic sarcoma cell line (ST88-14), which has been documented to have elevated levels of activated Ras.GTP.[95,96] The levels, when normalized to β-actin, are similar to human malignant astrocytoma cell lines (U87,U373) which also have elevated Ras activity (Figure 3A) and secrete abundant VEGF which is decreased by inhibition of Ras activity by the Ha-Ras-Asn-17 dominant inhibitory mutant or the FTI, L-739,749, Figure 5. In addition, we recently published the clinical, pathological and molecular presentation of an aggressive NF-1 neurogenic sarcoma.[70] This particular tumor, whose Ras activity was measured and found to be elevated, was highly angiogenic and rapidly metastasized. Invasion of tumor cells into the tumor vascular channels leading to infarction of the tumor and subsequent acute presentation was noted. Expression of VEGF at both the mRNA and protein level in this tumor and other nerve tumor samples whose Ras activity has been measured are underway, to investigate the interrelationship of the mitogenic Ras pathway and VEGF expression.

Discussion and Conclusions

Astrocytomas: Understanding and potentially inhibiting the common signal transduction pathway(s) utilized by the various activated RTKs in human malignant astrocytomas may be of therapeutic advantage compared to inhibition of each RTK separately. For example, the protein kinase-C (PK-C) pathway has been implicated in astrocytoma proliferation, leading to clinical trials with PK-C inhibitors in recurrent GBMs with equivocal results.[138-142] There are several reasons to be interested in Ras-mediated signaling in human malignant astrocytomas expressing activated RTKs. First, the Ras-Raf-MAP kinase pathway is presently the best understood signaling pathway linking RTKs on the cell surface to the nucleus.[92,100,102,118] Second, activation of Ras is important for proliferative or differentiating signals from a variety of RTKs, including PDGF-Rs and EGFR expressed by malignant astrocytomas.[101,103-106] Third, as demonstrated by our results, activation of Ras not only leads to tumor cell proliferation but also tumor vascularization, by transcriptional upregulation of the extremely potent and specific angiogenic factor, VEGF.[58,59] Hence, activation of Ras may be relevant to the proliferation of astrocytoma cells and to tumor angiogenesis, both of which appear critical for the growth of human malignant astrocytomas, which are one of the most vascularized of human neoplasms. Another reason to examine the role of Ras signaling in astrocytomas is the development of farnesyl-transferase inhibitors to block Ras activation. These agents reportedly have minimal toxicity and appear efficacious in inhibiting the growth of a variety of human tumors in nude mice, though their effect on human astrocytomas has not yet been reported.[119-121,143-146] Tumors without oncogenic Ras mutations, can also be growth inhibited by farnesyl transferase inhibitors, suggesting that if astrocytomas are dependent on Ras signaling they may be sensitive to these agents, despite the lack of activating Ras mutations in astrocytomas.

Human astrocytomas and derived cell lines, including the ones used in this study, are known to express PDGF-Rs and EGFR.[25,26,30,34,35,147-149] We demonstrate in this study that these RTKs expressed by the astrocytoma cell lines are capable of activating Ras. The adaptors Shc and Grb-2, are involved in activation of the nucleotide exchange factor mSos by activated RTKs, possibly by bringing mSos in proximity to Ras.GDP at the inner cell membrane, thereby converting it to Ras.GTP,[118,122-125,127,150-158] or by inducing direct mSos catalytic activation.[159] Upon stimulation, both EGFR and PDGF-R (data not shown) expressed by the malignant astrocytoma cell lines bound Shc proteins and induced tyrosine phosphorylation of the 46 and 52 kDa Shc isoforms. Upon stimulation, the SH2 domain of Grb-2 was able to bind to tyrosine phosphorylated Shc. In summary, these results demonstrate that PDGF-Rs and EGFR, and probably other RTKs expressed by human malignant astrocytomas, are capable of activating the Ras signaling pathway.

Levels of activated Ras.GTP were elevated in all of the four astrocytoma lines, and comparable to oncogenic Ras transformed fibroblasts, Figure 3A. NIH-3T3 cells rather than non-transformed human astrocytes were used as negative controls, as the latter are hard to obtain and sustain in culture to yield sufficient cell numbers to undertake ^{32}P-Ras loading experiments. To exclude culture induced artifacts, we utilized an enzymatic assay to measure Ras.GTP and Ras.GDP levels in flash frozen tissues.[10,98,136] Similar to the established cell cultures, Ras activity was markedly elevated in the GBM specimens, compared to non-neoplastic brain samples, Figure 3B. We have also recently started to explore whether the Ras pathway is utilized by the p140^{EGFR} mutant expressed by a large percentage of GBMs, and which has been demonstrated to be constitutively activated and provide growth advantage.[28,29] The p140^{EGFR} mutant which is not expressed by astrocytoma cell cultures but only in the actual tumors, was transfected into a human malignant astrocytoma cell line (U118) expressing normal EGFR. Although levels of Ras.GTP was elevated in the parental U118 cells similar to other malignant astrocytoma lines (Figure 3A), expression of the

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$p140^{EGFR}$ mutant increased basal levels of Ras activity in serum starved conditions even more (data not shown). The U118-$p140^{EGFR}$ mutant expressing cells are more tumorgenic in anchorage dependent/independent assays and preliminary nude mice experiments compared to the parental U118 cells, the latter in keeping with their increased VEGF secretion, Figure 5. Another piece of evidence in support of the $p140^{EGFR}$ mutant utilizing the Ras signaling pathway is their increased sensitivity to FTIs, compared to astrocytoma cells expressing only the normal EGFR (data not shown). Further experiments to characterize the Ras mediated signaling of the $p140^{EGFR}$ mutant receptor are presently ongoing, as is a study investigating whether there is a gradient of Ras activity between Grade 1 to Grade 4 (GBM) specimens using the enzymatic assay and specimens from the University of Toronto Nervous System Tumor Bank that we have established.

Expression of the Ha-Ras-Asn17 dominant inhibitory mutant decreased levels of Ras.GTP in the astrocytoma cells by approximately 50%, as measured in the U373 clone, Figure 3C. Decreased levels of Ras.GTP in the astrocytoma cells correlated with inhibition of the major mitogenic downstream effector of Ras, involving activation of the Ras-Raf-MAP Kinase pathway. Expression of Ha-Ras-Asn17 decreased proliferation in all astrocytoma cell lines, as observed in the pooled proliferation and colony formation assays and those undertaken on the isolated U373 clone. Anchorage independent growth of the U87 cell line (which has the most tumorgenic growth in nude mice of the four astrocytoma lines, unpublished observation), was decreased by blocking Ras activation with the Ha-Ras-Asn17 inhibitory mutant, Figure 4A. These results demonstrate that Ras activity is elevated in human malignant astrocytomas, and activation of this signaling pathway is important for tumor cell proliferation.

Farnesylation is the most critical post-translational modification step of Ras, essential for its localization to the inner cell membrane and subsequent activation by RTKs.[160,161] Farnesyl transferase inhibitors (FTI) have demonstrated minimal toxicity in animal models, while reducing growth of a variety of human tumors, with malignant astrocytomas not yet tested.[97,119-121,143-146] Surprisingly, growth inhibition was independent of whether or not the tumors harbor activating oncogenic Ras mutations.[162,163] For example, human neurogenic sarcoma cell lines are inhibited by these drugs,[97] though elevated levels of Ras.GTP in these cells are secondary to decreased levels of neurofibromin, one of the two major mammalian Ras-GAPs,[95,96] and not due to activating mutations of Ras. Our preliminary data with L-739,749, a FTI developed by Merck Research Laboratories, demonstrates that pharmacological inhibition of the Ras signaling pathway inhibits proliferation of human astrocytoma cells, Figure 4B. In addition, these and other strategies directed at inhibiting the Ras signaling pathway may also block tumor induced angiogenesis. As demonstrated by our results in Figure 5, the abundant VEGF secretion by malignant astrocytomas was decreased by genetic or pharmacological inhibition of the Ras pathway. Hence, these agents may have a greater *in-vivo* effect in decreasing overall tumor growth compared to their documented *in-vitro* anti-mitogenic effects, since they may inhibit not only tumor cell proliferation but also tumor induced angiogenesis. Current work in my laboratory is directed to evaluating FTI in animal models of human malignant astrocytomas, to ultimately determine their potential therapeutic role in this presently terminal human cancer.

Neurofibromas: Previously, Ras activity could only semi-quantitatively be measured in cell cultures using the ^{32}P-Ras loading technique, as utilized for the malignant astrocytoma cell lines, Figure 3A. The enzymatic assay (Figure 3B) developed in collaboration with Dr. Gerry Boss, yields results that are quite comparable to the ^{32}P-Ras loading technique.[10,98,136] This assay of course has wide applicability in other tumor systems where Ras activity is increased through oncogenic mutations, increased activation (such as in malignant astrocytomas), or decreased inactivation (such as in NF-1 neurofibromas). Some limitations of the assay exists however. 1) Relatively large quantities of tissue are required, as amounts less than approximately 2c.m.^{3} did not provide reliable measurements. 2) Specimens should be flash frozen (within 30 sec. in these experiments), although the exact time before significant phosphatase and protease activity leads to degradation of the samples is not known. 3) Measurements of Ras bound guanine nucleotides are from the entire tumor, and do not specify the levels specifically found in the tumor cells only, as distinct from infiltrating and surrounding cells. Hence, both tumor and non-tumor cells (a variable and occasionally significant proportion of the cells in some tumors) within the specimens, contribute to the measured values of Ras bound GTP and GDP. This obstacle could be overcome by the development of activation-specific antibodies, capable of recognizing specifically Ras.GTP. 4) Another potential source of variability between tumors is the extent of acellular areas, which may significantly alter the measured levels of Ras bound guanine nucleotides if expressed in terms of protein. This variability is minimized by expressing levels of activated Ras in the specimens as a percentage (%GTP/GDP + GTP), or absolute amounts of Ras.GTP in terms of μg DNA, to standardize for the cellular content between the specimens. Similar conclusions regarding the levels of activated Ras in both the peripheral nerve tumors and astrocytoma experiments were reached using both these methods.

Although NF-1 tumors are predicted to have elevated levels of Ras.GTP due to loss of neurofibromin, a major mammalian Ras-GAP involved in inactivating Ras.GTP to Ras.GDP, this was previously demonstrated in NF-1 neurogenic sarcoma cell lines and not in tissues.[95,96] The results of this study measuring Ras activity in peripheral nerve tumor specimens are in agreement with the cell culture data. Levels of Ras.GTP were elevated approximately 15X in the NF-1 neurogenic sarcomas, compared with non NF-1 Schwannomas, Figure 6. In addition to verification that levels of Ras activity were elevated in the actual NF-1 neurogenic sarcoma specimens, the enzymatic based assay allowed a determination of Ras.GTP levels in benign NF-1 neurofibromas, which do not grow in culture. Compared to non NF-1 Schwannomas, levels of Ras.GTP were increased approximately 4X in these NF-1 neurofibromas, Figure 6. Neurofibromin Western blot analysis (Figure 7) failed to demonstrate any detectable levels of neurofibromin by the NF-1 neurofibroma. Immunohistochemistry although less conclusive was also supportive, since less overall neurofibromin-staining was observed in the NF-1 neurofibromas where many cells did not positively stain compared to the non NF-1 Schwannomas (data not shown). We hypothesize that these neurofibromin negative cells are the actual transformed cells that form the NF-1

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neurofibromas and contribute to the elevated levels of activated Ras.GTP. Double labeling experiments with neurofibromin and cell type specific antibodies, to characterize the cellular subpopulations in neurofibromas, are underway to help resolve this issue.

Therefore these experiments demonstrate increased Ras activity in NF-1 peripheral nerve tumors, not due to oncogenic mutations but as a result of loss of neurofibromin, a major inactivator of activated Ras.GTP. In addition to the loss of both NF-1 alleles which lead to the NF-1 neurofibromas as per the "two hit hypothesis" of tumor suppressor genes,[164,165] additional genetic aberrations such as loss of p53 gene has been implicated or are being sought in the malignant neurogenic sarcomatous transformation which occurs in 3-5% of the deeper plexiform neurofibromas in NF-1.[11] The increased growth rate, tumor angiogenesis, and metastasizing capability of NF-1 neurogenic sarcomas compared to their benign counterparts are reflected in the increased levels of activated Ras.GTP, Figure 6. We hypothesize and demonstrate in preliminary experiments (Figure 8), that similar to malignant astrocytomas the increased Ras activity in NF-1 neurogenic sarcomas is also accompanied by transcriptional up-regulation of VEGF, the most potent and specific angiogenic factor implicated in tumor angiogenesis and metastasis.

These experiments have demonstrated in cell culture experiments and in operative specimens the involvement of the Ras mediated signaling pathway in two nervous system tumors. Although oncogenic mutations of Ras are not present in astrocytomas and peripheral nerve tumors, there is a functional activation of this critical mitogenic signaling pathway. Aberrant Ras activity leads not only to an increase in cellular proliferation in these two tumor systems, but as our preliminary experiments demonstrate, may play a vital role in regulating VEGF mediated tumor angiogenesis. Survival curves of human malignant astrocytomas and neurogenic sarcomas have not improved despite advances in neurosurgical capabilities, due to limitations posed by the molecular biology of these tumors. The experiments in this report and others that are ongoing in our laboratory, are the first steps in evaluating the potential use of modulators of the Ras signaling pathway in the management of these presently terminal cancers.

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