Meeting Report

New Approaches to the Molecular Biology, Classification, and Therapy of Nervous System Tumors

A Workshop of the National Institutes of Health Pathology B Study Section

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Tumors of the nervous system are a diverse group of neoplasms, spanning a wide array of histological types and affecting all age groups. Most research has focused on gliomas and medulloblastomas, the most common malignant lesions of adults and children, respectively. Many of these tumors have been extensively studied at the genetic level, and considerable effort has been directed at associating different subtypes and grades with particular genetic alterations. Knowledge of these genetic changes have led to improvements in clinical classification of these lesions as well as better understanding of the molecular events underlying tumor formation and progression. Most are highly invasive tumors which prevent their surgical cure. There has, therefore, also been considerable interest in understanding the mechanisms of tumor invasion, and in determining the expression of cell surface receptors, glycolipids, and other molecules that could mediate invasion. Against this backdrop of progress in nervous system tumors, the Pathology B Workshop entitled "New Approaches to the Molecular Biology, Classification and Therapy of Nervous System Tumors" was held on January 29, 2001 in Tucson, AZ. The five presentations reviewed the pathological, genetic, and molecular diagnostic features of malignant gliomas, the

role of signaling pathways in glioma growth and therapy, studies of glioma invasion, the biochemistry and biology of cell surface molecules in glioma growth and differentiation, and a prospective peptide vaccine for malignant gliomas.

Dr. David Louis (Massachusetts General Hospital and Harvard Medical School) discussed the latest issues in molecular classification of human gliomas. The proper classification and grading of brain tumors is the foundation upon which different tumor types are separated into distinct therapeutic and prognostic groups. For instance, some brain tumor types can be cured by surgery alone and others by a combinations of surgery, chemotherapy, and radiation therapy, while others remain incurable. For over 70 years, tumor classification and grading rested on the histological examination of tissues. Beginning in the 1960s to the present, electron microscopy and then immunohistochemistry provided a better means for classification and grading. Nonetheless, the most current World Health Organization scheme widely acknowledges the numerous remaining problems in accurate brain tumor classification, and has begun to recognize the potential of molecular analysis in this endeavor.

Diffuse gliomas are the most common type of malignant brain tumor, and are classified based on their line of differentiation (astrocytic, oligodendroglial, or oligo-astrocytic) and graded according to their histological degree of malignancy (World Health Organization grades II, III, and IV). The most malignant, and most common, form is known as glioblastoma, and is designated grade IV. Different constellations of genetic alterations have been associated with specific types of malignant gliomas, with

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particular grades of malignant glioma, and with differential sensitivities to therapy.

The astrocytic gliomas and oligodendrogliomas have generally distinct genetic underpinnings. For instance, the formation of World Health Organization grade II astrocytoma is associated with inactivation of the TP53 tumor suppressor gene and loss of chromosome 22q, whereas oligodendrogliomas frequently display allelic losses of chromosome arms 1p and 19q. Information on the less common and more controversial oligoastrocytomas has not been as forthcoming, but has suggested that most of these tumors have genetic alterations characteristic of either the astrocytic or the oligodendroglial gliomas. Once lower-grade tumors progress to grade III anaplastic gliomas and to glioblastoma, the genetic events overlap among the astrocytomas, oligodendrogliomas, and oligoastrocytomas. For instance, both high-grade astrocytomas and oligodendrogliomas have frequent losses of chromosome 10 and deletions of the CDKN2A/CDKN2B gene complex on 9p. From a translational point of view, the most intriguing observations have been that molecular genetic analyses can aid in tumor classification. For example, in glioblastomas, *EGFR* gene amplification almost never occurs in glioblastomas with TP53 mutation or loss of chromosome 17p. The pathway that includes TP53 inactivation most often involves progression in younger adults from a lower-grade astrocytoma so-called "secondary glioblastoma." Those glioblastomas with *EGFR* amplification, however, typically occur in older patients who do not have a history of preceding lower-grade astrocytoma so-called "primary glioblastoma." While these initial studies of glioblastoma subsets have not revealed clear correlations of genotype with tumor behavior, they raised the possibility that new approaches to diagnosis and treatment could be based on objective biological parameters: a possibility that has now been realized for anaplastic oligodendroglioma. Anaplastic oligodendrogliomas are distinguished by their often remarkable chemosensitivity, with approximately two-thirds of cases responding dramatically to procarbazine, lomustine (CCNU), and vincristine (PCV) chemotherapy. While no clinical or pathological feature predicts chemotherapeutic response accurately, allelic loss of chromosome 1p and 19q is a powerful predictor of chemotherapeutic response and longer recurrence-free survival post-chemotherapy in patients with anaplastic oligodendrogliomas. Such data imply that differential clinical behaviors reflect two independent genetic subtypes of anaplastic oligodendroglioma. From a clinical perspective, they further indicate that patients whose tumors have different genotypes can be treated in significantly different ways. The results demonstrate that molecular genetic analyses may eventually guide therapeutic decision in patients with particular types of malignant glioma.

A few other major advances could also impact on brain tumor classification in the near future: expression profiling and molecular neuro-imaging. Expression profiling of brain tumors has been approached already from a number of directions, including initiatives from the National Cancer Institute and National Institute of Neurological

Disorders and Stroke (NINDS) such as the Brain Tumor Genome Anatomy Project. Early attempts to use such profiling for classification appear promising. Furthermore, neuro-imaging, which has already revolutionized the clinical practice of neuro-oncology, now stands on the verge of being able to image biological processes. When this comes to fruition, neuro-imaging itself may constitute an integral component of brain tumor classification and grading.

The role of ganglioside signaling in tumor development was the subject of Dr. Stephan Ladisch's (Children's National Medical Center) presentation. In contrast to rapidly increasing knowledge about mechanisms of control of cell proliferation which underlie autonomous proliferation of tumor cells, much remains to be understood about the manner in which tumors cells and the host interact such as to permit tumor formation *in vivo*. A likely important aspect of this interaction is the influence of tumor cells on the host microenvironment. One way this may occur is by their release of signaling molecules and one class of putative signaling molecules is the gangliosides which are cell membrane glycosphingolipids.

The release and transfer of tumor gangliosides which would be essential for cell signaling by these tumor-derived molecules, is now well established. Following initial studies demonstrating the shedding of cell membrane gangliosides by mouse lymphoma cells, *in vitro* and *in vivo*, the shedding of these immunosuppressive molecules has been demonstrated in multiple tumor systems, including murine lymphoma cells, human leukemia cells, neuroblastoma cells, and medulloblastoma cells, for example. Critical to the hypothesis that these shed molecules interact with host cells is evidence for their actual transfer from tumor cells, where they are synthesized, to host cells, where they may exert their activity. Dr. Ladisch has used a modified Transwell system in which he demonstrated that ganglioside molecules were shed by tumor cells and were taken up by normal fibroblasts on the contralateral side of the porous Transwell membrane, satisfying the conditions for potential interaction and activity in the host.

Significant experimental evidence correlating alterations in ganglioside metabolism with the formation of tumors in experimental systems (for example, degree of membrane sialylation with tumorigenic potential) has led to studies hoping to provide more direct evidence on this point. These include findings that addition of small quantities of highly purified tumor gangliosides to a tumor cell inoculum *in vivo* augmented the frequency of tumor formation in mice, and that the shedding of gangliosides by the highly aggressive human tumor, neuroblastoma, was highly correlated with poor outcome. These studies have underscored the potential importance of understanding potential mechanisms by which tumor gangliosides might affect tumor formation.

The first functional role for gangliosides was that of their modulatory effects on the immune response. *In vitro*, the addition of gangliosides to murine and human leukocyte cultures was found to suppress the cellular immune response as measured in several assay systems. Importantly, inhibition of the cellular immune response was not

accompanied by direct toxicity of these molecules, suggesting a mechanism of interference with one or several of the multiple systems involved in the immune response. A differential (greater) inhibition of immune responses induced by specific antigens, versus mitogen-induced responses, suggested that the antigen-presenting cell could be an important target of ganglioside inhibition. This was confirmed and the effect has been localized to one occurring after the initial steps of antigen processing and presentation on the cell surface. Importantly, while many studies had been performed using model systems, until recently, evidence demonstrating an effect of tumor gangliosides on the tumor-specific cellular immune response implicated in cancer was lacking. Most recently, studies undertaken in a syngeneic murine lymphoma model demonstrated that both the primary and secondary immune responses *in vivo*, as well as the generation phase of cytotoxic T lymphocyte and the proliferative response of splenocytes were inhibited by the administration of highly purified tumor gangliosides. Overall, these results implicate tumor gangliosides in inhibition of the specific antitumor immune response *in vivo*.

The development of inhibitors of glycosphingolipid metabolism (and specifically of glucosylceramide synthase activity) has provided a useful probe for the role of gangliosides in modifying cell function. By treatment of cells with certain of these inhibitors, depletion of membrane gangliosides could be achieved. When fibroblasts were treated in this manner and depleted their ganglioside content, the inhibition of glucosyl ceramide synthase and subsequent depletion of membrane gangliosides resulted uniformly in blunting of the cellular proliferative response to a number of growth factors, having in common receptor tyrosine kinases. Enrichment of membrane of the same cells by gangliosides resulted in enhancement of growth factor-mediated cell proliferation confirming the above observations that membrane enrichment in gangliosides somehow enhances growth factor-mediated signaling; the mechanism is currently under investigation.

The overall findings of inhibition of the immune response and enhancement of the growth factor-mediated proliferation of normal fibroblasts all support a potential biological role of gangliosides in enhancing tumor formation through signaling. To directly test this hypothesis a murine melanoma model was used to evaluate the consequences of ganglioside depletion of B-16 melanoma cells, on their ability to form tumors *in vivo*. Strikingly, a 90% depletion of cellular gangliosides did not cause any cellular cytotoxicity but markedly reduced the tumor angiogenesis inhibitor. *SPARC* was found to be expressed in the invading tumor cells, but expression was not observed when invasion was inhibited in the presence of the angiogenesis inhibitor. Thus, three different "models" of invasion indicated that *SPARC* was expressed in invading cells.

Recently, an *in vitro* model of invasion was used to determine whether *SPARC* functionally contributes to the invasion process. U87 glioma cells express very low levels of endogenous *SPARC* and grow as well-circumscribed tumors *in vivo* in rat brain. *SPARC* was trans-

ected into U87 cells using the TET-OFF system. *SPARC*-transfected clones demonstrated changes in morphology in culture and transfected *SPARC* mRNA and protein were eliminated in the presence of doxycycline. The U87T2 clone (transfected with regulator plasmid alone) and *SPARC*-transfected clones were used in a spheroid confrontation assay to assess invasion *in vitro*. The results indicated that increased *SPARC* expression was associated with increased invasion of the tumor cells into the fetal brain aggregates. Thus, *SPARC* functionally contributes to brain tumor invasion as assessed by this *in vitro* assay. These clones will serve as useful reagents to continue our investigations into the mechanism(s) by which *SPARC* promotes invasion *in vitro* and *in vivo*.

The extracellular matrix (ECM) and mechanisms of glioma cell invasion were the subject of Dr. Candace Gladson's (University of Alabama) talk. The ECM of astrocytic tumors promotes and modulates a variety of cell functions, such as cell attachment, migration, proliferation, survival, and signaling. Recent studies indicate that there are extensive and complex interactions among ECM molecules and that these can modify the function of the participating molecules, interactions between the proteoglycan, phosphacan, and the ECM protein, tenascin, being an example. Thus, interacting components in the ECM and cell surface receptors likely cooperate in regulating cell function and tumor invasion. As tumor cells are capable of remodeling their ECM through synthesis of ECM proteins and proteoglycans, as well as up-regulating integrin receptors and proteoglycans on their cell surface, tumor cells are capable of controlling their own destiny.

p125 focal adhesion kinase (p125FAK) is a cytoplasmic tyrosine kinase that is activated on engagement of integrin cell adhesion receptors, and initiates several signaling events that modulate cell function *in vitro*. To determine the biological role of p125FAK in malignant astrocytic tumor cells, U-251MG human malignant astrocytoma cells were stably transfected with p125FAK cDNA using the TET-ON system, and stable clones were isolated that exhibited an estimated up to 18-fold increase in p125FAK expression on administration of doxycycline. The induction of p125FAK resulted in a two- to three-fold increase in cell migration, increased p130CAS phosphorylation, localization of exogenous p125FAK to focal adhesions, and a two-fold increase in soft agar growth. To determine the role of p125FAK *in vivo*, clones were injected stereotactically into the brains of SCID mice. A 4.5-fold estimated increase in p125FAK expression was induced by administration of doxycycline in the drinking water. Analysis of xenograft brains demonstrated that, on induction of p125FAK, there was a 1.6- to 2.8-fold increase in tumor cell number, and an increase in mAb proliferating cell nuclear antigen-labeling of tumor cells in the absence of a change in the apoptotic index. Compared to normal brain, the expression of p125FAK was elevated in malignant astrocytic tumor biopsies from patient samples. These data demonstrate for that p125FAK promotes tumor cell proliferation *in vivo*, and that the underlying mechanism is not associated with a reduction in apoptosis.

PI 3-kinase has been shown to play an important role in the signaling pathway necessary for cytoskeletal reorganization in non-astrocytic cells. The role of PI 3-kinase in U-251 MG adhesion and migration was studied. Attachment of U-251 MG cells to vitronectin, fibronectin, laminin, and collagen was inhibited in a concentration-dependent manner by two specific inhibitors of PI 3-kinase (wortmannin and LY294002). Attachment to vitronectin, fibronectin, and laminin was more sensitive to inhibition of PI 3-kinase than attachment to collagen. Similarly, migration toward these substrates showed differential sensitivity to inhibition. Attachment of the cells to these matrix proteins resulted in an increase in PI 3-kinase activity, as compared to that of cells in suspension, with attachment to vitronectin resulting in the greatest increase in PI 3-kinase activity. p125 focal adhesion kinase (p125FAK) was found to coimmunoprecipitate with PI3-K from the NP40-soluble cell fraction of cells in the early stages of adhesion to vitronectin and fibronectin, but not during adhesion to collagen. The expression of p125FAK protein and the level of phosphorylation were similar on adherence to all three substrates. These data indicate that the sensitivity of U-251MG cell attachment and migration to PI3-K inhibitors is substrate-dependent, and that complex formation of PI3-K and p125FAK correlates with this sensitivity to PI3-K inhibitors. This data suggest a role for PI3-K and p125FAK complex formation in PI3-K activation.

Dr. Albert Wong (Thomas Jefferson University) then discussed novel signaling pathways in gliomas. A naturally occurring variation of the EGF receptor (EGFRvIII) has been identified in his lab that is frequently found in glioblastoma. A unique signal transduction pathway downstream of EGFRvIII has been found. While this receptor is constitutively active and transforming, it does not use the Ras and MAP kinase as does the normal EGF receptor. Instead, EGFRvIII signals through PI 3-kinase and JNK to effect transformation. This led to the discovery that JNK activity is elevated in a high percentage of glial tumors. Moreover, JNK is actually responsible for the prevention of cell death in tumor cell lines and promotes phenotypes related to tumorigenesis but not in normal cells and so JNK actually may play the opposite role in tumors cell than it does in normal cells. The AKT kinase, while strongly activated by PI 3-kinase, does not show constitutive activation in cells overexpressing EGFRvIII, yet does show activation in these same cells in response to PDGF. To understand the mechanism by which JNK promotes cell survival, it was found that expression of Bcl-X_L protein is increased in these cells. An inspection of this promoter shows that it has several sites for c-jun binding, the phosphorylation target of JNK. EMSA showed that these sites did indeed bind c-jun and reporter assays confirmed increased transcription in the presence of wild-type JNK and a decrease with dominant-negative JNK.

The discovery of EGFRvIII led to the finding of a novel signaling protein called Grb2 associated binder-1, or

Gab1. This protein is tyrosine phosphorylated by several RPTKs including the EGF, insulin, Met, and TrkA receptors. When phosphorylated, Gab1 in turn activates several signaling pathways. It was found that the presence of Gab1 can potentiate neurite outgrowth in PC-12 cells via a pathway that depends on PI 3-kinase and ERK1/2. Gab1 also promotes cell survival by nerve growth factor. The pleckstrin homology domain of Gab1 binds phosphatidylinositol 3,4,5-phosphate and not PI 4,5 P₂. The PH domain appears important for the membrane localization of Gab1, the subsequent activation of PI 3-kinase, and phenotypes such as the induction of branching tubules. To further understand Gab1 function, embryonic stem cells with a disruption of the Gab1 gene have been isolated and then mice bearing this deleted gene have been bred. Mice that are Gab1^{-/-} homozygous are embryonically lethal at approximately day 12 to 13 with vascular and cranial abnormalities, as well as petechiae. Mice that are Gab1^{+/-} appear to be normal. To further study Gab1^{-/-} cells, immortalized mouse embryo fibroblasts were derived. The basal activation of JNK is highly impaired in ^{-/-} cells and moderately decreased in ^{-/+} cells as compared to ^{+/+} cells. Interestingly, this pattern is also observed after two stress stimuli that activate JNK, raising the temperature to 42°C and H₂O₂ treatment. These stimuli also result in Gab1 tyrosine phosphorylation. On the other hand, JNK activation by osmotic stress via sorbitol is not affected and there was a more modest effect with nocodazole and anisomycin. Thus, Gab1 is essential for certain stimuli that couple to JNK but not others.

EGFRvIII has been found to be present in a wide variety of solid tumors including greater than 70% of breast and ovarian tumors. In metastatic breast lesions, it is found in 90% of tumors. Thus, EGFRvIII represents an interesting target for the treatment of tumors. In animal models, vaccination with a peptide corresponding to the alteration present in EGFRvIII could both prevent tumor formation and induce the regression of tumors that expressed this mutant receptor. To develop protocols that can be used in humans, Dr. Wong's lab investigated the use of granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant to enhance CD8⁺ activity. Experiments showed that this was as effective as oil-based adjuvants. A clinical trial based on the EGFRvIII peptide is now under regulatory review.

In summary, this workshop provided a broad overview of exciting new developments in the field of nervous system tumors. Clearly, there has been considerable progress in using molecular tools to classify tumors, to identify mechanisms responsible for invasion and metastasis, and the mechanisms underlying enhanced growth and decreased apoptosis. Many of these studies have pointed out potential novel therapies that can be readily tried in patients. Undoubtedly, the day when such tumors are considered routinely curable is soon approaching.