Glycolbiology and Brain Tumors

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Brain tumors are presently the leading cause of cancer death in children under the age of 20, only recently surpassing acute lymphoblastic leukemia (ALL). In addition, they are the third leading cause of cancer death in young adults ages 20-39 (1). They are phenotypically and genotypically diverse, with over 120 different types of brain tumors currently classified. Glioblastomas represent 23% of all primary brain tumors, rapidly approaching the incidence of meningiomas, which are the most common primary brain tumor and represent 26% of all primary brain tumors (2). Glioblastoma multiforme is nearly uniformly fatal, with median survival between 9 and 12 months from initial diagnosis.

Glycobiology and Brain Tumors

There are many cell-type specific, developmentally regulated and tumor-type specific cell-surface proteins and lipids containing covalently-linked carbohydrates (glycoconjugates). These glycoconjugates play a key role in regulating cell-cell recognition, adhesion, and migration, and include molecules such as integrins, selectins, cell-adhesion molecules (CAMs) and cadherins. They can modulate critical processes such as growth factor receptor function, intracellular protein trafficking, protease activity and secretion.

It is now clear that virtually all types of human cancers have altered patterns of glycosylation and express aberrant glycosyl moieties on their cell-surface glycoconjugates. Moreover, a significant number of studies have shown that

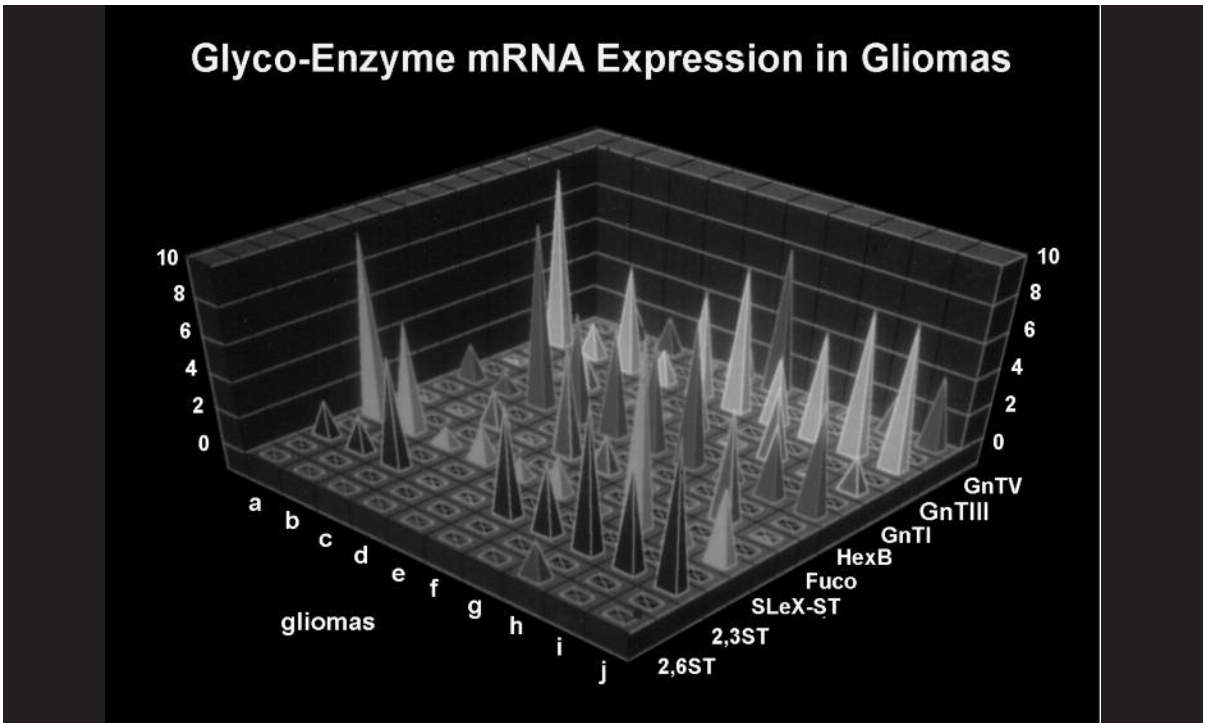

Figure 1: *This figure was created by compiling Northern blot data obtained from ten random glioblastoma specimens and using a variety of glycosyltransferase and glycosidase cDNA probes. Thus, the x-axis points, a-j, represent mRNA isolated from individual gliomas, the y-axis is an arbitrary number assigned from 1 to 10 obtained by scanning the autoradiograms for a measure of mRNA expression and the z-axis depicts each of the glyco-genes assayed. The purpose of creating this figure was to look at the patterns, if any, that one might observe by comparing a variety of glyco-genes against a random panel of gliomas. The values for gene expression are semi-quantitative at best but since the Northern blots were run with essentially identical amounts of total RNA it was concluded that these relative values would nonetheless give an overall sense of pattern of expression. Several conclusions have been drawn from this figure. First, it is clear that there is essentially no α2,6ST mRNA expressed in any gliomas-data confirmed from the analysis of many tumors and tumor types as described in Table 1. Second, each tumor expresses different types and quantities of glyco-gene mRNA, ranging from negligible to the maximal values measured. Third, for each glyco-gene examined versus individual tumors, there is appreciable variability in amount expressed. Interestingly, α2,3ST has been shown using immunocytochemical techniques to be overexpressed in gliomas [39]. And we have also reported that β1,6-N-acetylglucosamine-bearing N-glycans are expressed in gliomas but not in astrocytes from normal adult brain. However, in a separate panel of gliomas, different from the one used in this figure, both GnT-III and GnT-V were found to be variably expressed [42]. z-axis legend: α2,6ST = CMP-N-acetylneuraminate-β-galactoside α2,6-sialyltransferase, X17247; 2,3ST = CMP-N-acetylneuraminate-β1,4-galactoside α2,3-sialyltransferase, L23768; SLeX-ST = α2,3-sialyltransferase VI, NM_006100; Fuco = galactoside 2-α-L-fucosyltransferase, NM_000148; HexB = β-N-acetylhexosaminidase, NM_000521; GnTI = UDP-N-acetylglucosamine: α3-D-mannoside β1,2-N-acetylglucosaminyltransferase I, NM_002406; GnTIII = UDP-N-acetylglucosamine: β-D-mannoside beta-1,4-N-acetylglucosaminyltransferase, NM_002409; GnTV = α1,6-mannosyl-glycoprotein β1,6-N-acetylglucosaminyltransferase, NM_002410.*

these carbohydrates play key functional roles in oncogenic transformation, tumor progression and metastasis [reviewed in (3)]. Early descriptive work by Traylor and Hogan (4), examining a series of human glioblastomas, showed that total ganglioside concentration was reduced in tumors and that there was an increase in simpler ganglioside structures and a reduction in complex, polysialogangliosides. For example Yates and co-workers (5) have shown that GD1b ganglioside correlated with tumor grade in astrocytomas. And a series of reports by Ladisch and co-workers (6) and Nakamura et.al (7) has shown that human neuroblastomas, medulloblastomas and astrocytomas shed gangliosides that can be detected in patient serum and cerebrospinal fluid. Li et al. (8) showed that shed ganglioside GD2 was markedly immunosuppressive and as such may facilitate tumor formation and progression. Cell-surface glycoproteins, too, have been identified that are associated with the invasive potential of malignant gliomas, with studies by Paulus et al. (9) showing that β1 integrins play a key role in modulating glioma invasivity.

It is well established that the biosynthetic machinery-the glycosyltransferases, glycosylhydrolases, and the genes that regulate their expression-has been significantly altered in all forms of oncogenic transformation [for recent review see (10)]. Surprisingly, however, there have been very few such studies with brain tumors. Moskal and co-workers (11) reported on the expression of α2,6 sialyltransferase in a variety of human brain

tumors, the altered expression of α2,3 sialyltransferase mRNA in malignant gliomas (12), the ability of 2,6 sialyltransferase gene transfection to inhibit glioma invasivity *in vitro* and *in vivo* (13, 14), the ability of N-acetylglucosaminyltransferase III and V to play a role in regulating glioma invasivity in a stable transfectant of a human glioma cell line (15), as well as the ability of a variety of glycosyltransferase gene transfection studies on the ability to enhance cell death induced by staurosporine, C2-ceramide or etoposide (16).

Case	2,6ST	SNA mRNA	2,6ST
Tumors of neuroepithelial tissue			
Astrocytic tumors			
Astrocytoma (fibrillary)	0/4	0/4	0/1
Anaplastic astrocytoma	0/4	0/4	0/1
Glioblastoma	0/8	0/8	2/4 (0/4)
Pilocytic astrocytoma	0/1	0/1	
Oligodendroglial tumors			
Oligodendroglioma	0/4	0/4	
Anaplastic oligodendroglioma	0/2	0/2	
Ependymal tumors			
Ependymoma	0/5	0/5	
Myxopapillary ependymoma	0/1	0/1	
Choroid plexus tumors			
Choroid plexus papilloma	2/2	2/2	
Choroid plexus carcinoma	1/1	1/1	
Embryonal tumors			
Medulloblastoma	0/5	0/5	
Tumors of cranial and spinal nerves			
Schwannoma	0/3	0/3	0/1
Tumors of the meninges			
Tumors of meningothelial cells			
Meningiomas			
Meningothelial type	10/10	10/10	6/6 (5/6)
Transitional type	7/7	7/7	3/4 (1/4)
Fibroblastic type	0/3	2/3	1/2 (0/2)
Anaplastic meningioma	2/2	2/2	0/2
Tumors of uncertain origin			
Hemangioblastoma	1/3	0/3	
Haemopoetic neoplasms			
Malignant lymphomas	2/2	2/2	
Tumors of the anterior pituitary			
Pituitary adenoma	1/3	0/3	
Local extensions from regional tumors			
Craniopharyngiomas			
Adamantinomatous type	0/2	0/2	
Squamous papillary	2/2	2/2	
Chordomas	5/5	5/5	
Metastatic tumors			
Adenocarcinomas	2/4	2/4	0/2
Squamous cell carcinoma	1/1	1/1	0/1
Clear cell carcinoma	1/1	0/1	0/1
Mixed mesodermal tumor	0/1	0/1	0/1
Adenosquamous carcinoma	1/1	1/1	

Parenthesis shows the number of cases which expressed a high level of -2,6ST mRNA

Table 1: *istochemical examinations and Northern analyses of human brain tumors (SNA, Sambucus nigra agglutinin)*

Xu et al. (17) have reported that β1,4 galactosyltransferase-I, -II, and -V are overexpressed in human astrocytomas and Hoon et al. (18) have shown that ganglioside GM2/GD2 synthetase mRNA can be used as a marker for detecting metastatic neuroblastoma cells in bone marrow.

There have been many reports over the years in which some aspect of glycoconjugate biochemistry (e.g., inhibitors of biosynthesis, addition of cell-surface glycosphingolipids, addition of oligosaccharides or glycopeptides, etc.) has been manipulated leading to an inhibition of tumor growth, metastasis, etc. (19). Of all of these studies, however, only two therapeutic candidates have progressed into clinical trials; swainsonine, an inhibitor of the Golgi-associated α-mannosidase II leading to the inhibition of or alterations in normal N-glycan biosynthesis on many glycoproteins (20) and a GD2-based immunotherapy specifically targeting gliomas (21) that, while showing no toxic side effects, was unable to stimulate antibody formation or effect any tumor regression. A recent review by Rebbaa et al. (22) discuss the modulation of growth factor receptors in brain tumors by complex carbohydrates, yet another interesting pathway for the development of therapeutics.

Glyco-gene expression in brain tumors

Our program, aimed specifically at developing glycobiology-based therapeutics for malignant brain tumors is based primarily on the idea that glioma invasivity can be disrupted by altering the aberrant cell-surface glycosylation patterns found in these cells. We hypothesized that the most direct way to do this would be by directly manipulating glycosyltransferase and/or glycosylhydrolase gene expression in these cells. Our initial strategy was to attempt to identify a glycosyltransferase gene, for example, that was markedly altered in its expression in primary specimens of human brain tumors compared to normal human brain. By starting with the measurement of gene expression changes, the identification of multiple therapeutic targets would be possible: from the transcriptional regulation of gene expression to post-translational modification of the gene products themselves. Clearly for each altered gene there are an abundance of approaches to modulating its expression and the function of its product(s).

To date there are approximately twenty sialyltransferases that have been cloned

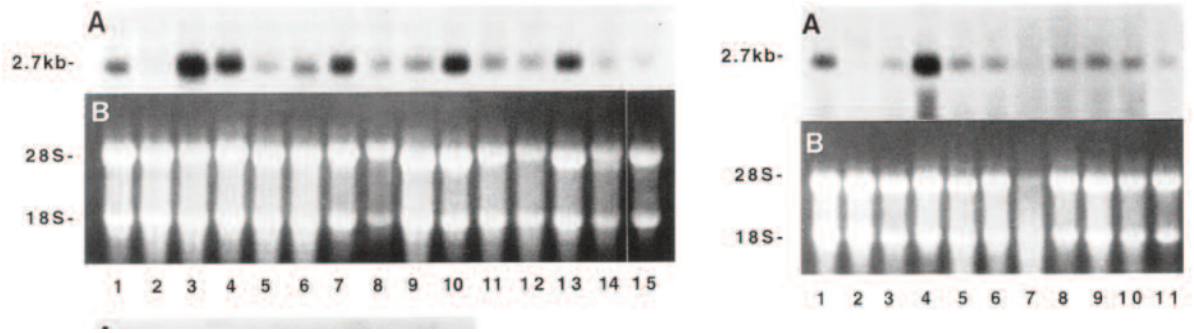

Figure 2: The expression of α2,3ST in glioma specimens (left panel) and brain metastases (right panel). *30 μg of total RNA per lane were used for Northern analysis. Upper panel lane 1: normal human brain, lanes 2-14: clinical glioma specimens, lane 15: U373MG human glioma cell line. Lower panel lane 1: normal human brain, lanes 2-10: clinical specimens of brain metastases, lane 11: U373MG human glioma cell line. All glioma specimens expressed α2,3ST mRNA (A) and 7 out of 9 metastases expressed α2,3ST mRNA. Ethidium bromide staining of total RNA (panel B).*

including O-linked and N-linked as well as the entire series a and b ganglioside sialyltransferases (23). They comprise a structurally related family of molecules that display substrate specificity, tissue specificity, and are all developmentally regulated (24). Studies by Recchi et al. (25) and Marcos et al. (26) showed that sialyltransferase expression in breast cancer cells is markedly altered and that the modulation of their expression can impact tumor cell behavior *in vivo* as well. Alterations in the expression of terminal sialic acid residues on glycoconjugates are typically found in cells undergoing or that have undergone oncogenic transformation (27, 28). Increased cell-surface sialylation has been associated with invasivity, metastatic potential, adhesion to endothelial cells and extracellular matrices and resistance to T-cell-mediated cell death (29).

In our first series of studies, glycosyltransferase and glyosylhydrolase gene expression was evaluated in freshly dissected brain tumor specimens using Northern analyses. Figure 1 shows a synopsis of some of these data. The x-axis of this figure represents ten arbitrarily chosen malignant, grade IV gliomas. The y-axis is the quantity of gene expressed and the z-axis gives the name of the gene measured. The purpose of depicting our data in this way was 3-fold. First, it gave us some sense of the patterning of each gene in a sample of tumors and addressed the question: "What is each gene's expression profile?". Second, it gave us a sense of the patterning of the ensemble of genes, addressing the question: "Is there a pattern that emerges by examining the gene expression patterns of more than one gene at a time?". And third, "What do the relative quantitative values of the gene expression patterns look like compared to each other?". Glioblastomas are complex tumors comprised of heterogeneous cell types. Glyco-genes are abundant-approximately 400-and are differentially expressed and developmentally regulated. Thus, it seemed likely that a complex pattern of gene expression might be expected in this family of tumors as well as a different pattern for each tumor. The data depicted in Figure 1 showed that indeed both the qualitative and quantitative expression of the glyco-genes that we measured were indeed complex and quite variable from tumor to tumor. However, it was also clear that the α2,6 sialyltransferase (α2,6ST) transcript that we measured was virtually absent from all gliomas measured.

This finding led us to our second set of studies in which we directly evaluated the expression of α2,6ST mRNA and the cell-surface expression of α2,6-linked sialic acids in a variety of brain tumors. These results are shown in Table 1. Epithelial-like tumors such as meningiomas, chordomas and craniopharyngiomas often expressed α2,6ST and α2,6-linked sialic acids. However, glioblastomas, oligodendrogliomas, ependymomas, medulloblastomas, and brain metastases were essentially devoid of: (1) detectable α2,6ST mRNA (2) α2,6ST immunohistochemical staining; and (3) cell surface α2,6-linked sialic acids (11).

The lack of expression of the α2,6 ST in malignant gliomas and the fact that the α2,6ST together with the α2,3 sialyltransferase (CMP-NeuAc:Galβ1,3(4)GlcNAc α2,3 sialyltransferase (30); α2,3ST) are the two enzymes responsible for effectively all terminal sialylation of N-linked glycoprotein oligosaccharides led us to the next series of experiments. We examined α2,3ST mRNA expression in panels of primary human

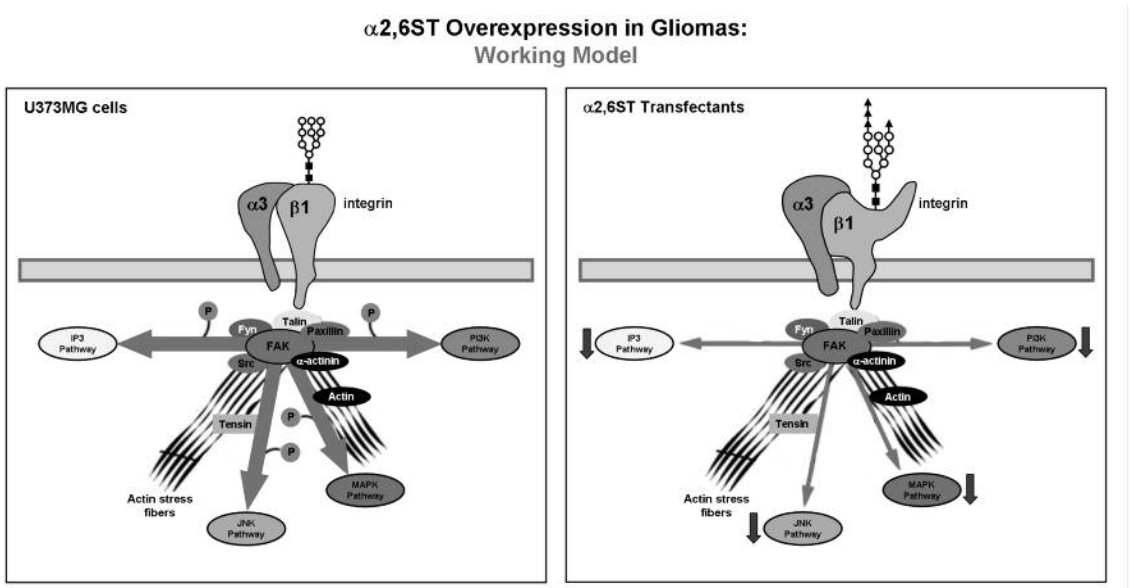

Figure 3: Sialylation-dependent α3β1 integrin signaling.

- *In parental, tumorigenic, U373MG cells, p125fak is present in a phosphorylated form and leads to activation of intracellular signaling cascades mediated by the IP3, PI3K, JNK, and MAPK pathways.*
- *Activation of these pathways impacts the increased adhesivity and invasivity in these cells.*
- *Expression of α2,6ST via stable transfection;*
  - *increases cell surface α2,6-linked sialic acids specifically on the β1 subunit of the α3β1 integrin receptor,*
  - *decreases adherence to fibronectin and collagen matrices,*
  - *decreases adhesion-mediated phosphorylation of p125fak and alters the pattern of focal adhesions,*
  - *decreases intracellular signaling through the IP3, PI3K, JNK, and MAPK pathways, and*
  - *decreases invasivity in vitro and tumorigenesis in vivo.*

brain tumors, cell lines and fetal astrocytes along with the expression of α2,3-linked cell surface sialic acids (12). Figure 2 shows Northern blots from these experiments. It was concluded from these studies and others, using lectins to examine the expression of α2,3-linked sialic acids (12), that gliomas markedly over-express terminal sialic acids compared to normal human brain controls and that it is the α2,3ST as opposed to the α2,6ST that is the principal enzyme involved in this increase in terminal glycoprotein sialylation.

The next step toward the development of our glycobiology-based brain tumor program was to create a model system that reflected the results described above and was based on two working hypotheses: (1) alterations in glioma cell-surface glycosylation should affect tumor cell invasivity and (2) altering the expression of glycosyltransferase gene expression in glioma cells should modify cell-surface tumor cell glycosylation patterns. We thus began by creating a stable, α2,6ST-expressing, human, glioma cell line and evaluating it for a number of properties. We chose the cell line, U373MG because it is derived from a human glioma, is tumorigenic, and does not express α2,6-linked sialic acids. Moreover, adhesion of this cell line to fibronectin or collagen matrices is mediated by the α3β1 integrin receptor since no other -integrins could be detected. This was important because α3β1 integrin expression is increased in glioblastomas compared to normal brain and likely plays an important role in glioma invasivity.

The results from these studies can be summarized as follows: stable transfectants were created that now expressed: (1) α2,6ST mRNA; (2) measurable amounts of α2,6ST enzyme activity; and (3) cell-surface, α2,6-linked-sialic acids. These transfectants showed a significant reduction in adhesivity to the extracellular matrix molecules fibronectin and collagen compared to mock transfected controls and the parental glioma cell line. The α3β1 integrin was found to contain α2,6-linked sialic acids and the tyrosine phosphorylation of p125fak was blocked in the transfectants despite increased expression of $p125^{fak}$ mRNA (13). Integrins interact with the extracellular milieu and act as signal transducers that can mediate glioma cell migration by regulating the function of focal

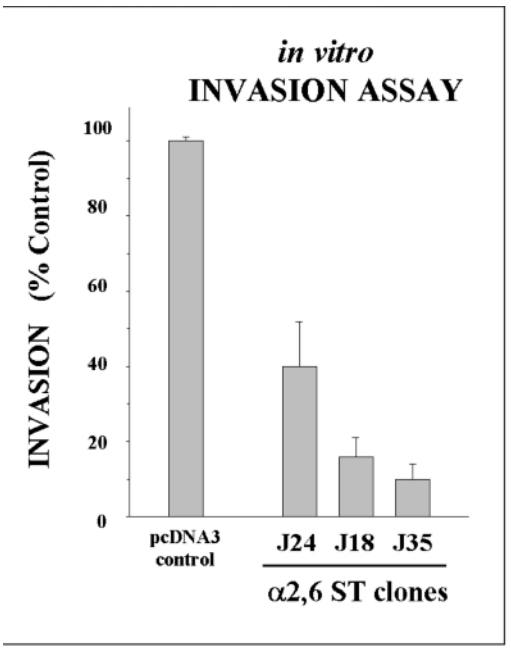

Figure 4: In vitro invasion assay of the α2,6ST-transfected U-373 MG glioma cells. *Biocoat Matrigel Invasion Chambers (Collaborative Research, Bedford, MA, U.S.A.) were used to evaluate the relative invasivity of the transfected subclones compared with pcDNA3 "mock"-transfected controls. The Invasion Chamber consists of two compartments separated by a filter precoated with Matrigel (contains laminin, type IV collagen, entactin, and heparin sulfate). Cell invasion was measured by counting the number of cells that pass through the Matrigel and the 8 micron filter pores. 4 x 104 cells/well were plated into the upper compartment and incubated for 24 hours. 0.5 ml of U373MG conditioned medium was placed in the lower compartment to facilitate chemoattraction. Cells that migrated through the Matrigel were fixed, stained, and the membranes mounted on glass slides and cells counted. Data is expressed as percent transfected cells migrated versus mock-transfected control cells. The data are average ± SEM (bars) values of two separate experiments done in triplicate.*

adhesion proteins such as $p125^{fak}$ through control of their phosphorylation state (13). Thus, our results suggested that by simply stably expressing the α2,6ST gene, we could indeed alter the adhesivity/invasivity of these tumor cells via a well characterized molecular mechanism (Figure 3 and (13)).

In the next set of studies (Figure 4), the α2,6ST U373MG stable transfectants were evaluated for their invasive potential using Biocoat Matrigel Invasion Chambers, as described in the legend. Clearly, various clones of the α2,6ST expressing stable transfectants showed marked inhibition of invasivity compared to parental or mock transfectants. These results were robust enough to merit *in vivo* studies which are shown in Figure 5.

Using the severe combined immunodeficient, SCID mouse model (the details of the methods are described in the Figure 5 legend) we found that U373MG clones expressing the α2,6ST gene showed virtually no brain tumor formation whereas parental cell or mock transfectant-injected SCID mice typically had quite large tumors by comparison. The right-hand panel shows tumor cross sectional area as a percent of control versus tumor cell type injected. And the left-hand panel shows a typical SCID mouse brain six weeks after intracranial injection of either parental cells or transfectants, as indicated. Again, it can be seen that SCID mice injected with the α2,6ST expressing stable transfectants have no detectable tumor formation (14).

From these results it was felt that our approach had clear therapeutic potential. The α2,6ST transfectants completely suppressed human glioma invasivity in vitro and tumor formation itself in vivo. Mechanistically the data strongly suggested that we had modified the glycosylation of the key integrin in gliomas, 3 1 which, in turn, had altered its signal transducing capabilities and modified cell-extracellular adhesion properties of the tumor cells.

Glycobiology-based microarrays for novel therapeutic target identification

The steady-state expression of the oligosaccharides associated with a specific cell-surface glycoconjugate is the result of the concerted expression of all of the glycosyltransferases involved in its biosynthesis and the glycosidases, involved in its degradation (31). In a system as complex as brain tumorigenesis and invasion it seems as important to determine global context or pattern of these changes as it does to determine the aberrantly expressed, individual glyco-related gene changes found in such systems.

Microarray-based approaches have emerged as one of the cornerstone technologies in rapid throughput gene expression analyses and have made significant impact defining aberrant gene expression in a multitude of tumor systems. In order to begin to grasp the intrinsically complex regulation of genes responsible for the synthesis and degradation of specific carbohydrate structures that are essential for mediating glioma invasivity, we have created a microarray core facility to provide a comprehensive platform of glycoconjugate metabolism-associated oligonucleotides assuring the most up-to-date coverage of these gene families.

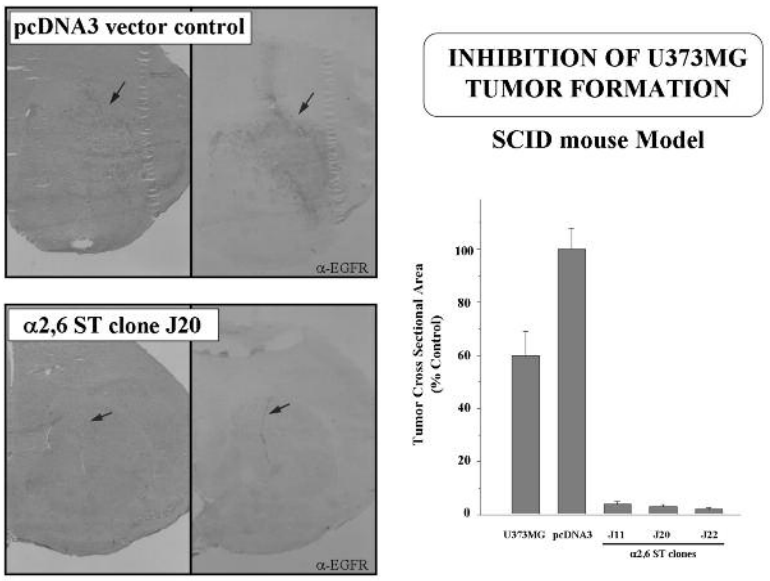

Figure 5: Intracranial tumorigenicity of α-2,6ST transfectants. *Glioma cells (1.25 x 106) were injected into the basal ganglia of SCID mice (at 6 weeks of age) with the assistance of a stereotactic frame. Six weeks later, the brains were harvested, and sections were stained with H&E (left panels) or antihuman EGF-receptor antibody (right panels). The control pcDNA3 vector-transfected U373MG cells formed large tumors (arrows). The α2,6ST transfectants formed no tumors, and only the needle-track was stained (arrows). Differences in tumor size among the animal groups were determined by χ2 analysis of the maximum cross-sectional area of each tumor. Ten mice/transfectant were used in each group: pcDNA3 vector-transfected U373MG cells as a control, and three α2,6ST-transfected U373MG clones (J11, J20, and J22).*

The 359 genes comprising our Human Glycobiology microarray are compiled from currently available NCBI/EMBL/TIGR human sequence databases and the Consortium for Functional Glycomics-CAZY databases. Unique sense 45-mer oligonucleotides corresponding to mRNAs of each gene used as probes are individually synthesized, purified and immobilized via a 5'-amino linker onto aldehyde-coated microarrays. Total RNA is reverse transcribed and used as the substrate for RNA amplification and labeling using the indirect aminoallyl methodology based on the Eberwine protocol (32). To circumvent the inherent biological heterogeneity of clinical GBM specimens, we employ a universal reference design (33) and comprehensive statistical analysis platforms to facilitate acquisition of expression profiles from a necessarily large number of biological and technical replicates. We have found that our high quality, application-specific, low density microarray platform provides an efficient strategy for such an endeavor.

Focused microarray analyses comparing a panel of Grade IV gliomas with a panel of aged-matched normal brain specimens yielded 11 significant genes more highly expressed in gliomas compared to normal brain and 25 genes more highly expressed in normal brain compared to gliomas (34). Clearly there are many significant differences in expression of genes associated with glycoconjugate biosynthesis and degradation, many of them novel and all of them potential targets for the development of therapeutics for the treatment of brain tumors. The current and future direction of our program includes: (1) increasing the number of primary tumors analyzed by our microarrays, (2) evaluating human glioma cell lines by microarray analysis: Cell lines can provide powerful model systems to study the regulation of a given gene or associated gene family, (3) confirming and extending our microarray data with in situ hybridization studies and quantitative RT-PCR analyses, and (4) continuing to evaluate therapeutic candidates using viral vectors in preclinical mouse models.

Clinical significance

The data that we have obtained to date strongly suggest that a clinical trials program should be undertaken. Both the data that we have obtained in primary tumors and multiple tumor models together with the well established role that glycoconjugates play in modulating tumor metastasis and invasivity support this. However, significant challenges remain, for it will be difficult to find the right clinical setting to undertake a human Phase I gene-based clinical trial for malignant brain tumors and optimizing the delivery system to insure effective gene delivery to tumor cell targets will have to be addressed.

Acknowledgments

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