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Genetic Analysis of Intracranial Tumors in a Murine Model of Glioma Demonstrate a Shift in Gene Expression in Response to Host Immunity

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Abstract

For the study of malignant glioma, we have previously characterized a highly tumorigenic murine astrocytoma, SMA-560, which arose spontaneously in an inbred, immunocompetent VM/Dk mouse. Using this cell line as a model of murine glioma, we performed DNA microarray analysis of autologous normal murine astrocytes (NMA) and SMA-560 tumor cells grown in monolayer culture or intracranially in syngeneic immunocompetent or immunocompromised hosts in order to determine whether tumors grown *in vitro* recreate the complex genetic regulation that occurs *in vivo*. Our findings support our hypothesis that glioma phenotype *in vitro* may be quite different *in vivo* and significantly altered by *in situ* growth factors and other invading cell populations.

Keywords

brain tumors; host response; DNA microarray; gene profiling

Introduction

Despite the negligible incidence of metastasis, aggressive surgery and the application of novel treatments, malignant gliomas remain one of the most lethal forms of cancer (Wheeler et al., 2004). This is due in large part to malignant brain tumors being relatively resistant to all forms of conventional or standardized therapy (Pulkkanen and Yla-Herttuala, 2005). Recent studies aimed at developing anticancer agents for glioma have begun to focus more on drugs that target specific molecules or processes selectively involved in glioma growth, survival and progression, rather than general cytotoxic agents (Ochiai et al., 2004; Sampson et al., 2000;

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Sampson et al., 2005). In general, the advantages of targeted therapy include decreased healthy tissue toxicity and the availability of molecular markers that serve as indicators of drug action, as well as improved patient morbidity and mortality.

Yet, despite best efforts, targeted therapy for malignant glioma has not been universally successful. This lack of success can be attributed to a number of critical factors, including well-documented tumor cell heterogeneity (Liang et al., 2005; Nutt et al., 2003), antigen modulation (Cavenee, 2002; Louis, 2005) and the existence of multiple, redundant survival pathways (Heimberger et al., 2002; Learn et al., 2004). Thus, it would appear that further development of target-based approaches to treating solid tumors such as malignant glioma requires a better understanding of the underlying biology that determines the expression of potential targets and their resistance (Mellinghoff et al., 2005).

Experimental systems utilizing monolayer culture for therapeutic development have been indispensable in identifying both cytotoxic agents and mechanisms of resistance. For many cancers, these experimental models have provided significant information in regards to antigens and mechanisms mediating tumor survival and response to therapy. Investigations into the biology of brain tumors have also routinely used cell lines grown or maintained *in vitro* to gain information. However, when glioma cell lines are evaluated in monolayer culture, their sensitivity to treatment is surprisingly similar to cell lines originating from other cancers that typically respond to therapy (Deschavanne and Fertil, 1996). This would suggest that brain tumor cells grown *in vitro* undergo selection away from their *in vivo* characteristics such that a unique growth environment that includes the blood-brain barrier and circulating immune cells plays a significant role in restricting drug availability or regulating cell phenotype. Yet, the assumption that tumor cells cultured *in vitro* simulate the phenotype of the tumor cells *in vivo*, especially in the case of glioma, has come under review recently (Camphausen et al., 2005; Lee et al., 2006). As such, a central question that has resulted from this debate has become if tumor cells grown *in vitro* do not utilize the same genes *in vivo*, what affects this change, which genes are then expressed and why these genes? It is our contention that determining the expression signatures from normal astrocytes and tumor cells grown *in vitro* versus *in vivo* from a spontaneous malignant astrocytoma model in the mouse would help to generate a more complete understanding of brain tumor biology and the factors producing these changes. Thus, for the investigations described here, we hypothesize that glioma expression profiles *in vitro* may be quite different *in vivo* and significantly altered by *in situ* growth factors and other invading cell populations. Testing this hypothesis could provide evidence for which models may be most appropriate for specific types of glioma investigation.

In order to define the differences in gene expression that exist between normal astrocytes and brain tumors, we have previously developed and utilized here a cell line, SMA-560, derived from an astrocytoma that arose spontaneously in an inbred, immunocompetent VM/Dk mouse (Ashley et al., 1998; Sampson et al., 1997). This transplantable astrocytoma mirrors human gliomas phenotypically and morphologically, secretes immunosuppressive cytokines secreted by human gliomas, and, because this tumor arose spontaneously, it may most closely resemble human tumors of this type. Therefore, we believe that this model may be the most relevant to the clinical condition, as purely *in vitro* or xenograft models and screens for glioma have thus far yielded limited applicability to human disease. For the investigations described herein, we used DNA microarray analysis to compare and contrast the expression profiles of normal astrocytes and SMA-560 tumors grown as a monolayer or *in vivo*. By using either syngeneic immunocompetent mice or athymic nude mice, we could determine the effect growth in a syngeneic and immunocompetent surrounding had on intracranial (i.c.) tumor gene expression. Hierarchical clustering and principal components analysis reveal that specific genes regulating tumor cell response *in vitro* are not the same as those for tumor cells *in vivo*. Furthermore, these data indicate that statistically validated expression signatures of cells grown *in vitro* versus *in*

vivo are dependent upon the experimental model and influenced by the presence of stromal and infiltrating cells.

Materials and Methods

Cell Lines, Tissue Culture, Intracranial Tumor Implantation and Tissue Extraction

For the purpose of these investigations, the previously characterized highly tumorigenic murine astrocytoma, SMA-560, which arose spontaneously in an inbred, immunocompetent VM/Dk (H-2b) mouse, has been established as a serially passaged tumor cell line and shown to be free of mycoplasma contamination (Sampson et al., 1997). All tissue culture was performed in antibiotic-free zinc option medium (Invitrogen, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Invitrogen), as previously described (Learn et al., 2004). For implantation of tumors and as described previously, cells were resuspended in 2.5% methylcellulose, and the lethal tumorigenic dose of 1×10^4 SMA-560 tumor cells was injected i.c. into immunocompetent VM/Dk mice or athymic nude BALB/c mice (8-12 weeks in age, 20-22 g in weight; Jackson Laboratory, Bar Harbor, Maine) in a volume of 5 μ l by using a 250- μ l Hamilton syringe and injector (Hamilton Co., Reno, NV) with an attached 25-gauge needle (Heimberger et al., 2000). The needle was positioned along the coronal suture, 2 mm to the right of bregma and 4 mm below the surface of the skull, by using a stereotactic frame (Kopf Instruments, Tujunga, CA). Intracranial tumors were harvested 17 days post-implantation, when all mice were still physically active and showed no overt signs of pathology or disease. At time of animal sacrifice, isolation of brain tissue for whole normal brain or brain with solid tumor was performed. For brain with tumor, brain was removed in total and a 2 mm³ section of tumor was sharply dissected from the surrounding right frontal lobe from where it was implanted.

Isolation of Normal Murine Astrocytes

Normal murine astrocytes from 3-day old VM/Dk pups were isolated as previously described (Nikceovich et al., 1999). Briefly, whole brain was removed from neonatal mouse pup cerebral cortices and mechanically disrupted in tissue culture plates containing media, resulting in a mixed glia culture. Cells from 4-5 mouse pup whole brains were plated into a 25 cm² flask. At this density, a small number of neurons and process-bearing microglia survive, but the majority of the population are astrocytes. Cells were maintained in DMEMH-/10%FCS/pen/strep/glutamine (Invitrogen) in 37°C/5% CO₂, with media changes every 2-3 days. Following one week in mixed glia culture, cells were agitated in an incubated orbital shaker at 220 rpm for 16 h at 37°C. Detached microglia cells were discarded, and the remaining adherent cells, the vast majority being astrocytes, were cultured for another 2-4 weeks to expand population size. To ensure purity of cells at time of harvest, a portion of each astrocyte isolation was analyzed by FACS with anti-GFAP (positive control, Molecular Probes, Eugene, OR) and anti-CD11b (negative control, BD Biosciences, San Jose, CA) antibodies and purity was determined to be $\geq 98\%$. A total of three separate, individual isolations were performed for these investigations.

RNA Isolation, Amplification and Generation of cDNAs, Probe Preparation and Microarray Hybridization

Total RNA from cultured normal murine astrocytes from neonatal VM/Dk pups or SMA-560 tumor cells cultured *in vitro* or *in vivo* as described above were isolated using an RNeasy RNA extraction kit (Qiagen Inc., Valencia, CA). For RNA isolation from SMA-560 monolayer culture, three different, low passage stock lines were used. Total RNA quality was assayed using an Agilent bioanalyzer (Silicon Genetics, Redwood City, CA, USA), per the Duke Microarray Core Facility (DMCF). Isolated total RNA passing quality control assessment was then amplified as previously described (Potti et al., 2006). Briefly, amplified mRNA was assayed on 'in-house' printed oligonucleotide DNA microarrays per the DMCF, using the

Operon Murine Genome Oligo Set Version 3.0 (Operon, Huntsville, AL) that possesses 32,640 optimized 70mers, representing approximately 24,000 genes. Therefore and as is customary, these printed arrays contain different sequences but functionally redundant oligomers for many annotated genes. Data analyses were performed using Genespring software v. 7.2 (Silicon Genetics). Total RNA (2 µg) from each sample and a reference RNA (Universal Murine Reference RNA, Stratagene, La Jolla, CA) was used in probe preparation. Briefly, reverse transcription is driven by an oligo (dT) primer bearing a T7 promoter using ArrayScript (Ambion, Austin, TX). The cDNA then undergoes second strand synthesis and clean-up to become a template for *in vitro* transcription with T7 RNA polymerase. To maximize RNA yield, Ambion's proprietary MEGAscript *in vitro* transcription technology is used to generate amplified RNA (aRNA). The antisense aRNA is then fluorescently labeled with Cy3 (reference) and Cy5 (sample). Sample and reference aRNAs were pooled, mixed with 1X hybridization buffer (50% formamide, 5X SSC, and 0.1% SDS), COT-1 DNA, and poly-dA to limit non-specific binding, and heated to 95°C for 2 minutes. This mixture was pipetted onto a microarray slide, a cover slip placed, and hybridized overnight at 42°C. The array was then washed at increasing stringencies, and scanned on a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Detailed protocols are available on the DMCF web site. All steps involved in RNA processing, probe preparation, microarray hybridization, and data processing used MIAME (Minimal Information About a Microarray Experiment)-guidelines established by the Microarray Gene Expression Data Society.

Confirmation of Microarray Results by Real-Time RT-PCR

To validate the hypotheses generated by the microarray study, quantitative RT-PCR was performed on selected candidate genes. Two genes were chosen according to multiple testing procedures and their differential levels of expression across all samples. The PCR primers specific to these genes were designed using ABI Primer Express Software Version 2.0 (Applied Biosystems, Foster City, CA). All of the primers were designed with the melting temperatures 58-60°C and resulting products between 100 and 150 bp. For each sample, a small aliquot of extracted total RNA was saved for quantitative RT-PCR (separate from the total RNA used for linear amplification). cDNA was transcribed from total RNA using Powerscript reverse transcription kit (Invitrogen) in a 20 µL reaction volume. One µL of cDNA was then used in triplicate reactions using iQ SYBR Green Supermix (Bio-Rad). The PCR conditions were as follows: 3 min at 95°C, 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s in the Bio-Rad iQ Real-time sequence Detection System (Bio-Rad). Expression levels of the murine genes interferon-activatable 202B (TaqMan Gene Expression Assay# Mm00839397_m1 from ABI) and Ia-associated Invariant Chain (TaqMan Gene Expression Assay# Mm00658576_m1 from ABI) were checked in all cells using the TaqMan Gene Expression Assay System. All gene expression levels were normalized with murine GAPDH (TaqMan Gene Expression Assay# Mm99999915_g1 from ABI).

Data Processing and Statistical Analysis

Genespring v. 7.2 (Silicon Genetics) was used to perform initial data analyses. Intensity dependent (Lowess) normalization was performed on the entire dataset. Based on triplicates of each condition, a threshold of 2-fold change in expression relative to control, and a 2-way ANOVA with a p-value cutoff of 0.05 was performed. Expression of each gene was reported as the ratio of the value obtained for each condition relative to control conditions after data normalization. For the microarray data, all analyses were performed as relative comparisons between normal brain tissues versus SMA-560 tumor tissues. Thus, genes that were upregulated in normal tissues were correspondingly downregulated in tumor tissues to the same extent relative to reference RNA levels and *vice-versa*, so that complete data sets are described.

Results

Hierarchical clustering analysis of normal astrocytes and SMA-560 tumors *in vitro* and *in vivo* reveals significantly different expression profiles dependent upon the experimental model

Investigations into glioma cell biology have often utilized various adapted cell lines and xenogenic models to dissect and define mechanisms underlying this disease and its pathology (Gutmann et al., 2002; Reist et al., 1995; Wikstrand et al., 1998). To this end, we have previously established and characterized the murine astrocytoma tumor cell line, SMA-560, that arose spontaneously in syngeneic VM/Dk mice and which closely recapitulates human glioma *in vivo* (Ashley et al., 1998; Sampson et al., 1997; Serano et al., 1980). For our investigations here we determined gene expression in normal astrocytes or SMA-560 tumor cells transplanted into athymic or syngeneic immunocompetent mice, two traditionally used experimental models. Our *in vitro* studies analyzed gene expression in normal astrocytes isolated from three day old VM/Dk pups versus established, low passage number SMA-560 tumor cell lines. Our *in vivo* work analyzed expression profiles from total normal brain versus isolated solid i.c. SMA-560 tumors from immunocompetent syngeneic VM/Dk mice or athymic nude BALB/c mice 17 days post tumor challenge. Briefly, specific cell populations were isolated and total RNA was purified and used in DNA microarrays as described in the *Materials and Methods*.

Following standard quality control analysis and external validation by quantitative real-time RT-PCR, resulting microarray data were filtered on level of gene expression in both normal astrocytes and SMA-560 tumors: for *in vitro* comparisons (NMA versus SMA-560 tumor cell lines), a total of 11,253 genes; for *in vivo* studies (whole normal brain versus i.c. SMA-560 tumors), 10,443 genes in immunocompetent mice, and 11,848 genes in athymic nude mice. Significantly different values between normal astrocytes (NMA or whole normal brain) and SMA-560 tumor cells grown under two different conditions, *in vitro* or *in vivo*, were determined by ANOVA ($p < 0.05$; 7,041 genes *in vitro*, 3,328 in immunocompetent background, and 297 in athymic nude background). Based on this, we performed an unsupervised analysis on all samples by hierarchical clustering (Figure 1; complete gene lists for this and all analyses described are available at <http://data.cgt.duke.edu/Learn.php>). The utility of this type of analysis helps to define coordinate regulation of similarly related genes, often termed 'gene trees,' within a group or population. In analyzing our data, coordinate regulation of various 'branches' of these gene trees was as expected fundamentally different between normal astrocytes and SMA-560 tumors either *in vitro* or *in vivo*. A detailed analysis of the genes that make up these differential profiles is described later. These data support the notion that expression profiles in normal astrocytes and SMA-560 tumors exhibit fundamental differences at the level of transcription, as expected.

Scatter analysis of SMA-560 tumors quantifies statistically significant changes in gene expression that are dependent upon the experimental model

To further investigate the expression profiles generated from microarraying, scatter analysis was performed on normal astrocytes and SMA-560 tumors from *in vitro* or *in vivo* conditions. Again, significantly different gene expression between normal astrocytes and SMA-560 for all experimental conditions was determined by ANOVA ($p < 0.05$, Figure 2). For our *in vitro* studies, 4,308 genes were determined to be significantly different between NMA and SMA-560 cell lines by a factor of two or greater. A total of 2,016 transcripts were increased or upregulated 2-fold or more above reference RNA levels in NMA compared to the SMA-560 cell line, while 2,292 transcripts were increased 2-fold or greater above reference in the SMA-560 cell line in comparison to NMA. In immunocompetent mice, 1,326 genes were determined to be significantly different between whole normal brain and i.c. SMA-560 tumors by a factor of

two or greater. A total of 541 transcripts were upregulated 2-fold or more above reference RNA levels in whole normal brain compared to i.c. SMA-560 tumors, while 785 transcripts were increased 2-fold or greater above reference in i.c. SMA-560 tumors in comparison to whole normal brain. In athymic nude mice, 138 genes were determined to be significantly different between whole normal brain and i.c. SMA-560 tumors by a factor of two or greater. A total of 77 transcripts were upregulated 2-fold or more above reference RNA levels in whole normal brain compared to i.c. SMA-560 tumors, while 61 transcripts were increased 2-fold or greater above reference in i.c. SMA-560 tumors in comparison to whole normal brain. As described later, major differences in levels of transcripts between *in vitro* and *in vivo* conditions occur along biological pathways such as cellular communication, growth and maintenance, receptor binding and catalytic activity. Thus, we observed that in comparison to monolayer culture, there were substantially fewer transcripts upregulated two-fold or greater *in vivo* for whole normal brain or i.c. SMA-560 tumors in either immunocompetent or immunocompromised mice. In addition, we conclude that statistically validated gene expression between groups is fundamentally different and is a function of the experimental model in which the profiles are generated.

Principal components analyses demonstrate convergent gene profiles for normal astrocytes and SMA-560 tumors *in vivo*

Principal components analysis is a decomposition technique that produces a set of expression patterns known as principal components. Linear combinations of these patterns are assembled to represent the behavior of all genes in a given sample, characterizing the most abundant themes or building blocks that reoccur in many genes of that sample. Expression values determined by ANOVA produce principal components for normal astrocytes or SMA-60 tumors *in vitro* and *in vivo*, based on the first three principal components statistically generated by Genespring Software Analysis (Figure 3, **A.-F.**). We observe that due to their vastly different gene expression *in vitro*, NMA (**A.**) and SMA-560 (**B.**) cell lines segregate independently of one another in a three-dimensional model. In contrast, we find that principal components for whole normal brain (**C., E.**) and i.c. SMA-560 tumors *in vivo* (in both immunocompetent (**D.**) and athymic nude mice (**F.**)) project to similar regions in three-dimensional space, based on the smaller statistical differences between these populations and/or their utilization of similar genes and pathways *in vivo*. These data provide evidence that normal astrocytes or SMA-560 tumors are defined by the expression signatures strongly dependent on a specific experimental condition.

Analysis of expression profiles in normal brain and SMA-560 tumors demonstrates that expression of specific genes in normal brain or SMA-560 tumors is shared across experimental conditions

Following statistical analysis, we next quantified how gene expression occurred and was shared across multiple experimental conditions. We compared expression of normal astrocytes (NMA versus immunocompetent whole normal brain versus immunocompromised whole normal brain) or SMA-560 cells (cell line versus immunocompetent i.c. SMA-560 tumors versus immunocompromised i.c. SMA-560 tumors) *in vitro* and *in vivo* and found overlap between the various experimental conditions using Venn diagram analysis (Figure 4). In regards to expression of specific genes, we found a total of only 126 genes are commonly expressed among the various experimental conditions for normal astrocytes. However, for SMA-560 cells we found twice as much shared gene expression (a total of 256) among the various experimental conditions. Notably, many of these genes in SMA-560 cells (both *in vitro* and *in vivo*) are involved in immunomodulatory functions, which are more fully described later. As such, these data support the notion that expression of specific genes in particular cell types are important, regardless of experimental conditions.

Specific biological pathways are engaged in SMA-560 tumors, dependent upon the experimental model

Based on data presented in Figures 1 and 2, we next investigated specific 'gene tree' pathways and the numbers of genes involved in each pathway that uniquely produce the differential expression profiles we observe in SMA-560 tumors *in vitro* and *in vivo*. To begin, we performed gene ontology pathway analyses in which we analyzed the number of genes involved in annotated biological processes (Table 1), based on ANOVA. Using this type of analysis, we find substantial changes in the pathways for each cell type again based on experimental model. *In vitro*, there is considerable gene expression in these tumors along pathways of cell growth, receptor binding and catalytic activity, compared to normal astrocytes. In immunocompetent mice, these same pathways upregulate considerably fewer transcripts compared to that in monolayer culture, with the notable exception that immunoregulatory gene expression is increased. In athymic nude mice, statistically validated expression along all pathways is substantially decreased in comparison to both monolayer culture and immunocompetent mice. Such observations provide clues to this tumor's ability to respond to its environment and the specific gene expression that results in order to maximize growth, survival and/or resistance.

Gene expression in SMA-560 tumors is affected by the presence of T-cells

To further investigate the composition of the differential expression profiles observed in SMA-560 cells (cell line versus immunocompetent i.c. SMA-560 tumors versus immunocompromised i.c. SMA-560 tumors), we investigated the genes that notably defined the profile in each experimental model, i.e. *in vitro* versus immunocompetent mice versus athymic nude mice. In doing so, we began by identifying genes that were the most up- or downregulated in a population or were found to be similarly regulated and belonging to a group of genes that are involved in a particular biological process. Our analysis revealed that the largest cohort of genes identified, that fit both these criteria, are genes implicated in affecting immune regulation and function (Table 2). In panel A, we identified increased expression in i.c. tumors grown in immunocompetent VM/Dk mice for genes involved in the processes of antigen presentation, interferon-induced protein expression and intracellular signaling. Specifically, transcripts for MHC Class I and II, interferon-activatable proteins p202B, p204 and p205 as well as other interferon-induced proteins on average were upregulated 2 to 40-fold in these tumors, in comparison to levels in normal brain as well as SMA-560 monolayer culture. Interestingly, in panel B, several genes from the statistically validated list in panel A were also up regulated in i.c. tumors from athymic nude mice, but not to the same extent or level observed in i.c. tumors from immunocompetent mice. Most of the genes listed here are known to directly affect immunity, and give us reason to speculate that their coordinate expression in SMA-560 tumors could result from a robust, yet ineffective response to this tumor.

Discussion

In glioma as with most other cancers, the bulk of our current knowledge of tumor survival and therapeutic response has been generated from *in vitro* monolayer culture of cell lines derived from solid tumors. However, a recent study has called into question the validity of this type of investigation for the study of malignant glioma given the fundamentally different conditions encountered *in situ* for this disease (Camphausen et al., 2005; Lee et al., 2006). In the studies described here, we have attempted to address the potential influences that define a tumor cell's phenotype based on a limited number of experimental conditions. The brain provides a unique and difficult environment to simulate, and as such, our investigations are novel in that they take into account the influences of various *in situ* tumor milieus for a syngeneic glioma model. We believe that a basic understanding of how a model system affects our interpretation of the

biology of malignant glioma is necessary and required for the design and application of the most effective strategies for treating this disease clinically.

The data presented here support our hypothesis that SMA-560 mRNA expression profiles are a function of the experimental condition. Genetic analysis of these profiles is a powerful tool that helps to explain the cellular phenotypes observed in the various experimental models, while providing further evidence of how tumors may work to dysregulate a host response. We were able to determine statistically valid unique genetic signatures for these tumors that depend on their environment. For example, while normal murine astrocytes and SMA-560 cells utilize vastly different expression signatures for growth *in vitro*, both utilize many of the same genes *in vivo*.

Interestingly, many of the genes that define the *in vivo* tumors are manifest in immunoregulatory gene expression. SMA-560 tumors grown intracranially significantly upregulate transcript levels for major histocompatibility complex Class I and Class II as well as interferon-activatable proteins 202B, -204, and -205. Most of these genes are known to be upregulated by interferon-gamma (IFN- γ), which is likely produced by T-cells that infiltrate these tumors (Gingras et al., 1995; Strik et al., 2004; Tang et al., 2005). The possibility of CD4⁺CD25⁺CD45RO⁺FoxP3⁺ regulatory T-cell (T_{reg}) presence and/or activity may explain why these tumors are not immunologically rejected, yet retain the ability to respond to their environment. Recent studies from our laboratory have demonstrated an increased systemic fraction of immunosuppressive T_{regs} in patients with malignant glioma (Fecci et al., 2006a). We have also shown that local tumor suppression of T-cell immunity can be effectively mediated in part by T_{regs} (Fecci et al., 2006b). Thus, we propose that immunoregulatory gene expression by glial tumors may be a multifactorial, integrated response to both systemic signals and local environment.

Although numerous other genes are co-expressed at increased levels in i.c. tumors in both immunocompetent and immunocompromised mice, suggesting a central role for those genes in tumor growth and survival, the genes that are not shared between these two populations may provide clues as to how these tumors escape immunologic elimination. As such, 'novel targets' for glioma therapy may not only be classical tumor-specific or tumor-associated antigens, but rather more complex, interactive sets of genes and cells involved in tumor evasion of host immunity. For example, recent studies have shown that some tumors are capable of dysregulating entire immunologic signaling pathways (Orchard et al., 2005; Whiteside, 2006) and subsets of normal healthy cells in the host (Fecci et al., 2006a; Fecci et al., 2006b). To affect meaningful tumor therapy for glioma, targeting tumor-induced deficits at multiple levels will likely have to be addressed as well.

The data presented here support the notion that malignant brain tumors respond specifically to their environment by changing their genetic signatures. While the relevance and application of the findings addressed here to clinical disease remain to be defined, the data presented further support the premise that model systems need to be critically evaluated in the context of the results they produce.

References

- Ashley DM, Kong FM, Bigner DD, Hale LP. Endogenous expression of transforming growth factor beta1 inhibits growth and tumorigenicity and enhances Fas-mediated apoptosis in a murine high-grade glioma model. *Cancer Research* 1998;58:302–309. [PubMed: 9443409]
- Camphausen K, Purow B, Sproull M, Scott T, Ozawa T, Deen DF, Tofilon PJ. Influence of *in vivo* growth on human glioma cell line gene expression: convergent profiles under orthotopic conditions. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:8287–8292. [PubMed: 15928080]

- Cavenee WK. Genetics and new approaches to cancer therapy. *Carcinogenesis* 2002;23:683–686. [PubMed: 12016138]
- Deschavanne PJ, Fertl B. A review of human cell radiosensitivity in vitro. *International Journal of Radiation Oncology, Biology, Physics* 1996;34:251–266.
- Fecci PE, Mitchell DA, Whitesides JF, Xie W, Friedman AH, Archer GE, Herndon JEI, Bigner DD, Dranoff G, Sampson JH. Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma. *Cancer Research* 2006a;66:3294–3302. [PubMed: 16540683]
- Fecci PE, Sweeney AS, Grossi PM, Nair S, Learn CA, Mitchell DA, Cui X, Cummings TJ, Bigner DD, Gilboa E, Sampson JH. Systemic Anti-CD25 Monoclonal Antibody Administration Safely Enhances Immunity in Murine Glioma without Eliminating Regulatory T-cells. *Clinical Cancer Research* 2006b; 12:4294–4305. [PubMed: 16857805]
- Gingras MC, Roussel E, Roth JA, Moser RP. Little expression of cytokine mRNA by fresh tumour-infiltrating mononuclear leukocytes from glioma and lung adenocarcinoma. *Cytokine* 1995;7:580–588. [PubMed: 8580376]
- Gutmann DH, Huang Z.-y. Hedrick NM, Ding H, Guha A, Watson MA. Mouse glioma gene expression profiling identifies novel human glioma-associated genes. *Annals of Neurology* 2002;51:393–405. [PubMed: 11891838]
- Heimberger AB, Crotty LE, Archer GE, McLendon RE, Friedman A, Dranoff G, Bigner DD, Sampson JH. Bone marrow-derived dendritic cells pulsed with tumor homogenate induce immunity against syngeneic intracerebral glioma. *Journal of Neuroimmunology* 2000;103:16–25. [PubMed: 10674985]
- Heimberger AB, Learn CA, Archer GE, McLendon RE, Chewning TA, Tuck FL, Pracyk JB, Friedman AH, Friedman HS, Bigner DD, Sampson JH. Brain Tumors in Mice Are Susceptible to Blockade of Epidermal Growth Factor Receptor (EGFR) with the Oral, Specific, EGFR-Tyrosine Kinase Inhibitor ZD1839 (Iressa). *Clinical Cancer Research* 2002;8:3496–3502. [PubMed: 12429640]
- Learn CA, Hartzell TL, Wikstrand CJ, Archer GE, Rich JN, Friedman AH, Friedman HS, Bigner DD, Sampson JH. Resistance to tyrosine kinase inhibition by mutant epidermal growth factor receptor variant III contributes to the neoplastic phenotype of glioblastoma multiforme. *Clinical Cancer Research* 2004;10:3216–3224. [PubMed: 15131063]
- Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, Pastorino S, Purow BW, Christopher N, Zhang W, Park JK, Fine HA. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell* 2006;9:391–403. [PubMed: 16697959]
- Liang Y, Diehn M, Watson N, Bollen AW, Aldape KD, Nicholas MK, Lamborn KR, Berger MS, Botstein D, Brown PO, Israel MA. Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme. *Proceedings of the National Academy of Sciences of the United States of America* 2005;102:5814–5819. [PubMed: 15827123]
- Louis DN. Molecular Pathology of Malignant Gliomas. *Annual Review of Pathology: Mechanisms of Disease* 2005;1:97–117.
- Mellinghoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, Lu KV, Yoshimoto K, Huang JHY, Chute DJ, Riggs BL, Horvath S, Liau LM, Cavenee WK, Rao PN, Beroukhi R, Peck TC, Lee JC, Sellers WR, Stokoe D, Prados M, Cloughesy TF, Sawyers CL, Mischel PS. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors.[see comment]. *New England Journal of Medicine* 2005;353:2012–2024. [PubMed: 16282176]
- Nikcevich KM, Piskurich JF, Hellendall RP, Wang Y, Ting JP. Differential selectivity of CIITA promoter activation by IFN-gamma and IRF-1 in astrocytes and macrophages: CIITA promoter activation is not affected by TNF-alpha. *Journal of Neuroimmunology* 1999;99:195–204. [PubMed: 10505975]
- Nutt CL, Mani DR, Betensky RA, Tamayo P, Cairncross JG, Ladd C, Pohl U, Hartmann C, McLaughlin ME, Batchelor TT, Black PM, von Deimling A, Pomeroy SL, Golub TR, Louis DN. Gene expression-based classification of malignant gliomas correlates better with survival than histological classification. *Cancer Research* 2003;63:1602–1607. [PubMed: 12670911]
- Ochiai H, Moore SA, Archer GE, Okamura T, Chewning TA, Marks JR, Sampson JH, Gromeier M. Treatment of intracerebral neoplasia and neoplastic meningitis with regional delivery of oncolytic recombinant poliovirus. *Clinical Cancer Research* 2004;10:4831–4838. [PubMed: 15269159]

- Orchard J, Ibbotson R, Best G, Parker A, Oscier D. ZAP-70 in B cell malignancies. *Leukemia & Lymphoma* 2005;46:1689–1698. [PubMed: 16263570]
- Potti A, Bild A, Dressman H, Lewis DA, Nevins J, Ortel T. Gene-expression patterns predict phenotypes of immune-mediated thrombosis. *Blood* 2006;107:1391–1396. [PubMed: 16263789]
- Pulkkanen K, Yla-Herttuala S. Gene Therapy for Malignant Glioma: Current Clinical Status. *Molecular Therapy* 2005;12:585–598. [PubMed: 16095972]
- Reist CJ, Archer GE, Kurpad SN, Wikstrand CJ, Vaidyanathan G, Willingham MC, Moscatello DK, Wong AJ, Bigner DD, Zalutsky MR. Tumor-specific anti-epidermal growth factor receptor variant III monoclonal antibodies: use of the tyraminecellobiose radioiodination method enhances cellular retention and uptake in tumor xenografts. *Cancer Research* 1995;55:4375–4382. [PubMed: 7671250]
- Sampson JH, Ashley DM, Archer GE, Fuchs HE, Dranoff G, Hale LP, Bigner DD. Characterization of a spontaneous murine astrocytoma and abrogation of its tumorigenicity by cytokine secretion. *Neurosurgery* 1997;41:1365–1372. [PubMed: 9402588]discussion 1372-1363
- Sampson JH, Crotty LE, Lee S, Archer GE, Ashley DM, Wikstrand CJ, Hale LP, Small C, Dranoff G, Friedman AH, Friedman HS, Bigner DD. Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:7503–7508. [PubMed: 10852962]
- Sampson JH, Reardon DA, Friedman AH, Friedman HS, Coleman RE, McLendon RE, Pastan I, Bigner DD. Sustained radiographic and clinical response in patient with bifrontal recurrent glioblastoma multiforme with intracerebral infusion of the recombinant targeted toxin TP-38: case study. *Neuro Oncology* 2005;7:90–96. [PubMed: 15701286]
- Serano RD, Pegram CN, Bigner DD. Tumorigenic cell culture lines from a spontaneous VM/Dk murine astrocytoma (SMA). *Acta Neuropathologica* 1980;51:53–64. [PubMed: 7435141]
- Strik HM, Stoll M, Meyermann R. Immune cell infiltration of intrinsic and metastatic intracranial tumours. *Anticancer Research* 2004;24:37–42. [PubMed: 15015573]
- Tang J, Flomenberg P, Harshyne L, Kenyon L, Andrews DW. Glioblastoma patients exhibit circulating tumor-specific CD8+ T cells. *Clinical Cancer Research* 2005;11:5292–5299. [PubMed: 16033848]
- Wheeler CJ, Das A, Liu G, Yu JS, Black KL. Clinical responsiveness of glioblastoma multiforme to chemotherapy after vaccination. *Clinical Cancer Research* 2004;10:5316–5326. [PubMed: 15328167]
- Whiteside TL. Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention. *Seminars in Cancer Biology* 2006;16:3–15. [PubMed: 16153857]
- Wikstrand CJ, Reist CJ, Archer GE, Zalutsky MR, Bigner DD. The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. *Journal of Neurovirology* 1998;4:148–158. [PubMed: 9584952]

Figure 1.

Hierarchical cluster analysis of gene expression in normal astrocytes and SMA-560 tumor cells *in vitro* and *in vivo*. For these investigations, normal murine astrocytes (NMA) from immunocompetent VM/Dk mice and the syngeneic spontaneous murine astrocytoma tumor line SMA-560 were cultured *in vitro* and analyzed, as described in **Materials and Methods**. In addition, normal brain and brain following intracranial (IC) SMA-560 tumor challenge for 17 days in immunocompetent VM/Dk mice as well as in athymic nude mice were isolated and analyzed, as described in **Materials and Methods**. All samples were performed in triplicate. Profiles of genes determined to be statistically different by ANOVA between normal astrocytes and SMA-560 tumors cells are represented in the thermograms. Clustering analysis of these expression profiles produces gene trees (green lines), which demonstrate coordinate regulation of groups of similarly related genes. Each gene is represented by a single row and each sample is represented by a single column. The color heat map represents genes in a graded fashion along a spectrum of activation, extending from strongly upregulated genes (red) to downregulated genes (blue). Complete gene lists for this and all analyses described here are available at <http://data.cgt.duke.edu/Learn.php>.

Figure 2.

Scatter profiles of normal brain and SMA-560 tumor gene expression in vitro and in vivo. **A.** Gene expression in normal murine astrocytes versus SMA-560 tumor cells *in vitro*. 7,041 genes were determined to demonstrate significantly different expression between normal astrocytes and SMA-560, as determined by ANOVA ($p < 0.05$). 2,016 genes were upregulated 2-fold or more in normal astrocytes compared to SMA-560. In contrast, 2,292 genes were upregulated 2-fold or more in SMA-560 compared to normal astrocytes. **B.** Gene expression in immunocompetent VM/Dk mouse normal brain versus SMA-560 tumor cells grown IC for 17 days. 3,228 genes were determined to be significantly different between normal brain and IC SMA-560, as determined by ANOVA. 541 genes were upregulated 2-fold or more in normal brain compared to IC SMA-560. In contrast, 785 genes were upregulated 2-fold or more in IC SMA-560 compared to normal brain. **C.** Gene expression in athymic nude mouse normal brain versus SMA-560 tumor cells grown IC for 17 days. 297 genes were determined to be significantly different between normal brain and IC SMA-560, as determined by ANOVA. 77 genes were upregulated 2-fold or more in normal brain compared to IC SMA-560. In contrast, 61 genes were upregulated 2-fold or more in IC SMA-560 compared to normal brain. Complete gene lists are available at <http://data.cgt.duke.edu/Learn.php>.

Figure 3.

Principal components analysis for SMA-560 tumors grown in vitro and in vivo. Principal components analysis (PCA) for **A.** *in vitro* cultured normal murine astrocytes (n=3), **B.** *in vitro* cultured SMA-560 tumor cells (n=3), **C.** immunocompetent VM/Dk normal brain (n=3), **D.** immunocompetent VM/Dk brain challenged with SMA-560 tumor cells 17 days post-implantation (n=3), **E.** athymic nude normal brain (n=3), and **F.** athymic nude brain challenged with SMA-560 tumor cells 17 days post-implantation (n=3). For this analysis, gene expression values for each signature are extracted from all experimental samples and mean centered. Single value decomposition analysis is then applied across all samples, from which linear combinations of these patterns are then assembled to represent the behavior of all of the genes in a given sample, characterizing the most abundant themes or building blocks that reoccur in many genes in a sample.

Figure 4.

Venn diagram analysis of statistically determined shared gene expression in normal brain and SMA-560 tumors following *in vitro* culture or *in vivo* growth **A.** Two-fold or greater shared gene expression between *in vitro* cultured normal murine astrocytes and *in vivo* normal brain from immunocompetent VM/Dk as well as athymic BALB/c athymic mice, as determined by ANOVA. **B.** Two-fold or greater shared gene expression between *in vitro* cultured SMA-560 tumor cells and *in vivo* intracranial SMA-560 tumors in immunocompetent VM/Dk as well as athymic BALB/c athymic mice, as determined by ANOVA. Complete gene lists are available at <http://data.cgt.duke.edu/Learn.php>.

Table 1

Gene ontology pathway analyses of normal brain and intracranial SMA-560 tumors in immunocompetent VM/Dk mice. Enumeration of genes involved in specific biological processes in normal astrocytes and SMA-560 tumors *in vitro* and *in vivo*. Statistical analysis of genes expressed at levels 2-fold or greater and deemed significant by ANOVA in *in vitro*-cultured SMA-560 tumor cells as well as SMA-560 tumors grown i.c. for 17 days in immunocompetent VM/Dk or athymic BALB/c nude mice. Gene ontology pathway analyses were performed using Genespring Software (v.7.2) from Agilent Technologies, Inc.

Biological process (# of genes in list)	<i>In vitro</i>	Immunocompetent	Nude
Cell communication (1,143)	127 (8.8%)	104 (7.2%)	15 (1.0%)
Cell growth and/or maintenance (2,616)	196 (7.5%)	72 (2.8%)	6 (0.2%)
Death (138)	10 (7.2%)	5 (3.6%)	0 (0%)
Apoptosis regulator activity (74)	7 (9.5%)	1 (1.4%)	0 (0%)
Binding (1,937)	155 (8.0%)	49 (2.5%)	3 (0.2%)
Catalytic activity (2,705)	223 (8.2%)	74 (2.7%)	4 (0.1%)
Cell adhesion activity (73)	4 (5.5%)	6 (8.2%)	1 (1.4%)
Immunoregulatory activity (466)	46 (9.9%)	55 (11.8%)	4 (0.9%)
Signal transducer activity (2,193)	66 (3.0%)	41 (1.9%)	2 (0.1%)

Genes involved in modulating the tumor response to host defenses in SMA-560 cells *in vitro* and *in vivo*. **A.** Statistical analysis of immunomodulatory genes expressed at levels 2-fold or greater and deemed significant by ANOVA ($p < 0.05$) in SMA-560 tumor cells grown *in vitro* versus intracranially in immunocompetent VM/Dk mice. **B.** Statistical analysis of immunomodulatory genes expressed at levels 2-fold or greater and deemed significant by ANOVA in SMA-560 tumor cells grown *in vitro* versus *i.c.* in immunocompetent VM/Dk mice versus athymic nude mice. Complete gene lists are available at <http://data.cgt.duke.edu/Learn.php>.

Table 2

A.	Immunocompetent				Description
	Operon Oligo ID	In vitro Fold Change	In vivo p value	In vivo Fold Change	
	M300004234	2.77	0.0064	84.67	H-2 CLASS II HISTOCOMPATIBILITY ANTIGEN, INVARIANT CHAIN
	M200003398	13.93	0.0143	77.38	T-CELL SPECIFIC GTPASE
	M300005162	8.328	0.00355	62.37	INTERFERON-ACTIVATABLE PROTEIN 202B
	M200000049	11.86	0.00113	44.37	MONOCYTE CHEMOTACTIC PROTEIN, MCP-1
	M200000311	8.958	0.01	39.86	INTERFERON-GAMMA INDUCED PROTEIN CRG-2
	M300005165	12	0.000507	37.9	INTERFERON-ACTIVATABLE PROTEIN 205
	M300005166	18	0.0011	33.78	INTERFERON-ACTIVATABLE PROTEIN 204
	M300001903	7.889	0.00148	26.66	CYCLIN-DEPENDENT KINASE 1 (CDK1)
	M200004993	4.106	0.0121	26.2	INTERFERON GAMMA INDUCIBLE PROTEIN
	M300004062	2.61	0.000434	24.82	H-2 CLASS I HISTOCOMPATIBILITY ANTIGEN
	M200000303	3.792	0.00398	22.16	INTERFERON-G INDUCED GTPASE
	M200002418	7.248	0.00229	20.56	INTERFERON-INDUCED 56 KDA PROTEIN
	M200002687	45.67	0.00365	19.38	LY-6A.2/LY-6E.1 PRECURSOR (T-CELL-ACTIVATING PROTEIN)
	M200002417	3.334	0.00934	19.33	H-2 CLASS II HISTOCOMPATIBILITY ANTIGEN
	M200001244	6.044	0.0066	17.99	INTERFERON REGULATORY FACTOR 7 (IRF-7)
	M200000461	2.09	0.0275	11.38	INTERFERON REGULATORY FACTOR 1 (IRF-1)
	M300005163	94.32	0.000635	11.19	INTERFERON-ACTIVATABLE PROTEIN 203
	M300009668	7.804	0.00184	10.16	CCL5 (T-CELL SPECIFIC RANTES PROTEIN)
B.	Nude				Description
	Operon Oligo ID	In vitro Fold Change	In vitro p value	In vivo Fold Change	
	M200003398	13.93	0.0143	77.38	T-CELL SPECIFIC GTPase
	M300005162	8.328	0.00355	62.37	INTERFERON-ACTIVATABLE PROTEIN 202B
	M200000311	8.958	0.01	39.86	SMALL INDUCIBLE CYTOKINE B10 PRECURSOR (CXCL10)
	M300005673	31.58	0.00143	30.03	TUMOR STROMA AND ACTIVATED MACROPHAGE PROTEIN DLM-1
	M300017406	41.83	0.00867	23.04	INTERFERON-STIMULATED PROTEIN I5
	M200002418	7.248	0.00229	20.56	INTERFERON-INDUCED PROTEIN WITH TETRATRIPEPTIDE REPEATS 1
	M300009668	7.804	0.00184	10.16	SMALL INDUCIBLE CYTOKINE A5 PRECURSOR (CCL5)
	M200011929	3.893	0.00102	3.551	28 KDA INTERFERON RESPONSIVE PROTEIN