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Review

Immunological considerations of modern animal models of malignant primary brain tumors

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Abstract

Recent advances in animal models of glioma have facilitated a better understanding of biological mechanisms underlying gliomagenesis and glioma progression. The limitations of existing therapy, including surgery, chemotherapy, and radiotherapy, have prompted numerous investigators to search for new therapeutic approaches to improve quantity and quality of survival from these aggressive lesions. One of these approaches involves triggering a tumor specific immune response. However, a difficulty in this approach is the the scarcity of animal models of primary CNS neoplasms which faithfully recapitulate these tumors and their interaction with the host's immune system. In this article, we review the existing methods utilized to date for modeling gliomas in rodents, with a focus on the known as well as potential immunological aspects of these models. As this review demonstrates, many of these models have inherent immune system limitations, and the impact of these limitations on studies on the influence of pre-clinical therapeutics testing warrants further attention.

The Potential Promise of Immunotherapy for Primary Brain Tumors

Primary central nervous system (CNS) malignancies, though of low incidence in relation to many adult solid tumors, represent a disproportionately large fraction of cancer deaths due to their highly aggressive and fatal character. For example, Glioblastoma Multiforme (GBM), the most common and malignant brain tumor of adults, carries a median survival of less than 1 year. While current approaches to brain tumor therapy, including surgical resection, radiotherapy, and either systemic or local chemotherapy with either nitrosoureas or temozolamide, appear to prolong survival for patients with CNS cancers,

the modest effect of these therapies, and their associated morbidity, has left investigators in search of alternative and novel treatments to extend quantity and quality of life for affected patients [1].

The nearly infinite flexibility and remarkable cellular specificity of the human immune response makes immune based approaches an attractive option to current therapy, which either crudely target entire regions of the brain (e.g. surgery, radiation), or potentially interfere with the cellular metabolism of all dividing cells in the body (e.g. alkylating agents). However, immunotherapy is not without technical barriers, which have hindered its incorpora-

tion into the therapeutic arsenal for treating CNS tumors. One such barrier is the known paucity of surface antigens unique to glioma cells, against which an immune response could be mounted. Another is the significant degree of local and systemic immunosuppression known to occur in glioma patients.

Perhaps the most significant hurdle to translating immunotherapeutic concepts into effective treatments for primary brain tumor patients is the fact that animals generally do not spontaneously develop CNS neoplasms, and, consequently, pre-clinical studies rely on artificial systems for basing conclusions regarding approaches being considered for use in patients. It is crucial that tumors artificially created in animal hosts for the purpose of developing immune based therapies, faithfully recapitulate the antigenic and immunological reality that exists in brain tumor patients. Artefactual inaccuracies could falsely suggest the efficacy of ineffective treatments [2], or worse, lead investigators to disregard effective ones. Given the limitations of the existing artificial systems used in pre-clinical studies, a critical evaluation of immunological considerations associated with the approaches used to create brain tumors in animals is essential prior to using these models to evaluate immune based therapies.

Observed and Anticipated Immunological Deficiencies in Various Brain Tumor Models

While there exist a multitude of methods for introducing glial-type neoplasms into the rodent CNS, which histologically mimic human primary tumors, these methods can be described as belonging to one of two groups: 1) Tumors created by methods which do not target a specific gene, and 2) Tumors created by targeted mutation of genes known to be mutated in human tumors (i.e. gene specific methods) [3].

Non-Specific Methods

It has been known since the 1970's that repetitive intravenous administration of nitrosourea compounds such as methylnitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU) produces glial-type neoplasms in immunocompetent rats [4]. However, the long time required to induce neoplasms, and inconsistency of tumor development, led to a shift towards implantation of neoplastic cells propagated *in vitro* [4].

While the majority of these models involve the use of rodent glioma cells injected in syngeneic hosts, it is also possible to use human glioma cells *in vivo* via their implantation in athymic mice. The pan-immune alterations seen in these rodents obviously limits the use of the xenograft models in some immunologic investigations, namely studies involving T-cell related immunity. These models however do maintain some aspects of their native

immune systems and thus can be used to study some aspects of innate immunity [5], cytokine function [6], and natural killer cell function [7].

While rodent tumor cells implanted in rodent hosts have been widely used to study the interaction of brain tumors and the immune system, a number of major concerns with this approach have been reported. The first is these methods' dependence on cell culture for the production of neoplastic cells to implant. For example, we have shown that glioma cells long removed from their native histological milieu are immunologically different than similar cells immediately *ex vivo*, including changes in MHC and FasL expression and cytokine production; changes which apparently begin as soon as the first passage *in vitro* [8]. Consistent with these observations, expression profiling of patient tumors vs. corresponding cell cultures have revealed widespread changes gene expression once a tumor is subjected to *in vitro* growth conditions [9].

As well, while many of these models involve implantation of cells into animals derived from the cell-line originating strain, these cells still represent a graft, and unfortunately too often behave immunologically like foreign cells. Most syngeneic graft based models of brain tumors have been shown to induce an immunological response against implanted tumor cells [4]. For example, one of the original implantation models, the 9L Gliosarcoma model, was initially created in Fischer rats using serial MNU injections [10], and has been widely used to evaluate various immunotherapeutic therapies [11-15]. However, investigations have demonstrated the 9L model is relatively immunogenic, and that it is possible to immunize animals against these tumors using irradiated 9L cells, implying that they are viewed as foreign tissue [16]. We have demonstrated the occurrence of a similar phenomenon in the C6 glioma cell line, as rats subjected to simultaneous intracerebral and subcutaneous glioma cell implantation experienced a nearly 9 fold improvement in survival compared to those subjected to intracerebral implantation alone [2]. As well the 9L Fischer model has been demonstrated to induce a similar immune response. Other models such as CNS-1 cell implantation in Lewis rats have been found to induce less of an immune response [4]. Thus, variability in immune response occurs in a number of these models, and this should be taken into consideration when evaluating immunotherapies in these models.

There are significantly fewer syngeneic graft models in mice. GL261 is murine cell line which seems to be immunologically tolerated when implanted in C57BL/6 mice, and this model had been used in some immunological models with some success [17]. Similar to human tumors, GL261 cells have a relatively high fraction of CD133+ gli-

oma cells [18], which are a candidate for the "brain tumor stem cell [18-20]." This cell population has been shown to be relatively non-immunogenic [21], and thus these tumors may model the human condition fairly reliably [21]. The intact T-cell responses in these immunocompetent mice make this model an improvement over xenograft models for studying immunotherapy. The much broader range of reagents, and the much smaller size of mice make testing therapies in mice much easier than in rats, thus giving GL261 model a logistical advantage over other grafting models. Regardless, the implantation methods all suffer from the necessity to introduce foreign tissue into mice to create brain tumors, which likely will always have some immunologic effects.

Gene Targeted Methods

Mutational analyses of tissue from human brain tumors have revealed that various histopathological categories for

primary CNS neoplasia generally result from a limited number of mutation patterns. Recently, transgenic technology has allowed investigators to alter the function of specific genes of interest and thus exploit defined genetic lesions to produce more biologically correct models of CNS cancers that result from activation and/or inactivation of endogenous genes in rodent genomes. A brief summary of presently described models can be found in table 1.

While to the genetically modified mouse models are intended to more faithfully recapitulate human brain cancer in animals, little attention has been directed toward the potential flaws in the transgenic paradigm. Many of the genetic mutations required to produce a de novo murine brain tumor, simultaneously interfere with genes involved in a variety of critical immunologic functions. Specific to the current discussion of the immune system,

Table 1: A summary of existing animal models of brain tumors

	Tumorigenesis Method	Technique	Tumor	Animal	Ref
Implantation	9 L Gliosarcoma	Syngeneic Graft	GS	Rat	[17]
	C6	Syngeneic Graft	GBM	Rat	[2]
	T9	Syngeneic Graft	GS	Rat	[4]
	RG2	Syngeneic Graft	GBM	Rat	[4]
	F98	Syngeneic Graft	GBM	Rat	[4]
	RT-2	Syngeneic Graft	GBM	Rat	[4]
	CNS-1	Syngeneic Graft	GBM	Rat	[18]
	GL261	Syngeneic Graft	GBM	Mouse	[23]
	Human Tumor Cells (U87, U251)	Xenograft	GBM	Mouse	[5]
	Genetic	p53 +/-, NF-1 +/-	Germline mutations	Astro	Mouse
GFAP- p53 +/-, NF-1 +/-		Conditional KO	Astro	Mouse	[78]
GFAP- p53 +/-, NF-1 +/-, PTEN-/-		Conditional KO	Astro	Mouse	[78]
GFAP- p53 +/-, PTEN-/-		Conditional KO	Astro	Mouse	[87]
INK4a/ARF -/-, PDGF Overexpression		Germline mutation, RCAS	Astro	Mouse	[47]
INK4a/ARF -/-, EGF-R overexpression		Germline mutation, RCAS	Astro	Mouse	[48]
INK4a/ARF -/-, Ras, Akt overexpression		Germline mutation, RCAS	Astro	Mouse	[49]
Ras, Akt overexpression		RCAS	Astro	Mouse	[80]
Ras, Akt overexpression, PTEN -/-		RCAS, Conditional KO	Astro	Mouse	[80]
GFAP-V12 Ras, EGFRvIII		Astrocyte targeted mutation, Adenovirus	Astro	Mouse	[77]
GFAP-V12 Ras, PTEN -/-		Astrocyte targeted mutation, Germline mutation	Astro	Mouse	[56]
RAS, EGF-R targeted overexpression		Astrocyte targeted mutations	Astro	Mouse	[73]
PDGF-B overexpression		MMLV retrovirus	ODG	Mouse	[75]
PDGF-B overexpression		RCAS	ODG	Mouse	[76]
Rb inactivation, PTEN -/-		GFAP-Cre targeted conditional KO	ODG		[82]
INK4a/ARF -/-, PDGF overexp., PTEN -/-		Germline mutation, RCAS, Conditional KO	ODG	Mouse	[88]
P53 +/-, S100β promoter driven-v-erbB		Germline mutation, Oligodendrocyte mutation	ODG	Mouse	[26]
INK4a-ARF +/-, S100β promoter v-erbB		Germline mutation, Oligodendrocyte mutation	ODG	Mouse	[26]
p53 +/-, EGF-R overexpression		Germline mutation, Oligodendrocyte mutation	ODG	Mouse	[48]
Ptc +/-		Germline mutation or Conditional KO	MB	Mouse	[25]
Ptc +/-, p53 -/-		Germline mutations	MB	Mouse	[25]
Shh, n-Myc		RCAS	MB	Mouse	[89]
Rb +/-, p53 +/-		GFAP-conditional KO	MB	Mouse	[84]
BRCA2 -/-, p53 +/-		Nestin-conditional KO	MB	Mouse	[86]
Xrcc4 -/-, p53 -/-		Nestin-conditional KO	MB	Mouse	[81]
SmoM2		GFAP-conditional KO	MB	Mouse	[79]

(abbreviations (GS-Gliosarcoma, GBM-glioblastoma multiforme, Astro-astrocytoma, ODG-oligodendroglioma, MB-Medulloblastoma, KO-knockout)

is the observation that processes such as lymphopoiesis, the clonal expansion of activated lymphocytes, and the ability of leukocytes to respond to cytokines, rely on the proper functioning of the genes that have been modified in developing transgenic mouse models. This is especially problematic for approaches that involve inducing gliomagenesis by mutating the germ line, and in so doing produce an immunologically flawed paradigm with limited value for pre-clinical testing immunotherapies.

p53

The tumor suppressor p53 is a critical regulator of DNA repair, cell cycle regulation, and apoptosis, and is frequently mutated in human cancers, including a significant fraction of secondary GBM. A large number of currently described murine models utilize genetic inactivation of p53 to produce brain tumors. In general, such inhibition is achieved via either germ line p53 deletions, or by functional p53 inhibition utilizing transforming viral proteins.

The germ line approach has been utilized to produce a variety of CNS tumors in mice. For example, Reilly and colleagues found that GBM like lesions developed spontaneously in mice heterozygously deficient in both p53 and the neurofibromatosis-1 gene (*nf1*) [22]. Wetmore and colleagues reported that medulloblastoma development was accelerated in susceptible *Ptc +/-* mice by crossing them with p53 *-/-* homozygotes [23]. Additionally, Weiss and colleagues described a model of oligodendroglioma produced by crossing p53 *+/-* mice with mice which specifically overexpress EGF-R in oligodendrocytes [24].

Given its central regulatory role in multiple cell processes, it is not surprising that germ line loss of p53 has immunological consequence. Most striking is the very high incidence of spontaneous lymphoma formation in both p53 *+/-* and p53 *-/-* mice, consistent with their Li Fraumeni-like genotype [25]. This is likely due to the key role p53 plays in lymphocyte differentiation, as it mediates an important checkpoint in early thymocyte development causing arrest at the CD4-CD8 double negative stage [26,27], regulates the proliferation of pre-B-cells [28], and alters the patterns of expression of Fas on both precursor and mature lymphocytes [29]. Additionally, p53-deficient mice demonstrate impaired B-cell maturation and reduced immunoglobulin deposition in tumors, more rapid aging of the immune system, accumulation of memory T-cells [30], and significantly greater expression of cytokines such as IL-4, IL-6, IL-10, IFN- α [30], osteopontin, and growth/differentiation factor-15 (GDF-15) [31]. Paradoxically, loss of p53 also causes a number of proinflammatory changes at the cellular and organismal level [32]. As well, a large number of immunologically important molecules such as macrophage migration inhibitory

factor (MIF) [33], IL-6 [34], IFN- α [35], IFN- β [36], and NF- κ B [37] are known to mediate at least some of their effects through p53. In addition, thymocytes from p53 deficient mice demonstrate increased resistance to radiation induced apoptosis [38,39], and p53 deficiency alters autoantibody levels in models of autoimmunity [40] as well as reduces mast cell susceptibility to IFN- γ induced apoptosis [41]. Given these observations, it seems likely that the pan-suppression of p53 activity introduced by the use of germ line p53 inactivation alters immune system function in a number of significant ways in these animals, limiting the use of these models for evaluating the effect of anti-tumor immunotherapies. Other research groups have shown that CNS tumors can be produced by cell-targeted introduction of viral antigens that suppress p53 activity. Probably the most immunologically correct method for accomplishing this are conditional knockout methods (described below), although a number of other methods exist. For example, Chiu and colleagues demonstrated that mice possessing an SV40 T-antigen transgene (which functionally inactivates Rb and p53), driven by the brain specific FGF-1B promoter, develop poorly differentiated tumors of the medulla and 4th ventricle which closely resemble primitive neuroectodermal tumors (PNET) [42]. An alternate approach, described by Krynska and colleagues, also produced PNET-like tumors by creating mice transgenic for the early region of the CY variant of the JC virus, which encodes a T-antigen that inhibits both p53 and Rb. To some extent, these models represent an improvement over germ line based models because they limit the effects of p53 inhibition to specific cells. However the introduction of viral antigens expressed in tumor cells, has great potential to alter the interaction of the immune systems with these tumors [43].

INK4a/ARF

The tumor suppressor locus *INK4a/ARF* encodes two tumor suppressor genes: p16^{INK4a}, which prevents Rb phosphorylation by binding CDK4; and p14/p19^{ARF}, which prevents p53 degradation via MDM2 inhibition [44]. Loss of function mutation of one or both gene products encoded by *INK4a/ARF* is a common mutation in human cancer, including glioma [44], and accordingly numerous investigators have utilized *INK4a/ARF* silencing mutations to create CNS neoplasms in mice. Dai and colleagues demonstrated that oligodendrogliomas and oligoastrocytomas could be produced in *INK4a/ARF -/-* mice by forcing glial precursor cells to overexpress PDGF, using the RCAS system [45], which involves delivery of oncogene-encoding viral vectors to cells that have been engineered to express receptor for RCAS virus. Using the same system, this group has described the production high grade gliomas by combining *INK4a/ARF* deletion with astrocyte specific overexpression of EGFR [46], or Ras and Akt [47]. The immunologic significance of a tumor

expressing RCAS antigens has yet to be addressed, and because all of these models share the common trait of utilizing germline *INK4a/Arf* deletion to promote glial neoplasms, there are undoubtedly additional immunologic consequences of these models that would not be encountered in patients where *INK4a/Arf* inactivation was limited to tumor cells only. For example, in a manner similar to p53 deficient mice, *ARF* ^{-/-} mice are known to spontaneously develop lymphomas in the absence of other mutations [48]. This is not surprising, given the important role these genes play in cell cycle regulation in developing thymocytes [49,50]. As well, p14/p19^{ARF} plays a role in suppressing the respiratory burst in neutrophils [51,52].

Phosphatase and Tensin Homolog (PTEN)

PTEN is a tumor suppressor gene which inhibits cell proliferation and growth via suppression of the PI3-kinase signaling pathway [53]. Loss of function mutations of PTEN have been observed in approximately 50% of de novo GBM patients [54]. One significance of this observation was revealed by Xiao and colleagues who reported that crossbreeding PTEN +/- mice with a strain containing a GFAP driven truncated SV40 T antigen resulted in Rb, p107, and p130 (but not p53) inhibition, and significantly accelerated the development of GBM in the double transgenic progeny [54]. Here again, the use of PTEN germ line mutations is problematic for immunological studies using this model. Similar to other tumor suppressor genes, PTEN plays a critical role in lymphocyte development, serving to eliminate T-cells that do not produce an effective TCR re-arrangement [55]. Not surprisingly, PTEN +/- mice have been demonstrated to frequently develop T-cell lymphomas [55,56], as well as diffuse lymphoid hyperplasia [57,58]. In addition, PTEN appears to regulate leukocyte chemotaxis at a variety of levels, including regulation of CXCR4 expression [59], which directs actin polymerization during chemotaxis [60]. It is unclear whether or not T cells from these transgenic animals are fully functional.

Epidermal Growth Factor Receptor (EGF-R)

EGF-R is a member of the ErbB tyrosine kinase receptor family that is mutated or overexpressed in a variety of human tumors, including approximately 30-50% of primary glioblastoma multiforme [61] and in roughly half of oligodendrogliomas [62]. In addition to its role in neoplasia, EGF-R plays a pivotal role as a so called "master switch" which modulates a broad variety of immunological functions [63]. For example, EGF-R activation appears to sensitize neutrophils to the effects of TNF- α , leading to increased expression of the adhesion molecule CD-11b, increased IL-8 production, and improved respiratory burst by these "EGF-R primed" cells [64]. EGF-R mediates chemotaxis in peripheral blood monocytes and

monocyte derived macrophages [65], and is critical for the response of myeloid lineage cells to colony stimulating factors [66]. EGF-R activation stimulates release of IL-8 from cultured bronchial epithelial cells [67], and is hypothesized to play a critical role in the pathogenesis of inflammatory lung diseases such as panbronchitis and asthma [67,68]. EGF-R down-regulates CCL2, CCL5, and CXCL10, and increases CXCL8 in keratinocytes which likely propagates the pro-inflammatory state seen in autoimmune skin disorders [69]. Finally, EGF-R is required for cytokine dependent production of nitric oxide by the pulmonary vasculature [70].

To date, there have been several reports demonstrating the use of EGF-R overexpression to produce either oligodendroglioma or astrocytoma-like tumors in mice. Holland and colleagues reported that virus expressing EGFRvIII (a common mutant form of EGFR), and used to infect *INK4a-ARF* null astrocytes or glial precursors (via the RCAS system described above), produce gliomas in transgenic mice [46]. Weiss and colleagues demonstrated that oligodendrogliomas reliably occur in mice doubly transgenic for an S100 β promoter driven-*v-erbB* (a transforming EGF-R allele), and either *INK4a-ARF* +/- or *P53* +/- heterozygosity [24]. Ding and colleagues have reported the development of oligodendrogliomas and mixed oligoastrocytomas in mice carrying *RAS* and EGF-R transgenes driven by GFAP promoters [71]. In all three models, the use of glial specific promoters likely minimize the systemic effects of EGF-R overexpression on immune function. However the dependence of EGF-R models on the use of cross breeding with germ line mutants, likely introduces its own set of immunobiological consequences, as discussed earlier.

Platelet Derived Growth Factor (PDGF)

PDGF is a growth factor that is expressed in many normal tissues and mediates a variety of effects on cell growth and differentiation via induced dimerization-activation of its corresponding tyrosine kinase receptor, PDGF-R. Overexpression of both the PDGF isoform, PDGF-B, and the receptor PDGF-R frequently occur in gliomas, suggesting the potential role of a malfunctioning autocrine signaling loop in the pathogenesis of some of these tumors [72]. Existing PDGF based models typically utilize approaches that limit ligand overexpression to the peritumoral region, or at least the CNS. For example, Hesselager and colleagues found that using a MMLV retroviral construct to drive PDGF-B expression it was possible to induce gliomagenesis in neonatal mice brains, and in the absence of other mutations (though additional relevant mutations appeared to accelerate tumor growth) [73]. Dai and colleagues have demonstrated that oligodendrogliomas could be produced solely by introduction of PDGF-B

overexpression using the RCAS system, and that this process was accelerated by the addition of INK4a/ARF p53 germline mutations [74].

While both models have the desirable feature of causing gliomagenesis with minimal effects to the host immune system, little attention has been directed towards analyzing the effects of overexpression of a soluble leukocyte chemoattractant. It is important to know whether PDGF-driven tumors secrete similar levels of PDGF as their naturally occurring counterparts, and what effect PDGF overexpression has on local intratumoral inflammatory responses.

Tissue Targeting with Conditional Knockouts

Tissue specific overexpression of putative oncogenes of interest, using methods which link the gene of interest to a glial specific promoter such as GFAP, S100 β , or Nestin, provides an appealing approach towards the creation of spontaneously occurring brain tumors in animals that lack the pan-immune dysfunctions seen in many germline knockout animals [75]. Tissue targeted models involving deletion of tumor suppressor genes is more difficult, which is why most models to date have utilized germline knockouts to reduce tumor suppressor gene function. Conditional knockout models represent a promising new attempt to eliminate tumor suppressor function in a cell specific manner [76,77]. For brain tumors, this involves GFAP or Nestin driven expression of the bacteriophage protein Cre, which removes sections of DNA between E. Coli specific DNA sequences known as loxP domains [76]. By co-introducing Cre driven on tissue specific promoters, and the tumor suppressor gene of interest flanked by loxP regions, it is possible to knock out tumor suppressor genes of interest in the cell type of choice [76]. These techniques have recently been utilized to create a variety of transgenic brain tumor models using targeted conditional knockouts of p53 [78,79], PTEN [80], Ptc [81], and Rb [82]. Frequently, conditional knockouts used in combination with oncogenes overexpressed on tissue specific promoters or introduced using viral vectors can create a localized tumor genetically similar to human cancer in an immune competent animal [74]. While these and other similar models [83-85] certainly represent an improvement over germline mutation based models [86], the constitutive expression of a bacteriophage protein in the cell of interest, raises some concern regarding the immunogenicity of the tumor cells created in this manner, and deserves future attention.

Conclusion

Animal models represent essential tools understanding complex molecular and cellular interactions occurring in brain tumors, and for the evaluation of potential therapies. Rodents do not typically develop CNS neoplasms

spontaneously, and it is important that we understand the physiologic changes induced by the methods used to create these tumors, and adjust our interpretation of results obtained with these models accordingly. Significant improvements have been made over the last decade to induce gliomas using tissue targeted conditional deletions and cell specific oncogene overexpression. While existing models may represent improvements over chemically induced rodent syngeneic models, the immunologic effects of these methods are not entirely understood, and deserve more investigation.

Conflicting interests

The authors declare that they have no competing interests.

Authors' contributions

All authors read and approved this manuscript. MS provided the manuscript idea, and prepared the manuscript. IY also provided the manuscript idea, and helped prepared the manuscript. AK, MR, and SF helped with literature searches and manuscript preparation and editing. DJ helped edit the manuscript and contributed insight from his experience in the field. AP helped generate the manuscript idea and contributed significantly to the manuscript's final form.

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