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TARGETED THERAPY OF BRAIN TUMORS UTILIZING NEURAL STEM AND PROGENITOR CELLS

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1. ABSTRACT

Neural stem and progenitor cells derived from the central nervous system (CNS) of embryonic and adult mammals share three critical features: 1. Stem and progenitor cells are highly migratory, 2. Stem and progenitor cells have affinity for areas of CNS pathology, and 3. The pluripotentcy of neural stem and progenitor cells allows them to engraft and replace damaged tissues in the CNS. These properties suggest that transplanted neural stem cells might be used to deliver molecular therapy to diseased regions of the nervous system, and to regenerate lost tissues. One of the greatest challenges and potential promises of stem cell therapy is to direct therapy to pathological tissues comprised of cells which themselves are migratory. The ability of glioma cells to migrate extensively into normal brain parenchyma in part underlies the lethal nature of these tumors. A better understanding of the mechanisms by which neural stem cells migrate to specific sources of injury may allow us to harness these cells as vehicles for delivery of molecular therapies to impact survival in patients with recalcitrant gliomas.

2. INTRODUCTION

Gliomas, the most common primary brain tumor, can arise from either astrocyte or oligodendrocyte lineage to form astrocytomas or oligodendrogliomas(1). The incidence for both of these tumors has increased in both children and adults over the last twenty years. It is likely that improved detection is responsible for at least part of these new diagnoses. Still, after taking ascertainment bias into consideration, it seems clear that the US incidence of glioma is on the rise (2, 3). High-grade astrocytomas, the most common gliomas, often recur within six months of surgery, and despite radiation therapy and chemotherapy, 80% of patients die within a year. The median survival of patients with glioblastoma multiforme, the most malignant grade of astrocytoma, has remained less than one year despite concerted efforts to improve current therapies and develop novel clinical approaches (1). Additionally, several studies have shown that the various therapies currently used to treat CNS tumors may themselves injure the brain and cause cognitive impairment (4). Most oncologists would say that there has been no substantive advancement in treatment of high-grade astrocytomas in the last several decades.

Gliomas are by nature diffuse and infiltrating with no clear border between normal brain and tumor. Surgical resection is almost always followed by regrowth of tumor cells residing in adjacent regions of normal brain. There are no therapies that eradicate minimal residual disease without harming normal brain. To spare healthy brain, new therapies must be developed that target small numbers of tumor cells surrounded by normal cells; effectively engaging those tumor cells which have escaped the main mass of the tumor. The use of engineered cells

capable of delivering therapeutic molecules represent a promising technology to target molecular therapies selectively to tumor cells, while simultaneously repairing or replacing the surrounding damaged tissues. The current use of neural stem and progenitor cells to target therapies to brain tumor follows the precedent set by previous investigation of engineered tissue transplantation for delivery of therapy *in situ* (5).

3. PREVIOUS WORK UTILIZING CELLS FOR TARGETED THERAPY

A variety of cell types have been isolated, genetically manipulated *ex vivo*, and then transplanted back *in vivo* to deliver their "payload". These include neurons, astrocytes, and oligodendrocytes from the CNS and adrenal medulla, sciatic nerve, Schwann cells, and dorsal root ganglia from the PNS (6-13). Cells from non-neural tissues such as myoblast, fibroblasts, amniotic membrane, macrophage, and hematopoetic stem cells have also been used(14-18). Even tumor cell lines have been manipulated to take advantage of their hardiness and proliferative potential, i.e. pheochromocytoma, neuroblastoma, neuroendocrine, glioma, astrocytoma, meningioma, melanoma, Schwannoma, fibroblast and astrocyte cell lines(19-27).

It is only recently that neural stem cells have been utilized for gene therapy. Early work in this field included transplantation of neural stem cells producing neurotropins to combat neurodegeneration (28, 29), the use of stem cells to replace myelin in shiverer mice (30), and the engineering of neural stem cells as packaging cells for post-transplantation infection of surrounding neurons and glia (31).The main advantage of using neural stem cells, over other cells types mentioned above, is that the transplanted stem cells not only localize and integrate into the diseased area of the brain but also differentiate into functional neurons and glia (32). While the definitive mechanism(s) of stem cell attraction to areas of damage and disease in the brain has yet to be identified, it is likely to be related to changes in chemokines, trophic factors, and possibly extracellular matrix components (33).

4. STEM CELL CANDIDATES FOR TARGETED THERAPY OF BRAIN TUMORS

A functional definition for "stem cells" is cells that self-renew and can give rise to cells other than themselves by differentiation after asymmetric cell division (34-36). The main types of stem cells are embryonic stem cells derived from the blastocyst inner cell mass or adult tissue-derived stem cells. Adult tissue-derived stem cells are sub-classified based on their embryonic tissue of origin, i.e. neural, hematopoetic, and mesenchymal. These last three must all derive originally from embryonic stem (ES) cells as ES cells have the potential to generate all lineages and cell types. Although these divisions are recognized based on the tissue from which the stem cells were derived, several studies have indicated that there is functional overlap between these populations. For instance, both hematopoetic stem cells and neural stem cells have been shown to differentiate into reciprocal cell types(37, 38). Although exciting, this conclusion is regarded as somewhat controversial and should be expressed with the caveat that, *in vitro*, hematopoetic cells have been shown to fuse with embryonic stem cells to form tetraploid cells capable of differentiating into the same tissue lineages as ES cells (39). This suggests that hematopoetic stem cells adopt the characteristics of the cells they fuse with rather than differentiate due to inherent plasticity (40). Unfortunately, unlike their hematopoetic relatives, neural stem cells have yet to be characterized based on cell surface markers. Instead, their identity and location derive from functional studies which have left some ambiguity as to which among the several candidate cells is/ are the actual neural stem cell(s) (35).

A standard purification and enrichment for neural stem cells involves the isolation and growth of neurospheres. Typically, tissues thought to be potential reservoir for neural stem cells are isolated and dissociated. The dissociated cells are then grown in media containing the neural stem cell mitogens epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2). Proliferating neural stem cells grow as floating spheres referred to as "neurospheres". These spheres are collected and assayed for neural stem cell character both *in vitro* and *in vivo* (41). The basis for these functional assays is capacity for self-renewal, differentiation *in vitro* along glial and neuronal lineage, and formation of glia and neurons after transplantation into the brain of a developing embryo (32, 37, 38, 42) These experiments and, in a very few cases, fate mapping (43)have supplied us with out current understanding of location and identity of neural stem cells.

4.1. Embryonic and Adult Neural stem cells

Studies of early neural development demonstrated neural stem cells in the neural plate of the developing fetus, as well as in the early cortex, cerebellum, brain stem, sub-ventricular zone, and hippocampus(38, 42, 44-46) Thus far, regions of the adult brain known to contain stem cell populations include the lateral wall of the sub-ventricular zone and possibly the ependymal layer lining the ventricle, the dentate gyrus of the hippocampus, and the external granular layer of the cerebellum (35, 43, 47-49). Recent work by Rakic *et al.*, and Gould *et al.*, has demonstrated the presence of neural stem cells in the adult primate cortex. Both Rakic and Gould found glial lineages derived from these cortical stem cells but their neuronal potential is still a matter of contention(50, 51).

4.2. Non-neural stem cells: ES, embryonic progenitors, and adult progenitors

In addition to the above mentioned populations of neural progenitors, embryonic stem cells, stem cells of the neuroectoderm, and adult hematopeotic stem cells all have the ability to generate neural tissues after implantation into the brain.(38, 44, 52) Deacon *et al*. demonstrated that blastula-stage stem cells when transplanted intracerebrally form both dopaminergic and serotonergic neurons (38). Precursors from the neuroectoderm of embryonic day 10.5 mice differentiate into functional oligodendrocytes after transplantation into the neonatal brain(53). Finally, several

studies have shown that the brains of bone marrow ablated mice contained neurons and glia derived from adult hematopoetic stem cells after rescue with hematopoetic stem cell transplantation (37, 54).

5. NEURAL STEM AND PROGENITOR CELLS IN BRAIN TUMOR THERAPY

The number of candidate stem cell populations with neural potential is quite extensive. This is especially true if one considers that these cells may behave differently if derived from embryonic or adult tissue. An additional complication is that cells may have been immortalized to provide a continuing supply of material for study. It is clear that age and location of tissue source as well as passage number all affect the behavior of stem cells.

Studies of early CNS development have demonstrated stem cell segregation along the anterior/ posterior and dorsal/ ventral axes. This compartmentalization of stem cells is required for normal development of CNS structures and correct targeting and synaptogenesis of neurons(55-57). A requirement for compartmentalization suggests that even in the earliest points in neuroectoderm development stem cells begin to show variable specification. Although we are only beginning to understand the regional and temporal patterning of stem cells, the implications for their use in transplantation studies is profound. It is likely that the migration, targeting, and differentiation capacity of each population of stem cells will vary. This variance between neural stem cell populations will certainly confound drawing general conclusions about the suitability of neural stem cells for targeted therapy as well as replicating the findings of previous studies. Thus far, three studies testing four candidate stem cell populations have addressed their potential use in targeted therapy of brain tumors.

5.1. Primary progenitors versus immortalized progenitors

Only one study thus far has made a direct comparison of function between primary and immortalized neural stem cells. Benedetti *et al.* carried out parallel experiments comparing non-immortalized murine cortical progenitors and immortalized rat progenitors from the striatum for their ability to 1.) Inhibit tumor growth and 2). Induce an adoptive immune response against xenografted tumors. Immunocompetent mice and rats were allografted with the glioma lines Gl261 and C6, respectively. Primary neural progenitor populations (mouse) and immortalized progenitors (rat) were engineered to secrete interlukin-4 (which has been previously shown to induce immune reactivity to rat glioblastomas). These engineered progenitors were then either coinjected with the glioma cell line or were injected into the tumor 5 or 7 days after the tumor was established. As the progenitors were injected directly at the site of the tumor, no data concerning migration and targeting potential was acquired.

5.1.1. Isolation of primary progenitors

Benedetti *et al.*, dissected and then disassociated non-immortalized neural progenitor cells from the cortex of mice the day after birth. The cell suspension was plated in media containing both EGF and FGF. Neurospheres were collected, disassociated, and replated at day 4 using in the same conditions. Neurospheres were passaged every 3-4 days to produce a bulk culture. To generate a pool of cortical progenitors that secrete IL-4 (C57.npr.IL-4), neurospheres were disassociated and transduced with a Moloney-murine-leukemia-virus-derived retrovirus containing IL-4. Cells were selected in G418 for one week and IL-4 production was measured by immunoassay.

5.1.2. Production of immortalized progenitors

Primary progenitors cells were isolated from the striatum of Sprague-Dawley rats obtained using the same methods described above. These striatal progenitors were then immortalized by transduction with a temperature sensitive variant of the SV40 T antigen to produce the immortal line ST14A. *In vitro*, these cells grew at 33°C but not at 39°C and *in vivo*, transplanted cells from this line were demonstrated to differentiate into glia and neurons. ST14A cells were transduced with a Moloney-murine-leukemia-virus-derived retrovirus containing IL-4 and expression levels were assessed by immunoassay. From this pool, one clone, ST14A.IL-4.3, was selected for propagation.

5.1.3. Tumor growth inhibition

In vitro, the established glioma cell lines GL-261 and C6 were exposed to conditioned media from progenitor cell lines C57.npr and ST14A respectively. As mentioned above, both C57.npr and ST14A lack expression of IL-4. In the presence of progenitor cell conditioned media, both GL 261 and C6 tumor lines demonstrated decreased proliferation as measured by ^{3}H-Thymidine incorporation. Incorporation of label into GL261 cells was reduced by 84.7% after 24 hours in C57.npr.conditioned media as compared to normal medium. Conditioned media from ST14A cells incubated at 33°C and 39°C were tested for their ability to inhibit the proliferation of C6 cells. The media from the ST14A cells grown at 39°C caused a 50% decrease in the growth rate of C6 cells. The media from the ST14A cells grown at 33°C did not affect proliferation. Benedetti *et al.* observed that conditioned media from both stem cell cultures decrease tumor cell proliferation when the cultures contained differentiating progenitors. The conditioned media from proliferating progenitors lacked this activity. They hypothesized that progenitor cells secrete some factor as they differentiate which inhibits the growth of tumor cells.

5.1.4. Adoptive immunotherapy

Previous work by Benedetti *et al.*. demonstrated that delivery of IL-4 to the site of intracranially transplanted glioma can induce an immune response and tumor regression (58). Similarly, Benedetti and collegues found that mice coinjected with C57.npr.IL-4 cells and GL261 glioma cells lived significantly longer than control mice injected with GL261 alone. Six out of 7 mice inoculated with IL-4 secreting progenitors compared to 0 out of 7 control mice were alive 90 days after injection. Although not as effective, mice that were coinjected with C57.npr cells that lacked expression of IL-4 also survived

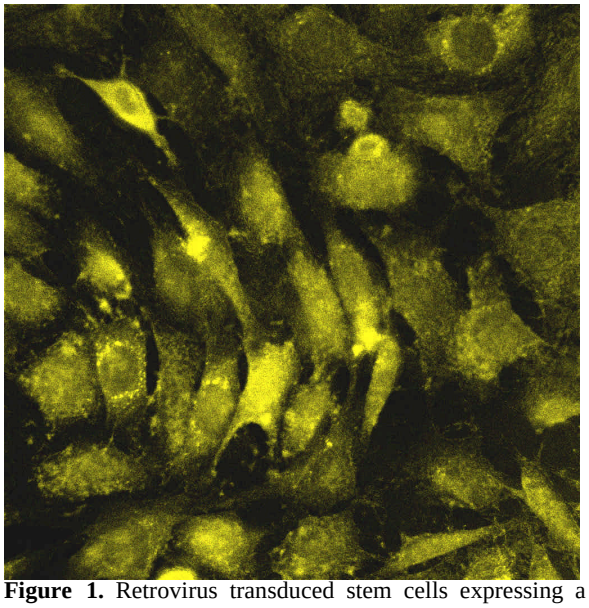

Figure 1. Retrovirus transduced stem cells expressing a foreign protein. C17.2 neural stem cells expressing yellow florescent protein one week after transduction.

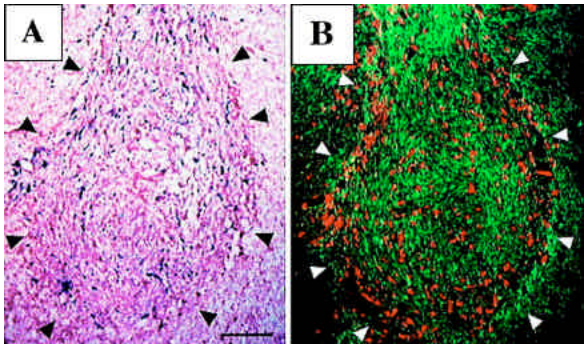

Figure 2. Brain tumor xenograft after stem cell transplantation. Adjacent tumor sections showing ß-galactisidase expressing NSC's distributed throughout the tumor mass 10 days post-transplantation. Tumor border indicated by arrowheads. Tumor stained with neutral red and NSC's revealed by X-gal staining (A). Double immunofluorescence where the CNS-1 tumor line, which expresses GFP, was stained with an anti-GFP antibody (FITC, green) and NSC's were identified by staining with anti-ß-gal antibody (Texas red) (B). scale bars: A, 90µm, B, 100 µm.

significantly longer than control mice. Similar observations were made in rats coinjected with C6 cells and ST14A.IL-4.3. The rats treated with IL-4 secreting progenitors showed 50% survival up to 90 days. Rats injected with ST14A and C6 cells also demonstrated enhanced survival as compared to control rats although significantly less than the animals treated with ST14A.IL-4.3. The increased survival seen in both mice and rats treated with progenitors and IL-4 secreting progenitors suggests that neural stem cells can effectively generate an adoptive immune response against glioma and that they have some endogenous mechanism for inhibiting tumor growth.

5.2. Migration and homing efficiency of immortal neural stem cells

Previous studies demonstrated that C17.2 neural stem cells (Figure 1) when injected into the brain of developing mouse embryos migrated and integrated throughout the brain. Snyder *et al.* demonstrated that these immortalized neural stem cells had tropism for areas of diverse pathology (infarct, blunt trauma, and demylination), and after migrating into the damaged tissue, differentiated into appropriate neural, astrocytic, and oligodendroglial lineages (30, 59).

5.2.1. Preparation of cell line

C17.2 cells represent an immortalized cell population derived originally from the external granule layer of the cerebellum of neonatal mice. The EGL was dissected and disassociated followed by culture in EGF and FGF containing medium. The resultant neurospheres were than transfected with retroviruses expressing V-myc and beta-galactosidase (LacZ). After initial screens of several immortalized lines, one line, C-17.2, was selected for further characterization.(42)

5.2.2. Migratory capacity

Aboody *et al.* and Herrlinger *et al.* both recently demonstrated that C17.2 home to xenografted gliomas in mouse and rat brains over a distance of several millimeters (Figure 2). In addition, Aboody showed that C17.2 migrate from one hemisphere to the other and even target a parenchymal tumor after injection into the mouse tail vein(60). These finding, while exciting, do not clarify the mechanism by which the stem cells migrate through highly cellular tissues without obvious potential spaces. Do stem cells migrate by destroying tight junctions or remodeling basement membrane? Do they travel along or within the vasculature of the brain? Do they require cell surface adhesion molecules or established migratory tracts? These are critical questions, the answers to which will determine the most effective way to deliver stem cells and capitalize on their native homing potential.

5.2.3. Modification to produce replication conditional herpes simplex virus-1 (HSV-1)

Herrlinger *et al.* modified C17.2 cells enabling them to produce replication-conditional herpes simplex virus type 1 (HSV-1). The infection and replication of this virus was restricted to dividing cells and causes cell death after 48-72 hours. C17.2 producer cells were infected with HSV-1 and then treated either with mimosine or mimosine in combination with gancylovir to arrest cell proliferation (61). The infected cells were then injected into a CNS-1 intracerebral tumor that had been established in the brains of nude mice. Three days later, X-gal staining produced by the cleavage of 5-bromo-3-indoyl-b-d-galactopyranoside by beta-galactisidase visualized the location of the C17.2 producer cells. C17.2 cells were found throughout the body of the tumor and juxtaposed to small beds of tumor cells that had escaped the main tumor mass and invaded the surrounding parenchyma. Immunohistochemistry for the herpes simplex virus tyrosine kinase protein (HSV-TK) demonstrated infection of cells both within the tumor and in the surrounding parenchyma (61). The combination of

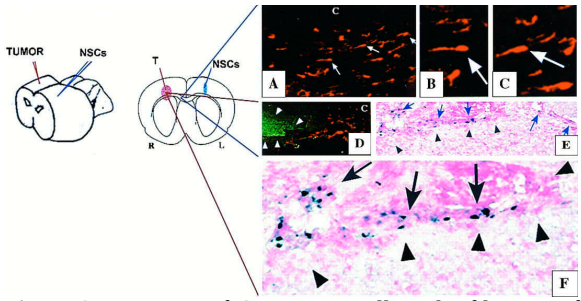

Figure 3. Migration of C17.2 stem cells and infiltration of CNS-1 tumor xenograft. (A) Corpus collosum "c" of hemisphere contralateral to site of NSC injection. Arrows indicate migrating NSC (red) with elongated morphology and leading edge process classically seen in migrating neural progenitors. (B-C) Higher magnification view of NSC indicated by arrows in (A). (D) NSC's (red) approach tumor (green) after migrating from the contralateral hemisphere across the corpus collosum. (E) and at higher magnification (F) NSC's (blue) have reached and invaded the tumor xenograft (neutral red) 10 days after contralaterlal injection.

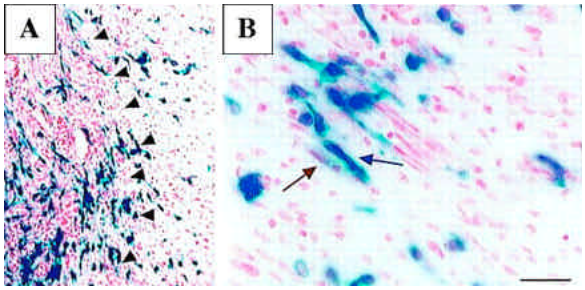

Figure 4. Co-localization of NSC's and invading tumor cells at the tumor border. (A) low power image of blue NSC's at the invasive edge of the CNS-1 tumor border (arrowheads). (B) high power view of blue NSC's adjacent to "spindle-like" red tumor cells (arrows) escaping the tumor and invading the surrounding parenchyma. Scale bars: A, 60µm, H, 15 µm.

replication-conditional virus with stem cells capable of homing to the main tumor mass and infiltrating tumor cells appears an effective means for targeting tumor while sparing the healthy parenchyma.

5.2.4. Delivery of cytosine deaminase to induce tumor cell death

Aboody *et al.* transduced C17.2 cells with cytosine deaminase (CD), an enzyme that activates the prodrug 3, 5-fluorocytosine (5-FC) by converting it into 5-fluorouracil (5-FU). 5-FU, a base analog, which interferes with DNA replication and causes cell death in proliferating cells. The C17.2CD cells were then injected into the brain of a nude mouse with an established CNS-1 tumor. Animals were injected either intratumorally, intraventricularly, at a distance from the tumor in either the ipsilateral or contralateral hemisphere, or IV into the tail vein (Figure 3). Localization of the stem cells was assayed 2-7 days later. C17.2CD cells were found mostly at the border of the tumor with some infiltration throughout the tumor body. C17.2CD cells were also seen to be in contact with tumor cells that had escaped the main mass of the tumor (Figure 4). Animals were treated with 5-FC i.p. (900mg/kg) over 10 days. Tumor mass was reduced by 80% in animals treated with C17.2CD as compared to control(60). The tumor tropism of C17.2 cells allowed for targeted delivery of the prodrug activating cytosine deaminase thereby enhancing the effect of the DNA replication dependent toxicity of 5-FU in tumor relative to surrounding parenchyma.

6. SUMMARY AND PERSPECTIVE

Studies to date include intratumoral injection of primary cortical progenitors, immortalized striatum progenitors, and immortalized cerebelluar stem cells. All three populations showed integration throughout the tumor mass in xenograft models. Benedetti described a stem cell endogenous inhibitory effect on tumor growth and demonstrated that both cortical and striatum progenitors can induce an IL-4 mediated adoptive immune response against tumor. Herrlinger illustrated the ability of stem cells to deliver virus with selective lethality to dividing cells thereby targeting tumor and sparing healthy parenchyma. Finally, the work by Aboody established the capacity of stem cells to seek out tumor throughout the brain and to deliver therapy to not only the main mass but also the invading tumor cells that would otherwise lead to tumor recurrence.

These studies clearly demonstrated the potential of stem cells to deliver therapy to brain tumors. The migration and homing studies by Aboody offer a potential means of treating invasive tumors that escape standard therapy. Yet additional work is necessary to answer the many questions that remain. What happens to stem cells after injection into the brain? What is the mechanism of migration and how efficient is the process of migration? What populations of stem and progenitor cells share this capacity? What happens to the stem cells after integrating into the tumor? Do they differentiate and form functional connections to the surrounding tissue? And, if as the study by Benedetti indicates, differentiating stem cells secrete an inhibitor of tumor growth, what might this factor or factors be? Also of great importance is the question of what cues are responsible for stem cell homing to tumor. Aboody began to address this question by showing that C17.2 cells migrate in a culture of tumor cells but not when cultured with fibroblasts. Additionally, only three populations of stem cells have been tested in this paradigm. It remains to be seen if all stem cells share a common tropism for tumor or whether there will be a finer differentiation in which certain stem cells have higher affinity for a given tumor type. The continued characterization of the remaining populations is a daunting but critical task.

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