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CHAPTER XI

Advances in stem cell therapy against gliomas

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ABSTRACT

Malignant gliomas are one of the most lethal cancers, and despite extensive research very little progress has been made in improving prognosis. Multimodality treatment combining surgery, radiation, and chemotherapy is the current golden standard, but effective treatment remains difficult due to the invasive nature and high recurrence of gliomas. Stem cell-based therapy using neural, mesenchymal, or hematopoietic stem cells may be an alternative approach because it is tumor selective and allows targeted therapy that spares healthy brain tissue. Stem cells can be used to establish a long-term antitumor response by stimulating the immune system and delivering prodrug, metabolizing genes, or oncolytic viruses. In this review, we discuss current trends and the latest developments in stem cell therapy against malignant gliomas from both the experimental laboratory and the clinic.

Stem cell-based therapy against gliomas

Gliomas account for about 60% of all primary central nervous system tumors with a very poor prognosis. Glioblastoma (GBM), the most malignant type of glioma, has a median survival of approximately 12-18 months [1, 2]. The characteristics of this malignancy include uncontrolled cellular proliferation, invasiveness with both long root-like processes and single invasive cells, areas of necrosis, resistance to apoptosis, extensive angiogenesis, and multiple genetic alterations (Figure 1) [3]. Standard-of-care treatment includes maximal surgical resection of the tumor followed by radiation and chemotherapy (temozolomide); however, as the poor survival rate indicates, these treatments have not been effective in preventing disease progression. Most patients die within a year of diagnosis from a new secondary tumor foci forming within 2 cm of the resected area [4, 5]. The location of the tumor (the brain) and its invasive nature prevent complete surgical removal, while radiotherapy cannot be given in a high enough dosage due to inevitable damage to the normal brain parenchyma. Since chemotherapeutics cannot efficiently cross the blood brain barrier (BBB), and glioma cells have a high tendency to develop resistance against these agents, the efficacy of this approach is limited. The heterogeneous nature of GBM cells, and the complex interaction between different types of tumor cells, stromal cells within the tumor, vasculature, and extracellular matrix (ECM), complicates matters even further since no clear target can be identified, and multiple

interlinked pathways exist, severely decreasing treatment efficacy. Recently, it has been shown that a small population of tumor cells, called cancer stem cells, is responsible for tumor/glioma growth, resistance and recurrence. These neural stem-like cells (also called glioma stem cells; GSC) have the ability of self-renewal and differentiation into a diverse population of cells, both tumorigenic and non-tumorigenic, and display a profound interaction with the endothelial vascular niche. Although the working mechanism is not exactly clear, it is thought that GSCs promote microvascular angiogenesis through secretion of vascular endothelial growth factor (VEGF), while secreted factors from this same vascular niche allow them to maintain their undifferentiated state [6]. Once implanted in immunogenic mice, GSCs have the ability to recapitulate a phenocopy of the original malignancy [7]. Further, GSCs appear to be more resistant to conventional therapy as compared to their non stem-like cells counterpart due to their relative quiescence, and will remain at the tumor site, eventually causing a relapse [8, 9].

Over the past decade, stem cells (SC) have become increasingly popular as an alternative therapy for treating malignant gliomas. In 2000, Aboody *et al.* described the unique intrinsic capacity of neural stem cells (NSC) to “home” to the tumor site and migrate along metastatic/invasive tumor borders far from their initial site of transplantation, thereby raising the possibility of using NSCs as a therapeutic delivery vehicle in the brain [10]. Many research groups followed this example and, as of today, a wide variety of stem cell-based therapeutics has been tested. Aside from the homing mechanism that selectively targets tumor cells, some stem cells can effortlessly cross the BBB, are easily modified to carry therapeutic genes, have immunosuppressive properties that prevent a host immunoreaction after implantation, and seem capable of shielding therapeutics such as oncolytic viruses from the host immune response, thereby ensuring long term reservoirs of therapeutic virus at the tumor site.

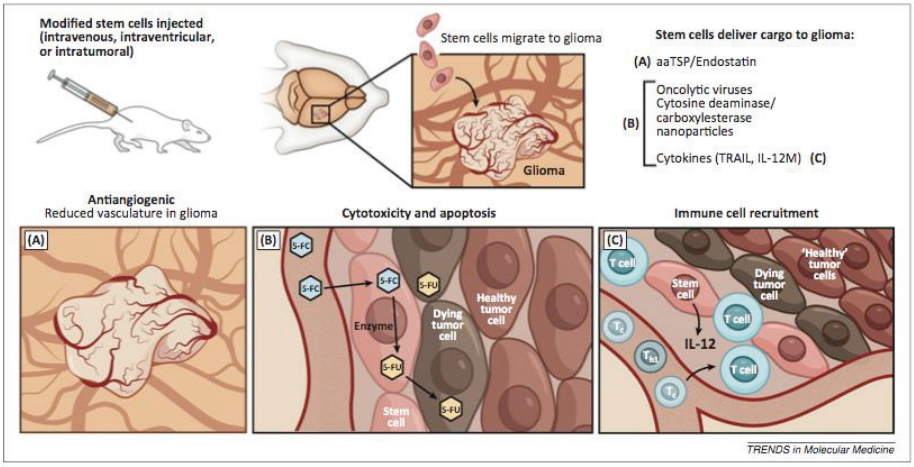

Figure 1. Overview of stem cell-based delivery of different therapeutics to gliomas. Several forms of therapy can be delivered by modified stem cells, including antiangiogenic factors such as aaTSP or endostatin (A), oncolytic viruses or enzymes capable of processing prodrugs such as 5-FC to cytotoxic compounds (B), and immune regulatory factors such as interleukin (IL)-12 that can recruit antitumor immune cells (C). Abbreviations: aaTSP, antiangiogenic protein thrombospondin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; T_{h1}, T helper 1 cell; T_{cx}, cytotoxic T cell.

NSCs are the stem cell type most commonly used for glioma therapy. They are the precursor cells of the central nervous system (CNS) and the only endogenous stem cells to the brain. These cells can self-renew and, due to their multipotent nature, can differentiate into neurons, astrocytes, and oligodendrites. NSCs have a very strong glioma tropism, especially targeting tumor border and hypoxic zones, and can cross the BBB, making excellent carriers for therapeutics such as viral particles, prodrugs, and cytokines [10]. An interesting feature of NSCs is their ability in targeting not only the primary tumor mass, but also the invasive GSCs, providing a chance in eliminating the driving factor of glioma progression and recurrence. NSCs not only target gliomas, but have also shown an equal tropism for breast cancer and melanoma brain metastases [11, 12]. The mechanism underlying this tumor tropism is not yet fully understood, but it is assumed that various chemo attractants and cytokines released by the tumor microenvironment are critical. Because NSCs do not display major histocompatibility complex type II (MHCII) on their cell surface, no host immunoresponse is evoked upon transplantation [13]. In addition, the secretion of

immunomodulating cytokines such as interleukin 10 (IL-10) further suppresses the local immune response, allowing the optimal delivery of a therapeutic payload with minimal neuro-inflammation [14]. NSCs could potentially be harvested from the adult brain, but this process is very complicated and time consuming. As an alternative, most studies use stable cell lines of immortalized NSCs originally obtained from embryonic cells, which often makes their use controversial due to ethical, regulatory, and political concerns.

Mesenchymal stem cells (MSC) are the most often studied alternative to NSCs for glioma therapy. These adult stem cells retain their stem cell characteristics, display similar tropism to glioma, and can cross the BBB. They can differentiate into any cell of the mesenchymal lineage including osteoblasts, chondrocytes, myocytes, and adipocytes [15]. MSCs are easily obtained from bone marrow, adipose tissue, peripheral blood, umbilical cord (UC) blood, or the placenta, and can be isolated by their expression of the surface markers CD73, CD90, CD105, CD146, CD271, STRO-1, and lack of expression of the hematopoietic markers CD34 and CD45 [16]. As with NSCs, local immunosuppression can be observed upon implantation [17].

Less frequently used cell types include embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs). The use of ESCs is heavily disputed due to their origin; they can only be obtained from embryonic or fetal tissue [18]. Unlike other cell types, ESCs can be modified by homologous recombination, not only eliminating the use of (often inefficient) viral transduction, but further allowing for vey specific genetic alterations yielding lines of cells that are stable and identical, ideal for clinical use [19, 20]. HSCs on the other hand are adult stem cells that can be easily obtained from peripheral blood or bone marrow. HSCs display tropism to brain tumors and therefore are becoming of interest for malignant glioma therapy [21]. Homing of these cells to the tumor site is mediated through attraction to two cytokines, tumor necrosis factor beta (TNFβ) and stromal derived factor alpha (SDF1α) [22]. Furthermore, the expression of E-selectin by glioma endothelial cells helps adhere circulating HSCs to the tumor tissue.

Currently, a wide range of stem cell-based therapeutic strategies is being investigated pre-clinically while a small portion of this research is being transitioned

to the clinic (Figure 2; Table 1). In this review, we summarize recent advances in the field of stem cell therapy for malignant gliomas and discuss future directions and challenges.

Box 1. Cell types used for glioma stem cell therapy

NSCs are the only adult stem cells endogenous to the human brain. They can differentiate into neurons, astrocytes, and oligodendrites. The subventricular zone (SVZ) of the forebrain is the area richest in NSC, but they can also be found in the striatum and the dentate gyrus of the hippocampus. NSCs are problematic to isolate and expand because only small numbers are available in the mature brain. A wide range of surface markers has been associated with NSCs, as well as expression of sox-1 and -2, pax-6 and nestin. A recent study shows selection based on expression of the surface markers $CD133^+/CD184^+/CD271^-/CD44^-/CD24^+$ allows for highly pure cultures of NSCs [23]. NSCs tend to grow in neurospheres *in vitro* and are cultured in specialized NSC growth medium containing Dulbecco's modified Eagle medium (DMEM)/glutamax, B27, insulin, glucose, penicillin/streptomycin, bGFG and EGF. Differentiation is promoted by epidermal growth factor (EGF) and fibroblast growth factor (FGF) [24].

MSCs are non-hematopoietic bone marrow (BM)-derived adult stem cells with the capacity to differentiate into cells of the mesenchymal lineage including osteocytes, chondrocytes, myocytes and adipocytes. Compared to NSCs, they are relatively easily isolated from BM, umbilical chord (UC) blood, placenta, adipose tissue and peripheral blood. Once cells are aspirated from BM, they are cultured in DMEM and fetal bovine serum (FBS) at 37 ^{0}C and 5% CO_2. MSCs, in contrast to the hematopoietic progenitor cells that are also derived from BM, adhere to tissue culture plastic within 24-48h. Isolation and selection occurs based on their adherent growth in culture, expression of the surface markers CD73, CD90, CD105, CD146, CD271 and STRO1 and lack of expression of CD34 and CD45 and HLA-DR.[16]

HSCs are bone marrow derived adult stem cells that give rise to blood cells of both the myeloid and lymphoid lineage, including thrombocytes, erythrocytes, monocytes, neutrophils, basophils, eosinophils, macrophages, dendritic cells (myeloid) and B- and T lymphocytes and Natural Killer (NK) cells (lymphoid). Cells can be obtained from the BM, umbilical cord, and peripheral blood. Pretreatment with granulocyte colony stimulating factor (GSCF) stimulates migration of HSCs to the blood and is often used. Selection takes place based on surface markers and is subject to ongoing debate. Currently the markers most widely accepted for human MSCs are $CD34^+/CD59^+/Thy\text{-}1^+/CD38^-/c\text{-}kit^+$ combined with a lack of lineage markers[25], but for mice MSCs different expression markers are used.

ESCs are pluripotent. They are the only stem cells with unlimited plasticity and replication

potential, which makes them highly attractive for research purposes. However, their use is highly disputed due to the source of origin. ESCs are derived from the inner mass of the blastocyst 4 to 5 days after *in vitro* fertilization (IVF) by immunosurgery and plated onto a layer of support cells consisting of mouse embryonic fibroblast (MEF) in special hESC medium consisting of DMEM with 20% KSR, bFGF, glutamine, non essential amino acids, penicillin/streptomycin and β-mercaptoethanol. This allows the embryonic cells to attach and to expand without the risk of differentiation. Differentiation occurs once the embryonic cells are removed from their support cells and are allowed to form embryoid bodies. Nanog and Oct4 transcription factors are often used to determine the phase of differentiation. As of lately, several protocols using synthetic polymers are now available culturing ESC in the absence of feeder cells or serum. However, Higuchi et al showed that none of these protocols have been able to prevent differentiation and pluripotency of ESCs in the long term, limiting their current value for the stem cell therapy field.[26]

iPSCs are somatic cells that are reprogrammed to become ESC-like through introduction of embryonic genes including Sox 3 and 4, Oct-4, myc and Klf4/LIN28 by viral vectors in a process that takes between 15 and 30 days. The infected cells are then cultured in ESC medium, and after 10 to 15 days colonies will appear, which can be expanded. These new stem-like cells express ESC markers are capable of differentiating into cells of the endoderm, mesoderm, and ectoderm and can replicate indefinitely.

Stem cells for cargo delivery

Genetic manipulation is one of the research strategies most often investigated for glioma, because it has an almost unlimited range of potential targets. Therapeutic genes stimulating the immune system, inducing tumor cell death, inhibiting angiogenesis, and limiting metastatic potential, have been extensively studied, and many different approaches and gene combinations have been used. However, gene therapy (and/or viral therapy) alone has not been able to live up to its full potential, due to activation and elimination by the host immune system, low transduction efficiency and gene expression, and a lack of even distribution throughout the target tissue. Since stem cells are known to display strong tropism to glioma, are capable of crossing the blood brain barrier, suppress the host immune system, and are easily genetically modified, they make ideal delivery vehicles for therapeutic agents, including genes. Most therapeutic strategies for malignant gliomas using stem cells involve the delivery of mainly four different types of cargos: cytokines, enzymes or prodrugs, oncolytic viruses, and nanoparticles.

i. Cytokine-based glioma therapy

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is one of the most commonly explored cancer therapeutics because it binds to death receptors found specifically on tumor cells, causing a widespread apoptotic effect with minimal cytotoxic effects on normal tissues (Figure 2); however, some cancer types, including GBM, display resistance to TRAIL-mediated apoptosis (Box 2) [27-31]. Three recent studies have used NSCs as a delivery vehicle for the secreted soluble variant of TRAIL (sTRAIL) by fusing the N-terminus of Flt3L (a ligand for Flt3L tyrosine kinase receptor) to the extracellular domain of TRAIL. Hingtgen *et al* designed a reporter system to non-invasively monitor the delivery, fate, and therapeutic effect of sTRAIL to GBM by fusing a luciferase reporter to sTRAIL [27]. NSCs delivered the fusion protein to the tumor site, and luciferase bioluminescence imaging allowed tracking of both NSCs and the delivery of sTRAIL to glioma tumors. With the continuous delivery of sTRAIL by NSCs, a decreased glioma burden was observed as soon as six days post-implantation. Given that glioma cells are known to develop resistance to TRAIL (Box 2), new ways are being explored to sensitize GBM to this therapeutic agent. Balyasnikova *et al.* explored the possibility of combining sTRAIL therapy with the proteasome inhibitor bortezomib and showed that survival significantly increases with this dual treatment [32]. NSC-mediated delivery of sTRAIL has also been combined with the kinase inhibitor PI-103, which inhibits the PI3 kinase (PI3K)–Akt–mTOR pathway and thus inhibits proliferation and tumor growth [31]. Inhibition of this pathway antagonizes the effect of sTRAIL, resulting in a more efficient induction of apoptosis and cell death. Both studies highlight the need for therapeutics capable of sensitizing glioma to TRAIL. Recently, Badr *et al.* characterized a family of cardiac glycosides, including lanatoside C, an FDA-approved compound that sensitizes GBM cells to TRAIL and showed that the combination of recombinant TRAIL and lanatoside C yielded an enhanced therapeutic effect [33, 34]. Given that this family of compounds is known to penetrate the brain, they can be easily applied in combination with the NSC-sTRAIL strategy for GBM therapy. Three additional studies used MSCs for sTRAIL delivery. In 2009, Sasportas *et al.* assessed the potential for using MSCs for treating glioma by investigating the cell fate, therapeutic efficacy, and genetic engineering of these cells [28]. In a proof of principle study, MSCs were engineered *ex vivo* to express sTRAIL [35]. These engineered MSCs migrated towards glioma, retained their stem-like

properties, and showed prolonged survival in the tumor surroundings, providing a basis to further develop MSC-based therapies for glioma (Figure 2). MSCs engineered to secrete sTRAIL also appear to be resistant to its cytotoxic effect, whereas a caspase-mediated apoptosis was induced in glioma cells. Shortly after, Menon *et al.* confirmed these finding [29] using MSCs transduced to express both sTRAIL and the mCherry fluorescent protein, demonstrating tumor specificity and retention in glioma cells both *in vitro* and *in vivo*. Moreover, significant survival was observed in the treated group as compared to control animals, suggesting that MSCs expressing sTRAIL could provide an interesting approach for anti-glioma therapy. Choi *et al.* applied the same strategy using human adipose tissue derived MSCs (hAT-MSCs) engineered to express sTRAIL and reported similar results [36].

Genetically modified MSCs can also be used to secrete molecules that do not directly target glioma, but which attract innate immune cells to the tumor, as shown by Ryu *et al.* [37]. MSCs engineered to express modified interleukin 12 (IL-12M), a proinflammatory cytokine that induces T-helper 1 and cytotoxic T cell immunity, yielded prolonged survival of glioma-bearing mice when injected intratumorally. Remarkably, control mice injected with USB-MSC-IL12M showed resistance to new tumorigenesis, suggesting a tumor-specific T cell immunity.

ii. Enzyme/prodrug-based glioma therapy

As an alternative strategy to the use of active drugs, which have the risk of targeting normal tissue, many studies have focused on the use of prodrugs that are activated exclusively at the tumor site, thereby increasing tissue selectivity. One of the most popular suicide gene therapy approaches relies on the herpes simplex virus type I thymidine kinase (HSV-TK) and the prodrug ganciclovir (GCV). Although excellent results have been reported in experimental settings, a lack of efficacy was observed in clinical trials [38-41]. Low transduction efficiency and the absence of a bystander effect are thought to be the main causes for this lack of success. To overcome these limitations, Ryu *et al.* designed a protocol using MSCs expressing HSV-TK (MSC-TK) combined with valproic acid (VPA), which upregulates gap junction proteins between MSCs and glioma cells, yielding an enhanced bystander effect [42]. This combined treatment significantly inhibited tumor growth and prolonged survival compared with mice treated with MSC-TK in the absence of VPA. Several studies

have tested a rat glioma model with NSCs (HB1.F3) transduced with the gene for cytosine deaminase (CD), which converts the prodrug 5-fluorocytosine (5-FC) into the active, inhibitory compound 5-fluorouracil (5-FU; Figure 2) [43, 44]. In contrast to the active drug 5-FU, the prodrug 5-FC can cross the BBB. Two separate studies have reported reduced tumor volumes and increased survival in CD/5-FC treated rats with glioma. [45] [41]. Joo *et al.* demonstrated both migration and homing of the HB1.F3 NSCs expressing CD to the tumor site as well as reduced tumor volume after breast cancer cells were implanted in one hemisphere of the mouse brain and CD-expressing NSCs were implanted into the contralateral hemisphere, followed by injection of the prodrug 5-FC [11]. Beyond demonstrating the feasibility of this treatment, this experiment showed that NSCs can not only home to primary brain tumors, but can also migrate towards metastases. However, the survival of animals was not significantly prolonged, suggesting that repeated administration of NSCs and prodrug is required. Further, a combination of NSC-encoding different therapeutic genes or the addition of conventional anticancer therapies to this treatment strategy might be needed. Two other studies reported the use of MSCs to deliver CD to brain tumors and showed an increased mice survival upon intratumoral injection of MSC-CD cells followed by 5-FC therapy (Table 1) [43, 44].

Lim *et al.* modified NSCs to express the rabbit carboxylesterase enzyme rCE, which converts the prodrug CTP-11 (irinotecan) into the active chemotherapeutic agent SN-38 (7-ethyl-10-hydroxycamptothecin), a potent topoisomerase I inhibitor [46]. Given that intratumoral injection is not favorable when multiple lesions are involved, as in the case for glioma, NSCs were administered systemically. After intravenous injection, rCE-expressing NSCs efficiently penetrated the brain targeting both the primary glioma site as well as infiltrating glioma cells (containing GSCs) that are known to be the source of tumor recurrence and patient death. However, the accumulation of NSCs in non-brain organs was also observed, but did not lead to any tissue damage or tumor formation, although follow-up studies might be needed to evaluate these effects on the long term. [46]. The authors speculate that the use of tumor trophic modulating agents and/or the use of multiple injections could enhance NSCs delivery to the tumor site, thereby increasing specificity and therapeutic effect. Using the same enzyme/prodrug therapy, Zhao *et al.* explored the use of NSCs engineered to secrete rCE enzyme and showed that this strategy yielded 200-fold

higher bystander effect on tumor cells in vitro and enhanced therapeutic effect on metastatic breast cancer in vivo[47]. This strategy should provide an enhanced therapeutic effect for malignant gliomas as compared to NSCs expressing endogenous rCE.

A hallmark of malignant gliomas is extensive angiogenesis with glioma stem cells needing a vascular niche for optimal functioning [6, 8, 48]. Yin *et al.* used MSCs to express the anti-angiogenesis factor (endostatin), the prodrug-activating enzyme rCE (activates CTP-11 into SN-38), or a combination of both [49]. *In vivo*, MSCs expressing endostatin and rCE led to the highest antitumor response, including reduced angiogenesis, increased cell death, and a reduced GSCs population. Choi *et al.* evaluated the characteristics and therapeutic potential of human adipose tissue-derived MSCs (hAT-MSCs) in a rat brainstem glioma model and found, similar to NSCs, that hAT-MCSs modified to express rCE has tumor tropism, drug activation, and increased life span [50]. In another attempt to target angiogenesis, van Eekelen *et al.* modified NSCs to express antiangiogenic protein thrombospondin (aaTSP-1) [51]. aaTSP-1 was shown to target glioma vasculature and to significantly reduce vessel density in a glioma brain co-culture containing endothelial cells, established glioma cells, and glioma stem cells. The decrease in tumor vessel density correlated with decrease in tumor progression and increased survival, most likely due to the disrupted interaction between endothelial cells and glioma stem cells.

iii. Oncolytic virus-based glioma therapy

Theoretically, oncolytic viruses have a significant potential for glioma therapy due to their specificity and high efficiency in killing tumor cells. However, current viral therapeutic strategies have not yet reached their full potential due to poor distribution at the tumor site, low infectivity of tumor cells, and the host immune response (Box 2). To overcome these limitations, Ahmed *et al.* evaluated NSCs as carriers for the targeted delivery of CRAD-S-pk7, a glioma restricted oncolytic adenovirus [14]. NSCs loaded with CRAD-S-pk7 injected intracranially inhibited tumor growth and increased median survival by 50%, as compared to animals treated with CRAD-S-pk7 alone, suggesting that NSCs can shield the virus from the host immune system before delivery to the tumor. Interestingly, the oncolytic virus seemed to enhance

NSCs migration towards the tumor site. In a follow up study by the same group, the FDA-approved NSC line HB1.F3-CD was loaded with CRAD-S-pk7 and a thorough characterization of this carrier system was performed [52]. NSCs loaded with CRAD-S-pk7 retained tumor tropism,
continued to replicate CRAD-S-pk7 for over a week after injection, and effectively distributed the CRAD-S-pk7 virus among glioma cells *in vivo*. Nonspecific delivery of adenovirus in the brain was drastically reduced and, due to local injection of NSCs, no migration of NSCs to distant organs was observed, showing that this oncolytic virus carrier system holds a great potential for glioma therapy.

iv. Nanoparticle-based glioma therapy

Following a different approach, several groups are using MSCs to deliver to gliomas nanoparticles, which can carry different therapeutic agents incorporated into the particle or attached to the surface. MSCs can circumvent the problem that nanoparticles have in crossing the BBB, typically yielding low targeting efficiency to brain tumors. In a proof of principle study, Roger *et al.* used poly-lactic acid nanoparticles or lipid nanocapsules loaded with coumarin-6, a lipophilic fluorescent dye used to assess the intracellular uptake of nanoparticles by stem cells that was successfully delivered to the tumor site [53]. In a follow up study, MSCs loaded with lipid nanocapsules containing the organometallic complex ferrociphenol (Fc-diOH), a drug with demonstrated cytotoxic effect on glioma cells both *in vitro* and *in vivo,* were shown to have an effective anti-cancer treatment [54]. Li *et al.* designed a high-efficacy targeting approach for nanoparticle drug delivery using MSCs expressing silica nanorattle doxorubicin (dox) on the cell surface [55]. The drug was efficiently delivered and resulted in a wider distribution and longer retention of dox at the tumor site, with subsequent enhanced glioma apoptosis as compared with free dox and silica-encapsulated dox control groups.

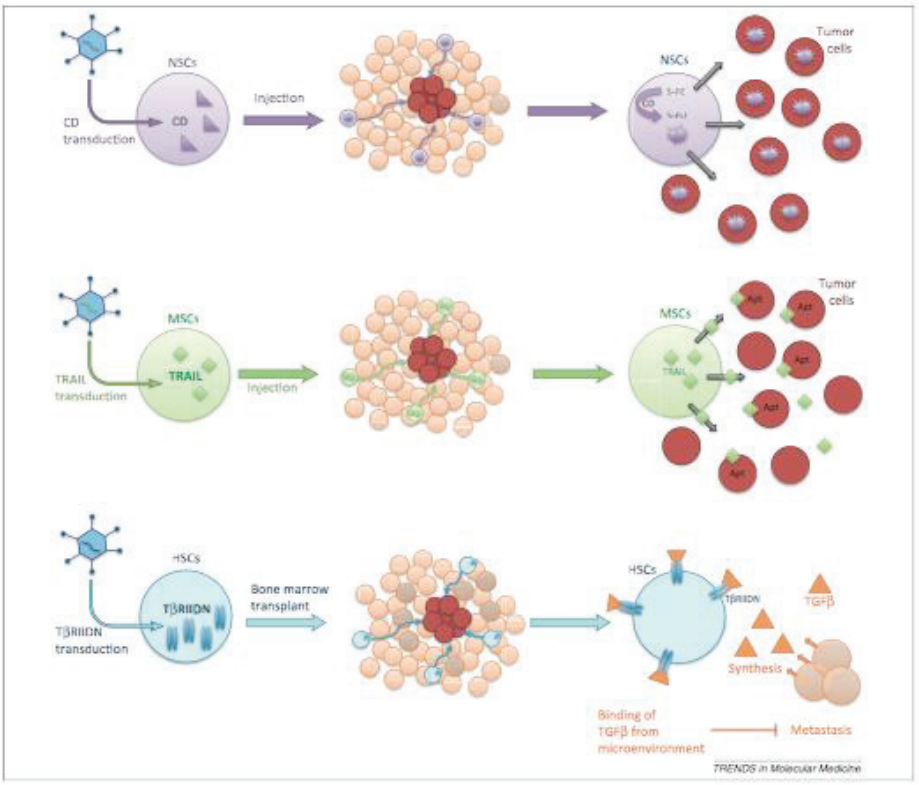

Figure 2. Examples of stem cell-based therapies against gliomas. Many variations on stem cell therapy are possible, and three are depicted here using mesenchymal, neuronal and hematopoietic stem cells (MSCs, NSCs, and HSCs, respectively). Abbreviations: TGF β, transforming growth factor β; Apt, apoptosis; CD, cytosine deaminase; TRAIL, tumor necrosis factor apoptosis-inducing ligand; TβRIIDN, dominant negative mutant of transforming growth factor β receptor II.

Routes of administration and enhancement of the stem cell model

Several studies have focused on developing alternative strategies to increase the therapeutic effect of SC-based therapy to brain tumors by enhancing delivery mode, tumor tropism, and cellular delivery vehicles (Table 1 and Box 2).

i. Routes of administration

Successful administration of stem cells is crucial for their antitumor efficacy. Both intratumoral or intravenous injections can effectively deliver stem cells, and either injection route is used in the majority of studies [56]. Panciani *et al.* proposed a different delivery route using injections into ventricles or spaces of the brain speculating that this injection mode may lead to the formation of a reservoir of therapeutic cells [57]. This study confirmed that intraventriculary transplanted MSCs do create a niche in the subventricular space and can be triggered to migrate to the site of tumor formation. A follow up study investigating the life span of implanted MSCs and their potential for finding and attacking GSCs and tumor recurrence is planned. Meanwhile, Bexell *et al.* studied long distance tropism and migration of MSCs after intratumoral and extratumoral implantations in a rat glioma model [58]. No evidence of long distance MSC migration to the tumor site through either the corpus callosum to the contralateral hemisphere or through the striatum to the ipsilateral hemisphere was observed, suggesting the use of MSCs is limited to certain delivery routes. Intratumoral injection resulted in a dense and tumor specific distribution, as previously reported [59].

Biodegradable synthetic extracellular matrices (sECMs) have been used in various rodent models to provide mechanical support that promotes stem cell survival and differentiation into neurons [60, 61]. Kauer *et al.* have evaluated the implantation of NSCs expressing sTRAIL encapsulated in sECM at the tumor cavity following tumor resection and found that the washout of NSCs by cerebrospinal fluid was reduced drastically [30]. Both migratory stem cells and sTRAIL could leave the ECM environment and reach the tumor site, but increased retention at the tumor site and a subsequent increase in sTRAIL secretion was observed, suggesting that coating stem cells with ECM may be a highly successful strategy for treating GBM [62].

ii. Factors that regulate glioma tropism

Stem cells are particularly attractive for glioma therapy due to their tropism to the tumor site. It is still not clear how this “homing mechanism” works, but growth factors and chemokines secreted or expressed by glioma cells are known to be important. Park *et al.* designed MSCs to overexpress the alpha chemokine receptor CXCR4 [63], a receptor that specifically binds SDF1α, a key cytokine mediator of glioma tropism

[64, 65]. CXCR4 overexpression enhances the migratory capacity of MSCs to gliomas both *in vitro* and *in vivo*; inhibition of either SDF1α or CXCR4 completely blocks migration. Kim *et al.* followed a similar approach and showed that upregulating of interleukin 8 (IL-8) secretion by glioma, or overexpression of the IL-8 receptor CXCR1 on the MSC surface, enhanced the migration capability of MSCs to the tumor. Inhibiting IL-8 significantly reduced migration, suggesting that CXCR1 is a major regulator in glioma tropism [66]. Velpula *et al.* showed that multiple cytokines are involved in recruiting MSCs to the glioma site, including IL-8, GRO, GROα, MCP-1, and MCP-2 [67], but more research is needed to completely unravel the mechanism of tumor site homing.

iii. Improved cellular vehicles

To date, the experimental use of ESCs for glioma therapy has been limited to the delivery of sTRAIL, owing to ethical, regulatory, and political concerns, and no recent studies have been published (Table 1) [68]. Recently, Lee *et al.* reported on the use of induced pluripotent stem cells (iPSCs) to generate NSCs [69] and showed that in this context, iPSCs and ESCs are functionally equivalent, but iPSCs can be relatively easy to generate from somatic cells and are not burdened by the ethical concerns. In this study, iPSCs cells were generated by transducing primary mouse embryonic fibroblasts with four transcription factors, Oct-4, Sox 2, c-Myc and Klf4; by culturing iPSCs in monoculture, NSCs were generated. To test the functionality and potential use for glioma therapy, these NSCs were transduced with a baculovirus containing the HSV-TK gene and injected in the contralateral hemisphere of tumor-bearing mice. Prolonged survival and inhibition of tumor growth was observed, indicating that iPSC-derived NSCs possess all characteristics required to serve as a cellular carrier for glioma therapy. The same research group recently published a new study evaluating the use of human embryonic stem cells to generate NSCs [70] in which the authors speculate that ESC-derived NSCs have the potential to produce limitless amounts of identical NSCs, while at the same time eliminating variability in the quality of therapeutic cells, allowing for better comparative analysis of different studies.

Endothelial progenitor cells are a subpopulation of hematopoietic stem cells that are known to migrate towards the neovasculature of certain cancers and integrate at the

tumor site and have also been studied as gene carries for the treatment of glioma [71]. Because EPCs can be easily collected from peripheral blood and display the appropriate tumor tropism, they make an interesting candidate for glioma stem cell-based therapy. Accumulation of EPCs at the tumor site has been confirmed by non-invasive imaging: Tc-99 single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) imaging of EPCs transformed with the human sodium iodide symporter (hNIS) gene or ferumoxides-protamide sulphate (FePro), respectively. Using a novel inducible lentivirus expression system under the stress controlled HSP70B promoter, Noyan *et al.* reported a proof of principle study that used a HSC-based gene therapy method to treat solid tumors using immunotherapy [72]. Hematopoietic stem and progenitor cells (HSPCs) were genetically modified to express the dominant negative mutant of the transforming growth factor-beta receptor II (TβRIIDN), which is known to neutralizes TGF-β signaling in the tumor microenvironment and can thereby suppress tumor cell metastasis (Figure 2) [73]. Mice received a bone marrow (BM) transplant with the modified HSPCs followed by subcutaneous injection of glioma cells. A massive antitumor immune response was reported and glioma tumor cell growth was prevented completely.

Table 1. Stem cell therapy against malignant gliomas

SC function	Approach	Transgene/modification strategy	Application	Refs
Cargo delivery	Cytokine	Expression of sTRAIL-luciferase fusion; NSC	Visualization of TRAIL-mediated therapy	[27]
		sTRAIL plus bortezonib; NSC	Glioma sensitization to TRAIL	[32]
		sTRAIL plus mTor inhibitor; NSC	Glioma sensitization to TRAIL	[31]
		sTRAIL; MSC	Proof of principle MSC-mediated TRAIL therapy	[28, 29, 36]
		IL-12 expression; MSC	Immunotherapy	[37]
	Enzyme/prodrug activation	aaTSP-1 expression; NSC	Anti-angiogenesis therapy	[51]
		rCE expression; NSC	SN-38 mediated therapy	[46]
		rCE expression; MSC	SN-38 mediated therapy	[50]
		Endostatin and/or carboxylesterase 2; MSC	Anti-angiogenesis therapy	[49]
		CD expression; NSC	5-FC therapy	[45, 41, 11]
		CD expression; MSC	5-FC therapy	[43, 44]
		HSV-TK and VPA; MSC	Enhanced efficacy of HSV-TK mediated therapy	[42]
	Oncolytic virus	CRAD-S-pk7 expression; NSC	Proof of principle	[14]
			Enhanced carrier system	[52]
	Nanoparticles	NPs loaded with coumarin 6; MSC	Proof of principle NP-mediated delivery system	[53]
		NPs loaded with Fc-diOH; MSC	NP delivery	[54]
		NPs carrying silicia nanorattle dox; MSC	NP delivery	[55]
	NSC delivery to glioma	Coating with sECM; NSC	Improved NSC delivery	[30]
Enhancement of the SC model	Routes of administration	Intraventricular injections	Improved delivery mode	[57]
		Intratumoral vs extratumoral injections; MSC	Proof of principle; improved delivery mode	[58]
	Factors regulating tropism	CXCR 4 overexpression; MSC	Enhanced glioma tropism	[63]
		IL-8 and/or CXCR 1 overexpression; MSC	Enhanced glioma tropism	[66]
		Overexpression of various cytokines; MSC	Enhanced glioma tropism	[67]
	Improved cellular vehicles	IPSCs generated from embryonic fibroblasts; ESC	Proof of principle	[69]
		NSC differentiation; ESC	Proof of principle	[70]
		EPC; hNIS and FePro expression; HSC	Proof of principle; imaging	[71]
		TβRIIDN expression; HSC	Proof of principle; Immunotherapy	[72]

Abbreviations: MSC mesenchymal stem cell; NSC neural stem cell; ESC embryonic stem cell; HSC hematopoietic stem cell; NP nanoparticle; sTRAIL secretable tumor necrosis factor apoptosis-inducing ligand; IL-12 interleukin 12; Fc-diOH ferrociphenol; dox doxorubicin; HSV-TK herpes simplex virus thymidine kinase; VPA valproic acid; CD cytosine deaminase; rCE rabit carboxylesterase; IL-8 interleukin 8; aaTSP-1 antiangiogenic protein thrombospondin; sECM synthetic extracellular matrix; iPSCs induced pluripotent stem cells; FePro ferumoxides-protamide sulphate; TβRIIDN Growth factor β-receptor II; 5-FC 5-fluorocytosine

Box 2. Glioma stem cell therapy in the clinic

City of Hope Hospital, NCT 01172964

In July 2010 the very first clinical trial using stem cells as therapeutics for malignant gliomas was started at the City of Hope Hospital, California. Patients with histologically confirmed grade III or IV glioma, or patients diagnosed with grade II glioma and radiographic findings of grade III/IV glioma were enrolled and had their tumor mass removed by craniotomy. At the time of debulking, they received intracranial injections with HB1F3.CD genetically modified neural stem cells (day zero). In the absence of disease progression or intolerance to the injected cells, patients received on day 4–10 oral dosages of 5-FC every six hours. Response to therapy, and adverse effects were evaluated by MRI on day 32, 60 and for every 2 months onwards. No results have been published yet, and as for now, 30 patients have been enrolled.

Study details as described on www.clinicaltrials.gov

- Primary Outcome Measures: determination of the safety and feasibility of intracerebral administration of genetically-modified neural stem cells (NSCs) in combination with oral 5-fluorocytosine.
- Secondary Outcome Measures: Relationship between intracerebral and systemic concentrations of 5-FC and 5-FU with increasing NSC dose level; Presence of 5-FU in the brain using 19F-MRS; Assessment of development of immunogenicity against NSCs; Obtain preliminary imaging data regarding perfusion permeability parameters and imaging characteristics as shown on magnetic resonance imaging (MRI) studies due to the presence of NSCs in the brain; Assessment of the fate of NSCs at autopsy when feasible

Clinical transition and/or obstacles to translation

i. Glioma stem cell therapy in the clinic

Although a vast amount of interesting and exciting research is being explored using stem cells as a therapeutic strategy for malignant gliomas, most of these studies are being performed in the laboratory setting. This indicates that although the bench results are promising, translating these therapeutic strategies to the clinic remains difficult with only a single clinical trial in progress (Box 2, 3).

Box 3. Barriers to glioma therapy

Blood-brain barrier (BBB): The BBB consists of a lining of tight junctions between the endothelial cells of the brain capillaries. These tight junctions restrict the passage of molecules from the blood to the brain extra cellular matrix, allowing only certain substances to pass. Antibodies, antibiotics, chemotherapeutic agents, and some stem cells are unable to cross, severely limiting the potential of systemic therapy for glioma.

Blood-tumor barrier: Angiogenesis with leaky vessel formation, necrosis, and the highly heterogeneous character of the glioma cell population makes it vey difficult to establish consistent distribution of vectors and other agents. Further, certain areas of the tumor are almost inaccessible, resulting in only a very limited effect of the applied therapeutics.

Tumor cells Invasion in the brain: As gliomas progress and invade the brain, an extensive modulation of the extracellular matrix occurs. This phenomenon complicates curative surgery and radiotherapy considerably and results in tumor recurrence after surgical resection, often leading to patient death.

Secretion of local cytokines and growth factors that might induce malignant transformation in stem cells: Glioma cells are known to secrete a wide variety of chemokines and GF such as matrix metalloproteinases (MMPs), plasminogen tissue inhibitor 1 (PTI1), VEGF, EGF, FGF insulin growth factor 2 (IGF2), hepatocyte growth factor (HGF), and IL6 that are capable of initiating malignant transformation of nearby stem cells, recruiting them for contributement to tumor proliferation and growth. This is of particular concern when one actively introduces SC at the tumor site for glioma therapy and therefore extensive research needs to be done to address these safety issues.[74]

Escaping immune surveillance: Glioma surface markers such as MCH surface expression are often downregulated allowing glioma cells not only to escape the host immune response, but also to protect themselves from newly designed drugs targeted specifically to glioma cells.[75]

Resistance to therapies such as TRAIL: Malignant gliomas such as glioblastoma are known to acquire resistance to therapies. In the case for TRAIL-based therapy, upregulation of the Bcl2 associated Athanoge (BAG3) genes and multiple other genes have been described to cause resistance at various points along the apoptotic pathway. New research is focused at finding molecules that sensitize GBM cells to TRAIL.[31, 32, 34]

Secretion of local immunosuppressants: This problem does not only hinder the efficacy of the host immune system against the tumor cells, but also makes it increasingly difficult to use immunotherapy for anti-glioma treatment.

At the City of Hope (California) by Aboody *et al.,* NSCs (HB1.F3-CD) genetically modified to express *E. coli* cytosine deaminase, which will convert the oral pro-drug 5-FC into the chemotherapeutic agent 5-FU at the tumor site, are being tested as was done in various animal models (Table 1) [11, 41, 43-45]. The modified NSCs are injected directly at the tumor site after surgical resection of the tumor mass. Oral 5-FC will be given every six hours between day 4 and day 10. Because NSCs have a strong tropism for glioma [10, 76], no toxicity to normal brain cells is expected while efficient elimination of GBM cells is expected. The primary aim of this trial is to test the safety and feasibility of the NSC-CD system in humans, with secondary objective to evaluate immunogenicity and pharmacokinetics.

ii. Improving techniques for clinic/trials

A major limitation of stem cell therapy in general is safety. Stem cells possess many characteristics that make them well suited as cellular transport vehicles but their capacity for unlimited self-renewal raises several concerns regarding patient safety. Spontaneous tumor formation in longstanding MSC cultures has recently been reported, and it was shown that after implantation, a small fraction of immortalized NSCs continue to proliferate [10, 77]. A 2009 clinical trial by Amariglio *et al.* for the treatment of ataxia telangiectasia with NSC injection reported the formation of multiple brain tumors in a patient four years after treatment [78]. The standardized use of suicide genes such as CD for each stem cell line would theoretically minimize this risk.

Aside from malignant transformation of stem cells, the secretion of growth factors and chemokines, and the direct local immunosuppressive effect of stem cells may modify the tumor microenvironment in such a way that tumor growth is promoted. The latter has been reported in other solid tumors after injection with MSCs [74, 79, 80], and MSCs have been shown to enhance the metastatic potential of breast cancer cells [81]. The tumor promoting role of MSCs, however, remains in dispute; several studies report a glioma-suppressing effect of implanted MSCs [82, 83], and MSCs used in the clinic to treat neurodegenerative diseases and stroke have been well tolerated with limited side effects. The discrepancy between various studies is yet another issue that needs to be solved before stem cell-based therapy can be successfully applied to glioma treatment in the clinic. For now, it remains very difficult to interpret study results and to compare data between various study groups,

given the large variability between the stem cells themselves and the methods employed by different groups. Better ways of cell selection and preparation are absolutely essential to design stable and identical cell lines that can create reproducible datasets and optimally functioning cell carrier systems, a characteristic that might be attributable to subgroups rather than the stem cell population as a whole. Furthermore, systematic comparison of stem cell migratory potential, the ability to target GSCs, survival, and efficacy of delivery are needed to identify the optimal carrier system and delivery route. Ahmed *et al.* recently reported that effective oncolytic virus delivery by NSCs was clearly superior to MSCs, although equivalent migration capacity was displayed [84]. However, although many groups make use of the enzyme/prodrug combination of CD/5-FC in either NSCs or MSCs, no comparative studies have been performed, which is a missed opportunity in the quest for an optimal carrier system. Many more examples could be discussed, and until these issues are resolved, it seems to be overly optimistic to expect an easy transition of stem cell glioma therapy to the clinic. The ability to target glioma stem cells rather than glioma cells in general might prove to be a crucial point since these cells are thought to be the cause of tumor recurrence and patient death.

Translation is also slowed by concerns regarding several limitations of current glioma models used to test these strategies in the laboratory. Although many pathophysiological similarities between the rodent glioma model and human tumors are observed, many models are based on xenografts in immunocompromised mice. Implanted tumor cells will not mimic the process of *de novo* tumorigenesis, and tumor-associated immunosuppression and immune-modulating events are not likely to be accurately reflected, resulting in a slightly different tumor microenvironment. Doucette *et al.* have proposed overcoming this limitation by using an RCAS/Ntv-a glioma model in which endogenous glioma develop and acquire tumor and stromal features similar to human tumors [85]. This may be an improvement over existing glioma models, but this study was also performed using immunocompromised mice, implying that many variables will remain unknown until clinical testing is completed.

To resolve some of these issues and obtain a true understanding of the working mechanism and antitumor effect of stem cell-based therapy, the development of adequate imaging tools is of the utmost importance. Not only do we need these tools to increase treatment efficacy, but the ability to track single stem cells and determine their fate, tropism, migration, interaction with the tumor

environment, and mechanism of action will answer important questions regarding safety and efficacy. Several imaging tools capable of tracking stem cells are currently available preclinically (e.g., bioluminescence imaging, fluorescence), but these techniques are not yet available for use in humans due to several concerns including (substrate) toxicity and sensitivity. Recently, Thu *et al.* developed a method to visualize NSCs by magnetic resonance imaging (MRI), using iron labeling (ferumoxide-protamine sulfate complex) of NSCs [86], and Menon *et al.* reported similar results after labeling human MSCs with ferumoxide [87]; tumor tropism remained unaltered. Similar approaches might provide a solution that is easily translated to the clinic; however, more research is needed to fine tune these techniques for application in humans.

Whereas new imaging tools are necessary to develop stem cell therapy, the availability and efficacy of stem cells and whether they serve as vehicles for therapy or have a direct therapeutic effect are issues that also remain to be addressed. Malignant gliomas are a rapidly progressing and ever changing cancer, and if too much time is needed to obtain a certain number of stem cells, the tumor might have acquired resistance to the therapy being explored (e.g., chemotherapeutics, TRAIL, etc.). Furthermore, when stem cells are passaged too many times during expansion, differentiation and phenotypic changes may occur that limit their therapeutic potential. The use of stem cells might also be disputed due to ethical concerns. Limited availability hinders not only research opportunities but also limits the benefit of the potential approach, given that a working strategy that is not readily available cannot provide a cure. Techniques that allow for the rapid growth and expansion of cells while maintaining their characteristics are of extreme importance, as is optimal cell delivery to the tumor site. Whereas clinical studies opt for a direct intratumoral injection, preclinical experiments are testing intranodal, intradermal, intraventricular, or systemic injections in an attempt to enhance delivery success.

iii. Appropriate patient selection - when will this method work?

Patient selection may play an important role in the efficacy of the chosen therapeutic approach. More and more evidence suggest that specific genetic mutations in glioma cells respond to different therapies, and therefore genotyping or discovery of new biomarkers for personalized medicine could yield to an enhanced treatment success. An example would be the status of O6- methylguanine DNA transferrase or MGMT,

a DNA repair enzyme that protects cells from damage caused by ionizing radiation and alkylating agents. The MGMT promoter is methylated in 40 to 45% of GBMs, which means the cells are unable to properly repair DNA damage [88, 89]. This group might benefit much more from a prodrug/enzyme-based approach as compared to patients without a methylated MGMT promoter tumor. Also, it is known that patients with an EGFR amplification rarely respond to chemotherapy at all, suggesting that the benefit of a CD/5-FC approach in this group will be minimal. This may not only potentially downplay the overall efficacy of this therapy, but may also falsely disqualify a successful approach by showing that results obtained in experimental studies cannot be repeated in the clinic.

TRAIL plays an important role in the experimental design of stem cell-based therapy against gliomas, however, the use of this therapeutic is not (yet) reflected in clinical trials. Some clinical studies using TRAIL for treating various cancers can be found, but, except for a small subset of patients, the therapeutic results of administering TRAIL have been disappointing and do not reflect the results obtained in animal models [90, 91]. Finding ways to identify the subgroup of patients that are responsive to TRAIL therapy or the discovery of adjuvants that help sensitize gliomas and other cancer cells to TRAIL might be needed before taking additional steps towards the clinic. With the discovery of lanatoside C as a TRAIL sensitizer, one of these hurdles has been overcome and since both agents are FDA-approved and have been used in the clinic separately, we expect a short transition to the first clinical trial. However, a proper comparison between carrier types and injection routes in experimental setting will be necessary to give this strategy a fair shot.

Conclusions

Stem cells provide a highly promising and innovative approach for the treatment of malignant gliomas. Provided that some of the discussed issues/limitations can be addressed, this therapeutic strategy could become of tremendous value in the search for a cure for tumors as heterogeneous and as difficult to reach as glioblastoma. Other exciting strategies such as gene therapy and oncolytic viral therapy, which by themselves have failed to establish clinically-relevant antitumor effects, are now given a second chance to prove their value for the treatment of brain

tumors. The combined approach of stem cells and gene/viral therapy has the potential to be of great benefit for glioma patients, and in this role, stem cell therapy could be used alongside surgery, chemotherapy, and radiation therapy, complementing each other to create a highly effective, integral antitumor therapy.

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