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Review

Gene delivery by embryonic stem cells for malignant glioma therapy

Hype or hope?

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Patients with glioblastomas have a poor prognosis and new treatments paradigms are needed. The futility of current treatments lies in part on the difficulty of delivering therapeutic agents to the tumor. In this article, we review and discuss current knowledge of adjuvant treatment for glioblastomas including gene therapy and viral vectors. Additionally, this review provides an update on recent progress, which has focused on improving delivery methods including the development of new approaches such as stem cells. Particular emphasis is given to laboratory data showing the use of embryonic stem cells used as vectors to deliver pro-apoptotic genes to glioblastoma cells.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of primary brain tumor.[1] In children, it is the second most common malignancy following leukemia and represents the leading cause of cancer death in children under the age of 15.[2] In adults, they account for approximately 50% of primary brain tumors.[3] The extensive infiltrating growth pattern of these tumors is the cause of their recurrence within six months of treatment.

Aggressive multimodality treatments with surgery, radiation and chemotherapy have lead to some improvement in the prognosis for patients with glioblastoma and high-grade gliomas. Recent Level IIb evidence supports the concept that aggressive surgical resection of GBM has a positive impact on survival.[4] However, the five year survival rate for patients with GBM is less than 5% and the median survival rate is less than 12 months.[1,5] The futility of present treatments in combating this disease is in part due to their inability to address the highly invasive nature of these neoplasms. Glial tumor cells intersperse themselves with normal brain parenchyma and typically give rise to tumor recurrence within the surgical site.[6] Targeting these tumor cells while sparing the normal cells may prove to be critical for the success of any potential therapeutic strategy.

The development of successful treatments for GBM needs to focus on how to eliminate the intracranial disease left behind at the time of surgery. Residual brain tumor cells may be protected from conventional adjuvant therapies by intrinsic factors, such as resistance to alkylating agents, and extrinsic factors, such as the blood-brain barrier.

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The Blood-Brain Barrier

The blood-brain barrier (BBB) is a membranic structure that acts primarily to protect the brain from the entrance of chemicals into the bloodstream.[7] In the body outside the brain, the walls of the capillaries are made up of fenestrated endothelial cells allowing substances to cross the walls.[8] In the brain, however, endothelial cells have tight junctions.[7] Additionally, the glial cells surrounding capillaries prevent the access of hydrophilic molecules. Finally, the endothelial cells are also capable of metabolizing certain molecules before they can pass through the vessel wall. One example is dopamine, which is metabolized by the endothelial cells and therfore cannot be administered as such.[9] These three factors constitute the blood-brain barrier, which prevents the movement across the vessel wall of all substances except those that are lipid soluble, such as oxygen and carbon dioxide.[10]

Overcoming the difficulty of delivering therapeutic agents to specific regions of the brain presents a major challenge to the treatment of most brain disorders. In its neuroprotective role, the blood-brain barrier functions to hinder the delivery of many potentially important diagnostic and therapeutic agents to the brain. Therapeutic molecules and genes that might otherwise be effective in diagnosis and therapy do not cross the BBB in adequate amounts. Mechanisms for drug targeting in the brain involve going either "through" or "behind" the BBB. Modalities for drug delivery through the BBB entail its disruption by osmotic means,[11] biochemically by the use of vasoactive substances, localized exposure to high intensity focused ultrasound (HIFU),[12] the use of endogenous transport systems, including carrier-mediated transporters such as glucose and amino acid carriers, receptor-mediated transcytosis for insulin or transferrin, and blocking of active efflux transporters such as p-glycoprotein.[13] Strategies for drug delivery behind the BBB include intracerebral implantation and convection-enhanced delivery.

Gene Delivery and Viral Vectors

Aggressive surgical resection has been shown to have a significant positive impact on prolonged survival in patients with GBM.[4] Therefore, therapies that are focused on the delivery of apoptotic

genes at the time of surgical intervention and implanted in the surgical cavity seem to have a solid clinical rationale.

Viral vectors have been considered the most effective in vivo gene delivery carriers. Retrovirus, adenovirus (Adv), and herpes simplex virus-1 (HSV-1) are the best-studied viral brain tumor therapy vectors.[14] Replication competent retroviruses have infection rates of 97% with tumor specificity and lack of spread to nontumor tissue. Insertional mutagenesis, however, is a potential disadvantage.[15] Adv and HSV-1, used in both replication competent and replication defective forms, offer numerous advantages including high transgenic capacity (Adv) and persistent gene expression (HSV-1).[16,17] Potential disadvantages, however, include short term transgene expression and potential toxicity for Adv as well as neurovirulence and recombination with wild type for HSV-1. Oncolytic viruses have also successfully been used for clinical trials[18] as they replicate selectively within tumor cells, therefore leading to increased intratumoral viral titers. To date, however, their clinical effectiveness for brain tumors remains to be substantiated.[15,19,20]

Gene therapy strategies currently in clinical trials use viral vectors to deliver therapeutic transgenes directly to normal and tumor cells within the central nervous system (CNS).[21] Viral vectors, however, have a number of theoretical and practical limitations, including limited diffusion into the brain parenchyma, poor transfection of some cell types and antigenicity. For retroviral and adeno-associated viral vectors that incorporate into the host chromosome, insertional mutagenesis with the potential to cause tumors, as summarized above, is also a disadvantage. The use of genetically modified cells to deliver gene therapy to the CNS may avoid some of these limitations.

Stem Cells: An Overview

A stem cell is a single cell that can replicate itself or differentiate into many cell types. Stem cells have two defining properties: (1) self-renewal—that is, stem cells can maintain themselves in an undifferentiated state through multiple cycles of cell division; and (2) potency—that is, when given the proper molecular cues, stem cells can produce differentiated cells with specialized functions.[22] Stem cells found at different stages of development exhibit varying levels of potency. Totipotent stem cells are produced in the first few cell divisions after the fertilization of an egg by a sperm; these unique cells eventually give rise to all cells of the embryo in addition to tissues needed for fetal growth, such as the placenta. As the cells of the "morula" continue to divide, they form a blastocyst—a hollow sphere of cells surrounding a group of about 30 additional cells known as the inner cell mass. Each cell of the inner cell mass is pluripotent or capable of differentiating into all of the more than 200 types of cells found in the body. Finally, as the embryo matures into a fully formed organism, many organs retain stem cells that are multipotent. Such cells can develop into a limited number of cell types and are used by the body throughout life to replenish and repair some tissues and organs.[23] The promise of stem cell research and regenerative medicine was boosted in 1998 when human embryonic stem cells (hESC) were isolated and successfully grown in the lab.[23] In theory, such cells can be directed to turn—or "differentiate"—into any type of cell in the human body (Fig. 1). Of note, differentiation toward the neural lineage requires fewer steps than to other tissues, such as cardiac, liver or lung.

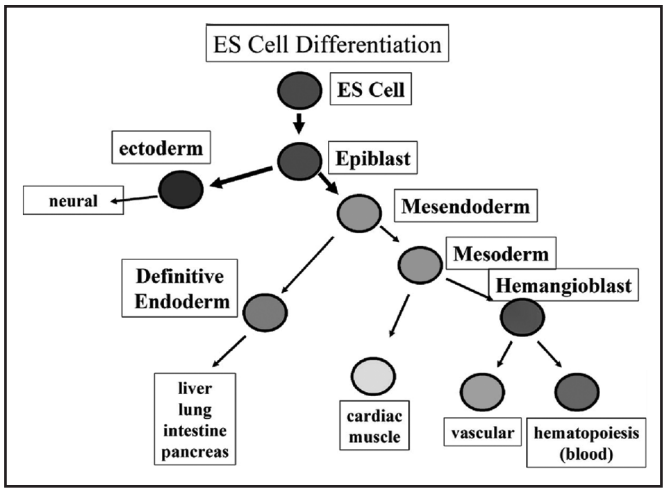

Figure 1. Diagram showing the differentiation steps from undifferentiated embryonic stem cells to the primary germ layers and specialized tissue. Note that the differentiation to CNS lineage is shorter than for other lineages.

Isolating pluripotent human stem cells has been a major challenge in biomedical research. Pluripotent hESC lines are derived from blastocysts that have been created by in vitro fertilization (IVF) techniques. To create these lines, stem cells are removed from the inner cell mass and grown in nutrients that allow the cells to remain in an undifferentiated state while replicating into larger numbers. Alternative methods of producing pluripotent human stem cells that do not entail the destruction of IVF-created blastocysts are being exploited. One such method, called "somatic cell nuclear transfer (SCNT)," involves taking the nucleus from a differentiated adult or "somatic" cell, such as a skin cell, and inserting it into a donated human egg from which the original nucleus has been extracted.[24] The egg, now carrying the genetic material of the adult cell, is then stimulated to form a blastocyst from which pluripotent inner cell mass cells can be harvested. "Altered nuclear transfer (ANT)" has been proposed as an alternative technique to create pluripotent stem cells. In ANT, either the somatic nucleus or the enucleated egg are modified such that the fused cell can give rise only to the pluripotent inner cell mass cells, but not to the cells that form the outer layer of a blastocyst.[25]

Multipotent stem cells have been identified in several adult tissues, including bone marrow, umbilical cord blood, skin, liver, skeletal muscle, brain and fat tissue. Although adult stem cells are normally capable of differentiating into only a few specific cell types within their tissue of origin, researchers are looking for ways to coax them into a wider variety of cell fates. If successful, such research could generate new sources of pluripotent stem cells or, at least, expand the therapeutic potential of multipotent cells. Importantly, adult stem cells have not yet been found in many tissues.

Stem cell research raises ethical, legal and social issues that have garnered much discussion among the research community and the public. Many of these issues are unique to stem cell research or have unique aspects, including: the appropriateness of using human embryos in stem cell research; proper methods of obtaining informed consent for donation of human embryos or gametes (i.e., eggs); the creation of chimeras that combine human and non-human cells; the establishment of institutional oversight of human pluripotent

stem cell research; and others. Understanding and addressing these issues require ongoing discussion among researchers, ethicists, health care providers, patients, advocacy groups, policy makers and the general public. Stem cell research, like all biomedical research, is expected to conform to the highest possible standards of public accountability, respect for research participants and scientific integrity.

Gene Delivery by Stem Cells: Why Embryonic?

Neural stem cells (NSC) have recently aroused a great deal of attention in the field of neuro-oncology as delivery vehicles for therapeutic agents. In order to be termed NSC as opposed to neural progenitor or precursor cells (NPC) they need to have the following functional properties: (1) self-renewal; (2) the ability to repopulate a degenerated CNS region; (3) multipotency, i.e., the ability to yield all three major neural cell types. NPCs are found in the fetal brain in the ventricular zone, midbrain and spinal cord. NSCs have the capability to migrate toward pathologically altered tissues, including stroke, trauma and tumors.[26]

Recent studies have demonstrated that NSCs display extensive tropism for experimental gliomas, distribute throughout the primary tumor bed and migrate to outgrowing tumor microsatellites.[27] These inherent tumor-tropic properties that can be exploited for the targeted delivery and distribution of anti-cancer agents to invasive and metastatic tumors.[28] The tumor-selective NSC-mediated delivery approach could therefore maximize local concentrations of anti-cancer drugs, while minimizing toxicity to normal tissues.

Recent data show that NPCs can carry genes to target malignant glioma cells.[29] Gene transfer can occur by transfection using calcium or liposomes. With both methods, however, the transfection efficiency does not exceed 15%. Electroporation, the temporary delivery of electric pulses to disrupt the phospholipid bilayer of the cell membrane, achieves higher transfection efficiency. The highest levels, however, are achieved by viral transduction. Retroviruses can be used only in dividing cells whereas adenoviruses and lentiviruses are effective in dividing and non-dividing cells. These approaches are limited by the use of potentially hazardous viral vectors, the need for multiple surgical procedures or large amounts of fetal tissue, and the restricted proliferative capacity of differentiated neural cell types.[30]

ESCs are totipotent cells obtained from the inner cell mass of the blastocyst stage embryo.[23] They have unlimited proliferative capacity, and unlike other cell types, can be genetically modified using homologous recombination, eliminating the need for viral vectors and permitting multiple, precisely determined genetic modifications.[31] Wild type and genetically modified ESC have been tested in several animal models of CNS disease, including demyelinating disease,[32] trauma[33,34] and Parkinson's disease.[35] ESC-derived astrocytes have potential advantages over other types of genetically engineered neural cells, such as NPCs. First, unlike pluripotent NPCs that could theoretically differentiate into functional neurons and interfere with existing neural circuits, ESC-derived astrocytes are fully differentiated. Second, unlike NPCs, ESCs can be permanently genetically modified using homologous recombination rather than potentially hazardous or transiently expressed viral vectors. Finally, ESCs have unlimited proliferative capacity; if stocks of undifferentiated human ESCs cells of various HLA types are established in the future, an unlimited supply of immunologically matched neural cells for transplantation would be available.

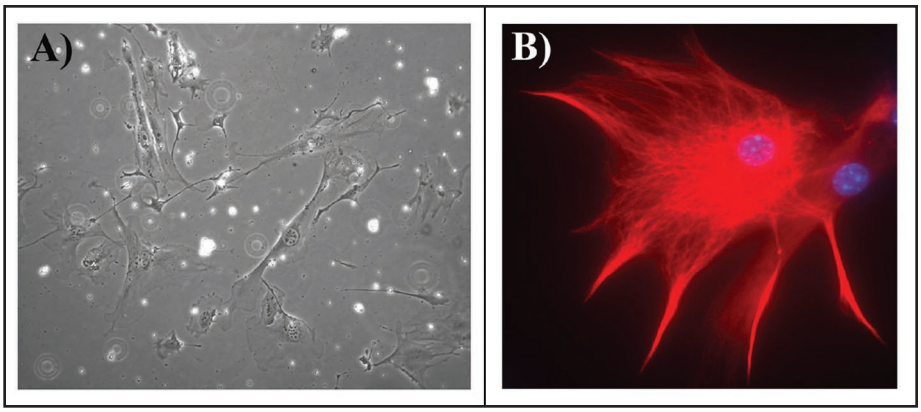

Figure 2. (A) A phase contrast micrograph of ESC-derived astrocytes showing stellate morphology. (B) Immunofluorescence microphotograph after GFAP labeling showing positive cells. These results are consistent with astrocytic differentiation. DAPI: nuclear counterstaining (*blue*).

Embryonic Stem Cell Differentiation to Astrocytes

Astrocytes are native to the CNS, which should maximize their survival and function after transplantation. They normally provide trophic and tropic support to neurons, and have important functions in protecting neurons from toxic levels of glutamate and potassium.[36] Therefore, transplanted astrocytes should be innocuous or protective within the host brain. Astrocytes are a highly secretory cell type and are able to generate large amounts of a transgenic protein. Finally, normal astrocytes have the ability to migrate along white matter tracts after transplantation into the brain.[33,37] This migratory capacity may be useful for delivery of gene therapy to infiltrative tumors like malignant gliomas.

We published a protocol[38] showing that we can generate mouse ESC-derived astrocytes in high numbers. In this study, we allowed undifferentiated murine ESCs to form embryonic bodies (EBs) at low density (1 x 10^4 cells per 60 mm dish) in serum-free media for 6 days, then partially dissociated the EBs and plated them on gelatin-coated tissue culture dishes. The EBs were then incubated in a sequence of cytokine-containing, serum-free media.

By 9 days after plating, confluent monolayer cultures of cells were seen. Cells growing at low density had either stellate or compact morphologies suggestive of Type I or II astrocytes, respectively. Differentiated cells have typical astrocytic morphology and express glial fibrillary acidic protein (GFAP), a marker of astrocyte differentiation and other astrocyte-selective markers, including the GLT-1 glutamate transporter and S100 beta[36] (Fig. 2). The expression of GFAP by ESC-derived astrocytes was assessed by flow cytometric analysis using fluorescence activated cell sorting (FACS). This showed that up to 98% of cells were $GFAP^+$. In parallel with the increased expression of astrocytic markers, we noticed a decrease in the expression of ESC markers.[38]

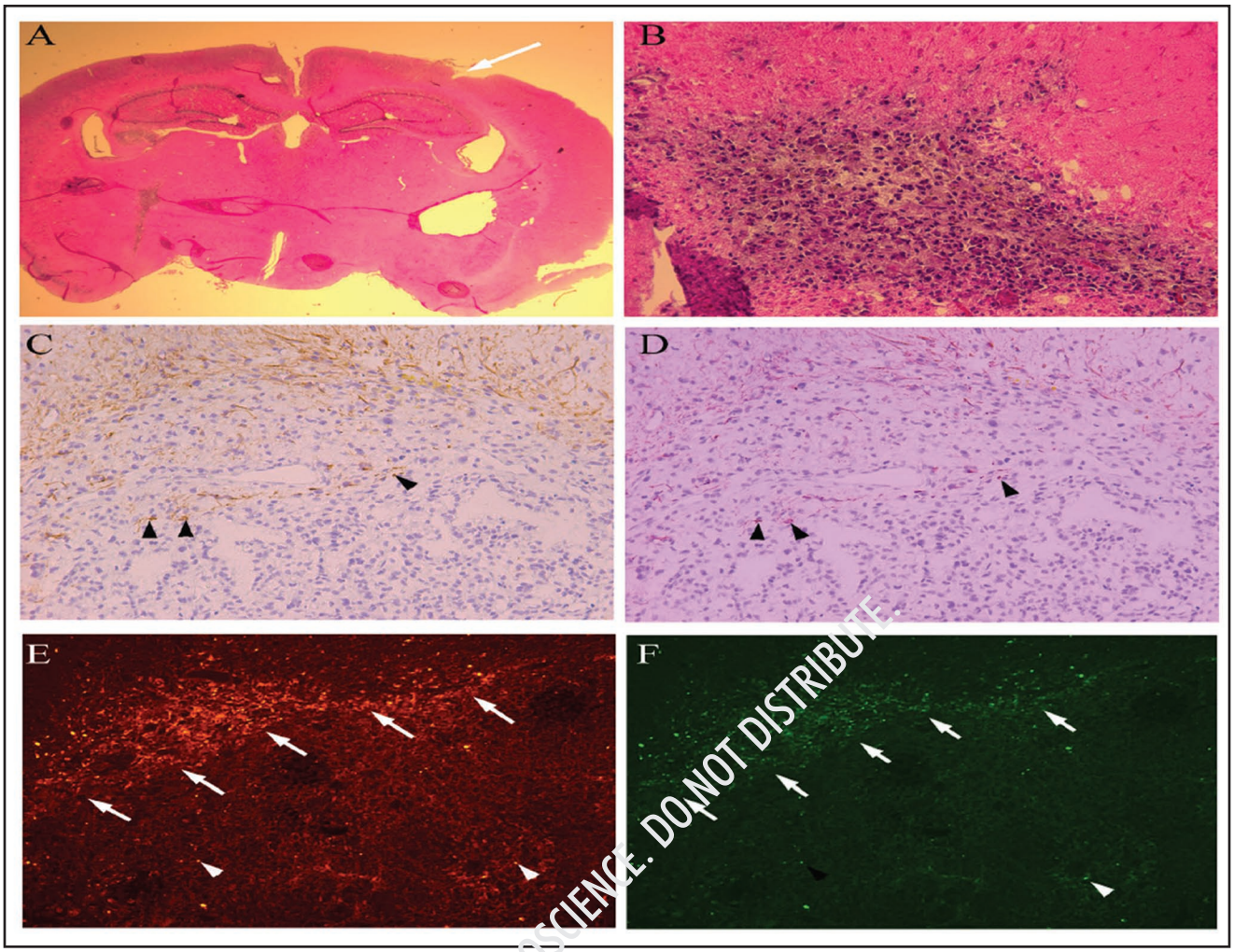

Figure 3. ESC-derived astrocytes conditionally expressing the pro-apoptotic gene, TRAIL, show homing 7 days after grafting. (A) H&E coronal brain section showing U87 tumor cells (*black arrow*) (20X mag in B) in the striatum opposite of the ESC-astrocytes graft (white arrow). (C and D) Fluorescence microphotographs showing (C) ESC-astrocytes tracked with PKH26 red at periphery of the tumor (*arrows*) and within tumor (*arrow heads*) and (D) after immunohistochemistry for mda-7/IL-24. Green: Alexa 488 nm; Red: PK dye 594 nm.

Engineering ESCs to Conditionally Express Pro-apoptotic Genes

The attractiveness of using ESC-derived astrocytes to carry conditionally expressed genes was discussed in the previous section. However, in view of the translational potential for these experiments, ESC-derived astrocytes must have the potential for conditional gene expression. The ability to conditionally express therapeutic transgenes is important in planning for safe and effective gene therapy, as many potentially therapeutic gene products could have unwanted adverse effects. A number of models for inducible transgene expression are available, including radiation-inducible, glucocorticoid-inducible and ecdysone-inducible systems. We used a tetracycline-inducible system[39] to regulate expression of our therapeutic transgene, because tetracycline and doxycycline (Dox) have minimal or no toxicity in animal and humans, and tetracycline-inducible gene expression by viral vectors have already been tested in animal models.[40] Since the first tetracycline-controlled transcriptional activation system was designed nearly a decade ago,[39] new variants, modifications and improvements have been steadily added to this powerful set of tools for temporal control of transgene expression in mammalian systems.

Tetracycline-based externally regulated systems have been successfully used to control the expression of numerous transgenes in cultured cells and in experimental animals. Both "tet-off" and "tet-on" (reverse tetracycline-controlled transcriptional activator system, rtTA) constructs are available for the regulation of transgene expression.[40] In the latter, a specific promoter directs the expression of rtTA in the cell. In the presence of Dox, rtTA binds to the tetracycline operator site (tetO) inducing transcription of the target gene. We chose to use the "tet-on" system because the induction of transgene by Dox is rapid and occurs within hours of Dox administration.

We published that we can engineer ESC-derived astrocytes to conditionally express the green fluorescent protein (GFP) gene.[38] Subsequently; we have shown that we can engineer ESC-derived astrocytes to conditionally express a gene with pro-apoptotic effects on malignant human glioma cells. The tumor necrosis factor-related apoptosis inducing ligand (TRAIL) gene was chosen as it induces selective apoptosis in tumor cells while sparing normal cells.[41,42]

Semi quantitative RT-PCR and immunohistochemistry (IHC) performed after the incubation of undifferentiated ESCs conditionally expressing TRAIL in the presence or absence of Dox demonstrated the Dox-inducible expression of hTRAIL and minimal "leaky" expression in the absence of Dox.[43] These results show that we can engineer ESC-derived astrocytes to express TRAIL under the control of a tetracycline (*tet*)-inducible promoter. Undifferentiated ESCs conditionally expressing TRAIL were then differentiated into astrocytes.[43] Eight days after plating the EBs, nearly 100% of the cells were differentiated and GFAP positive, confirming the astrocytic nature of differentiation. To verify the presence of inducible TRAIL, cells were incubated with or without 1 μg/ml Dox. RT-PCR, immunohistochemistry and flow cytometry demonstrated that Ainv-TRAIL ESC-derived astrocytes maintained inducible TRAIL expression after Dox induction. Thus, our data show that we can generate ESC-derived astrocytes conditionally expressing TRAIL under the tight control of a *tet*-inducible promoter (Dox).[43]

ESC-Derived Astrocyte Migration and Homing

The mechanisms of tropisms and targeting of gliomas by stem cells are not fully understood. Growing evidence demonstrates that chemokine signaling axes (homing) and matrix remodeling (enhancement of migration) represent major pathways for driving stem cell migration to glioma cells. Homing refers to the property of stem cells by which they detect a target and migrate through the tissue to reach that target. Homing appears to be mediated by the expression of chemokine receptors by stem cells.[27,44] The tumor cells secrete the chemokines that attract the stem cells.

The ability to migrate seems to be mediated at least in part by the secretion of metallo-proteinases by stem cells.[45] Several factors produced by glioma cells have been suggested to promote tropism for the stem cells, including vascular endothelial growth factor[46] and CXCR4.[47] A previous study has indicated that SF/HGF is the most powerful chemo-attractant released from gliomas.[26]

To confirm that ESC-derived astrocytes maintained homing capability, we performed transwell experiments and in vivo and in situ experiments to assess the capability of ESC-derived astrocytes to migrate toward the tumor when it is injected in the opposite hemisphere. For these experiments, athymic nude mice NCR nu/nu were stereotactically implanted with U87 malignant glioma cells into the left hemisphere. At the same time, ESC-derived astrocytes (10^6 cells/15 μl) labeled with PKH26 red dye were injected into the opposite hemisphere. Brains were harvested seven days after injection of ESC-derived astrocytes, frozen and coronal alternating 10 μm sections were stained with H&E, or left unstained for direct detection of ESC-astrocytes under fluorescent microscopy or for IHC of mda-7/IL-24.

We showed that the vast majority of implanted astrocytes had migrated towards the opposite hemisphere where the tumor was located. ESC-derived astrocytes were also visualized inside the tumor itself (Fig. 3). These results support our hypothesis that ESC-derived astrocytes maintain migratory characteristics of ESCs, as reported in other studies.[26,33] Additionally, these data show that these cells are capable of "homing", making these cells desirable for therapeutic approaches with malignant gliomas.

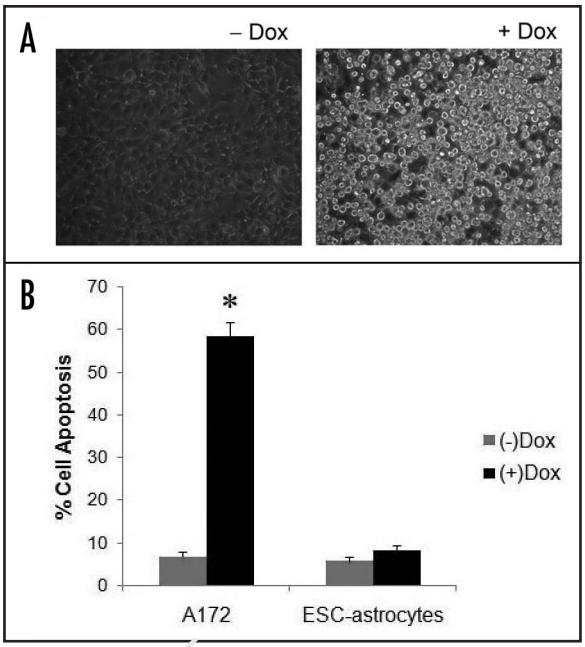

Figure 4. Pro-apoptotic effects of ESC-derived astrocytes conditionally expressing pro-apoptotic gene on human malignant glioma cells. (A) Phase contrast microscopy showing co-cultures of ESC-derived astroyctes conditionally expressing the pro-apoptotic gene, TRAIL, with human glioma cells (A172) in absence (-Dox) and presence (+Dox) of doxycycline induction. Note the significant number of apoptotic cells after Dox induction. (B) Bar graph showing pro-apoptotic effects of ESC-delivered TRAIL on human glioma cells in co-culture. Note the significant increase in apoptotic rate after Dox induction (Dox^+). This is not evident in ESC-derived astrocytes after Dox induction.

ESC-Derived Astrocytes Conditionally Expressing a Pro-apoptotic Gene Induce Apoptosis in vitro and Tumor Regression in vivo

To assess the in vitro pro-apoptotic effects of ESC-derived astrocytes conditionally expressing TRAIL, we performed co-culture experiments using ESC-derived astrocytes conditionally expressing TRAIL and A172 human glioma cells. Since TRAIL is a membrane-bound protein, cell contact was necessary.[43] In this experiment, we showed that 24 hours after induction with Dox, the apoptotic rate of the human glioma cells was 58% (Fig. 4).[43] This pro-apoptotic effect compares favorably to that observed using recombinant hTRAIL.[48-50]

In another recent study, we assessed the pro-apoptotic effects of transgenic TRAIL delivered by ESC-derived astrocytes on malignant gliomas in vivo. ESC-derived astrocytes conditionally expressing TRAIL were injected into the subcutaneous human glioma xenografts in the mouse. TRAIL expression was documented in the malignant glioma xenografts by RT-PCR and immunohistochemistry after external gene induction. A significant reduction in tumor volume occurred 48 hours after a single injection (14%) and double injections (31%) in the experimental groups. TUNEL staining revealed abundant apoptotic tumor cells in the experimental groups. Seven days after injection, the tumors had undergone severe necrosis with only scattered residual tumor cells at the periphery. Death receptor (DR4) expression increased significantly in the experimental

groups compared to controls.[51] Our data suggest that ESC-derived astrocytes conditionally expressing TRAIL should be considered as vectors to deliver gene therapy for malignant gliomas.

Summary

Brain tumor gene therapy using viral vectors has shown promise in animal model studies. Although gene delivery to human patients appears to be safe, these studies have not yet translated to benefits in the clinic setting and several issues related to delivery need to be resolved. Stem cells seem to be a promising alternative for gene delivery to brain tumors. Despite great progress in recent years, these are still early days in the medical application of embryonic stem cells to human disease problems. Many gaps remain in our understanding of the basic biology of both embryonic and adult stem cells. New types of adult stem cells continue to be identified, but the full differentiation potential has not been defined for many of these cells.

Early phase clinical trials are underway using adult stem cells for a range of conditions that includes traumatic brain injury, myocardial infarction, Crohn's disease, multiple sclerosis and graft-versus-host disease. Clinical trials using embryonic stem cells are being planned for spinal cord injury and other conditions. However, the potential benefits, as well as possible side effects, of transplanting these cells into human patients needs to be investigated further. Thus, much fundamental research is needed to translate stem cell science into effective human therapies. Even with the aforementioned challenges, the in vitro and in vivo pre-clinical evidence that ESC-derived astrocytes can be used as vectors for gene therapy in malignant human gliomas is compelling and warrants the kind of robust investigation that is currently under way.

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