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Gene therapy, cell transplantation and stroke

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1. ABSTRACT

The use of neuroteratocarcinoma cells for transplantation therapy in stroke has emerged as a strategy for cell replacement therapy that has begun its transition from basic science laboratories to a clinical setting. Procurement logistics and novel neuroprotective functions associated with these cells allow neuroteratocarcinoma cells to serve as efficacious alternatives to using fetal cells as donor cell grafts for stroke therapy, although the optimal transplantation regimen must still be determined. In particular, the limitations of current stroke treatments and management reveal an urgent need to examine the efficacy of experimental treatments, such as neural transplantation, in order to develop better treatment therapies. This chapter will discuss the characteristics of NT2N cells, the role of the host brain microenvironment, the need for more rigorous laboratory research and clinical trials for the intracerebral transplantation of NT2N cells in stroke, the mechanisms underlying the grafts' beneficial effects, and the need for immunosuppression. This chapter will highlight some of the most recent findings regarding NT2N cells.

2. INTRODUCTION

In recent years, basic science research efforts have established the efficacy of cell replacement therapy in animal models of neurological disorders, and many of these findings have begun their transition from the laboratory to a clinical setting. Limited clinical trials of neural transplantation have already been initiated in chronically ill patients. The use of neuroteratocarcinoma cells for transplantation therapy in stroke is one such approach showing great promise for transitioning to the clinic. Procurement logistics and novel neuroprotective functions associated with this cell line allow neuroteratocarcinoma cells to serve as efficacious alternatives to using fetal cells as donor cell grafts for stroke therapy, although the optimal transplantation regimen must still be determined. It has become clear that the "non-regenerative central nervous system" dogma is arguably a historical past; modern research instead demonstrates that diseased or aging brain cells can potentially be rescued and have their functions restored. Cell replacement therapy has emerged as the current translational research trend and in the future could provide a promising treatment intervention for various neurological disorders.

To date, stroke is the third leading cause of death, and stroke affects over a half-million people each year in the United States alone. In addition, there are currently about 3 million stroke survivors, many of which suffer from significant cognitive and functional disabilities. Rehabilitation therapy has helped some stroke survivors recover, but many patients, however, still experience permanent loss of independent function. The cost for rehabilitation and lost wages is estimated at $30 billion each year and thus represents a significant financial impact on society. Current stroke treatments are typically limited to supportive care and secondary stroke prevention, resulting in only limited improvement in cognitive and motor function. Intravenous tissue plasminogen activator administration has been effective in ameliorating the neurological deficits arising from acute stroke, although this treatment strategy is also problematic because of is extremely limited window of efficacy, which remains within 3 hours of stroke onset. It is apparent that current treatments, which are directed towards acute stroke, are rather limited in their application and efficacy. Unfortunately, no therapy has been proven effective for treating chronic stroke, which is associated with significant morbidity and mortality. These facts reveal an urgent need to examine the efficacy of experimental treatments, such as neural transplantation, in order to develop better treatment therapies, particularly for chronic stroke.

3. FETAL NEURAL TRANSPLANTATION

In recent years, delivery of exogenous proteins into the central nervous system (CNS) has utilized a strategy of cellular and gene therapy. Researchers in this field have focused on finding a transplantable and transfectable cellular platform to serve as a local delivery system for gene products of therapeutic value (1, 2, 3). A continuous secretion of the gene product may be necessary in order to achieve a sustained therapeutic effect from application of a gene product to affected regions of the CNS. This sustained delivery could be accomplished by transplantation of cells genetically engineered to express the therapeutic protein of interest (4).

Promising laboratory findings in animal models of Parkinson's disease (PD) and Huntington's disease (HD) treated with neural transplantation strategies have formed the scientific basis for proceeding with clinical trials (2, 5). More than 350 PD, HD, and stroke patients have already received intracerebral neural transplantation. These patients, however, have demonstrated variable degrees of clinical improvement owing in part to the low viability of the grafts (2, 6-11). Because graft survival is greatly altered by the host immune response, cells that can avoid immunosurveillance, particularly autologous cells such as the transplant recipient's own adrenal cells or stem cells, may limit graft rejection (12-18). Fetal cells persist as the most widely studied graft source for transplantation. Unfortunately, many logistical and ethical issues hinder the use of primary fetal cells in the clinic. Thus, a primary research endeavor in cell transplantation has concentrated on searching for a non-primary fetal graft source. A variety of cells have been used as alternatives to primary fetal cells, including cultured neuronal stem and progenitor cells, cells engineered to secrete neurotransmitters or neurotrophic factors such as immortalized cell lines, fibroblasts, and astrocytes, para-neuronal cells which naturally synthesize neuronal substances and/or have neuron-like properties, and bridge-inducing cell grafts which assist in the physical reconstruction of lost axonal pathways. Typically, these cells have been shown to partially reconstruct the neuronal circuitry and form functional synapses when intracerebrally transplanted (19-23).

Although transplantation of primary fetal cells may promote prolonged release of neuronal survival-promoting proteins, the use of cell lines or neural stem cells would be associated with less controversy. These cells are readily available, can be maintained in culture indefinitely, and can be sorted in homogenous population with their phenotypic features fully characterized. Thus, establishing human neuronal cell lines or neural stem cells as vehicles for cellular and gene therapy in the CNS disorders could be of great utility.

4. GENERATING NEURONS FROM CANCER CELLS

An embryonal carcinoma cell line (NT2 cells) derived from a human teratocarcinoma can be induced to differentiate into post-mitotic neuron-like cells referred to as NT2N neurons (1, 24-26). During a six-week retinoic acid (RA) treatment period, NT2 cells, which share many characteristics of neuroepithelial precursor cells, cease expressing neuroepithelial markers and instead develop neuronal markers (27,28). Subsequent exposure to mitotic inhibitors produces >99% pure populations of terminally differentiated NT2N neurons (29). Furthermore, NT2N neurons exhibit outgrowth processes and establish functional synapses. Mature NT2N neurons are virtually indistinguishable from terminally differentiated post-mitotic embryonic neurons. Of note, these neurons do not divide, and they maintain a neuronal phenotype over a long-term period (28). NT2 cells, unlike other germ-cell tumor lines, do not give rise to progeny committed to other well-defined neural or nonneural lineages in response to RA or any other differentiating agent (27,28). Based on this unique property of NT2 cells, they are considered as *in vitro* equivalents to CNS neuronal progenitor cells (1, 30). Interestingly, both NT2 cells and NT2N neurons can be genetically engineered, allowing for the expression of a gene product of interest *in vitro* and possibly *in vivo*, and these applications can be exploited to reveal the cellular and molecular biology of neurons (1, 30). Equally important is the demonstration that both NT2 and NT2N cells can be used as alternative graft sources for transplantation therapy in CNS disorders (Table 1).

5. NORMAL HOST BRAIN MICROENVIRONMENT AND CELL GRAFTS

Pioneering studies using NT2N neurons revealed that purified NT2N neurons survive, mature, and integrate well with the host nervous system following transplantation into the CNS of rodents (24, 25, 26). From a developmental cell biology perspective, such transplantation setup allows for the direct examination of the growth and maturation of human neuronal cells in an *in vivo* CNS environment that otherwise could not be fully investigated in an *in vitro* setting. The use of NT2N neurons offers many advantages over the use of human fetal neurons. For example, NT2N neurons appear to have a better graft survival by 15%, excellent *in vitro* and *in vivo* grafted cell homogeneity, and a high degree of host re-innervation (1, 25, 26). In support of the post-mitotic feature of NT2N cells, histological examinations have revealed no observable tumorgenecity, as well as neoplasticity in NT2N cell grafts over prolonged transplantation periods of over one year (24, 25, 26). Because the aforementioned studies used rodents as transplant recipients of NT2N human derived cells, the observed cross-species graft tolerance required suppressing the host immune response. Surprisingly, some non-immunosuppressed transplant recipients tolerated the grafts depending on the transplant target brain area. This observation led to the belief that specific brain sites may be more conducive than others for NT2N cell transplantation.

The role of the host microenvironment in proliferation and survival of grafted cells is a factor that is recognized but has not yet been fully elucidated. Although it has been established that NT2N cells attained features of fully differentiated neurons following treatment with RA and mitotic inhibitors, and these cells do not revert to neoplastic state after transplantation, concerns abound on the possibility quiescent mitotic capacity remains in grafted NT2N neurons that may be stimulated by the host microenvironment. The literature on studies examining transplantation of NT2N neurons reveals no indications that these grafted cells re-acquire mitotic features, at least when they are transplanted

Table 1. Summary of NT2 cell/NT2N Neuron Transplantation Studies

Milestones	Reference
Purified NT2N neurons survive, mature, and integrate well with host nervous system following transplantation into CNS of rodents	24, 25, 26
NT2N neurons have better graft survival than human fetal neurons (15%), excellent *in vitro* and *in vivo* grafted cell homogeneity, and high degree of host re-innervation; prolonged NT2N cell grafts have no observable tumorgenecity or neoplasticity	1, 24, 25
Proliferation and survival of parent NT2 cells affected by host microenvironment, anatomical site into which NT2 cells are implanted significantly influences survival, proliferation, and differentiation of NT2 cells, NT2 cells survive and differentiate into neurons when transplanted into caudoputamen	24,25,26
NT2N neurons differentiate into dopaminergic and GABAergic neuron-like cells	35, 36
Ischemia-induced behavioral dysfunctions ameliorated by NT2N neuronal grafts as early as 1 month post-transplantation, pre-transplantation viability and post-transplantation survival of NT2N neurons correlates highly with functional recovery of transplanted stroke animals	38,39,40
Functional effects and survival of NT2N neuronal grafts are dose dependent, transplantation of more viable NT2N neurons required to rescue larger stroke-induced brain damage	34
NT2N neuronal grafts still produce robust functional recovery at one month post-stroke	38,39,40
Cytokines and inflammatory signals highly elevated at early post-stroke period which could be harmful to grafted cells	43, 44
Chemoattractants produced by glial cells/macrophages following stroke, may guide grafted cells to site of injury	45,46,47
NT2N cell grafts migrate away from original transplant site in chronic stroke model	38,39,40
Cryopreservation of NT2N neurons has no deleterious effects on viability of cells prior to and after transplantation in stroke animals	39
Sustained motor and cognitive improvements and cell survival in NT2N neuronal transplanted animals only observed with systemic Cyclosporin-A (CsA) immunosuppression	38,39,40
Host rejection of NT2N grafts in non-immunosuppressed animals, these animals significantly improve compared to control animals at 6 months post transplantation but remain impaired compared to immunosuppressed animals that receive NT2N cells, immunosuppression with CsA enhances graft survival	48
No evidence that transplanted NT2N cells with or without immunosuppression have deleterious effects on host brain, NT2N neurons may have immunosuppressive properties	24,25,26,51
Functional improvement in stroke animals ascribed to neurotrophic effects of NT2N cells, neuroprotection by NT2N neurons could be mediated by neurotrophic factor mechanism, NT2N neurons have GDNF mRNA	38,57
After transplantation into nude mice NT2N cells can integrate and change phenotype into neurons similar to target neurons, percentages of TH-positive neuron-like cells in NT2N cells treated with RA cocultured with striatal extracts exceeds percentage of TH-positive cells induced in sister cultures exposed to retinoic acid alone, host microenvironment of adult mouse striatum has potential ability to induce grafted NT2 cells to differentiate progressively into fully mature adult CNS neurons	28,25,26
NT2N cells can be stimulated through application of neurotrophic factors and activating factors to express TH, subsets of NT2N cells express neuronal markers for dopaminergic and GABAergic neurons	58,59,60,61
NT2N cells transplanted in patients with basal ganglia stroke and fixed motor deficits, serial evaluations show no adverse cell-related serologic or imaging-defined effects	62
No evidence of neoplastic formation from NT2N transplants into striatum, no serious adverse events in transplanted stroke patients at one year post-transplantation	24
PET and histological data from NT2N transplanted stroke patients shows uptake increase in FDG in area of cell implantation and neurofilament immunoreactive neurons that resembled those seen in NT2N neurons *in vitro*, clinical improvement maintained in 6 of 7 patients who show initial increase in uptake of FDG	64,65

into the striatum. In contrast, evidence exists that proliferation and survival of parent NT2 cells are affected by the host microenvironment (24, 25, 26). The neoplastic potential of grafted NT2 cells has been explored by grafting these cells into different regions of the brains of subacute combined immunodeficient (SCID) mice and nude mice (24, 25, 26). It was observed that the anatomical site into which the NT2 cells were implanted significantly influenced the survival, proliferation, and differentiation of NT2 cells. Histological results revealed that the NT2 cells continued to proliferate and undergo an apoptotic-like cell death with minimal capacity to differentiate into neurons following implantation into the sub-arachnoid space and superficial neocortex. However, when NT2 cells were transplanted in the lateral ventricles, liver, and muscle, the grafted cells rapidly progressed into bulky, lethal tumors within 10 weeks after transplantation. The observed tumorgeneic and neoplastic state of grafted NT2 cells was in sharp contrast to the phenotypic features displayed by NT2 cells transplanted into the caudoputamen of SCID mice. Caudoputaminal grafted NT2 cells stopped proliferating, showed no evidence of necrosis or apoptosis, and did not form tumors after more than 20 weeks post-transplantation. Furthermore, neuronal phenotypic markers demonstrate that the majority of NT2 cell grafts in the caudoputamen differentiate into post-mitotic immature neuron-like cells. These marked differential histopathological effects produced by the caudoputamen and other brain transplant target sites suggest that the choice of host microenvironment for transplantation of NT2 cells critically influences the eventual survival, proliferation, and differentiation fate of grafted cells. These observations support the notion that the host microenvironment, in this case the caudoputamen, may promote signaling molecules or other cues, such as cell–cell contacts, which are capable of regulating the fate of grafted NT2 cells. Of note, RA has been shown to be present in both developing and adult rodent striatum tissues (31), and RA is also believed to potentially influence cell fate (32,33). As mentioned above, RA, and other mitotic inhibitors, are primarily used as factors in the differentiation process of NT2 cells into NT2N *in vitro*. Accordingly, the presence of RA in the striatum likely influences the non-tumorgenic fate of NT2 cells following transplantation into this brain area. Although the effects of the host microenvironment appear limited to NT2 cells, more in-depth examinations are warranted to investigate the influence of specific brain transplant target sites on survival, proliferation, and differentiation of NT2N neurons.

6. INJURED HOST BRAIN MICROENVIRONMENT AND CELL GRAFTS

Because progressive neurodegeneration ensues following the onset of many neurological disorders, as exemplified in stroke, critically identifying a conducive host microenvironment seems prerequisite for successful cell transplantation therapy. In stroke animal models, the reported NT2N neuronal graft survival rate of 15% (34) is a bit higher compared to fetal cell grafts, but this rate is still low considering that ischemic stroke is not limited to a specific cell population. Moreover, stratified ischemic zones exist, namely the predominantly necrotic core and the apoptotic penumbra. To produce therapeutic effects following an ischemic stroke, either via pharmacologic treatment or cell replacement therapy, the consensus is to target the ischemic penumbra rather than the core. Such preferential penumbral rescue is logical since apoptotic cell death accompanying the penumbra may be potentially reversed as opposed to the necrosis inherent in the core. Targeting secondary cell death mechanisms, as in the case of ischemia-induced apoptosis, suggests that the ischemic penumbra seems a more conducive host microenvironment than the ischemic core. Nonetheless, the brain damage that accompanies stroke, regardless of location in the penumbra or core, is characterized by the degeneration of many cell populations and brain structures. Accordingly, a higher number of cells with high viability and increased survival

ability must be transplanted into the ischemic regions. Despite the moderately conducive nature of the ischemic penumbra, the extensive brain area encompassing this region may require multiple brain targets to repair the damaged neuroanatomical circuitry. Multiple intracerebral transplantations may not be feasible, however, because of the trauma associated with such an invasive surgical procedure. In addition, different types of donor cells may need to be transplanted considering that many cell populations are destroyed in stroke. NT2N neurons have been shown to differentiate into dopaminergic and GABAergic neuron-like cells (35,36). Alternatively, RA-naive NT2 cells may possess some multipotent properties, such as those attributed to neural stem cells, and these features may be potentially advantageous for generating different cell populations. Indeed, it has been demonstrated that transplanted human neural stem cells can mature into the phenotype of cells that are undergoing cell death in brains of animals that were introduced to neuronal injury (37). Of note, NT2 cells can differentiate into neurons when transplanted into the caudoputamen. Thus, at least for focal caudoputaminal stroke, NT2N and NT2 cell grafts may both be beneficial. In the end, the pluripotent features of NT2 cells and the highly differentiated neuronal-like characteristics of NT2N cells may be directed towards specific stroke types to enhance their therapeutic effects. For example, a large striatal stroke may benefit from the multi-potent NT2 cell grafts, while a highly localized striatal stroke may appeal to the differentiated NT2N cell grafts. These hypotheses remain to be tested in the laboratory.

7. PRECLINICAL INTRACEREBRAL TRANSPLANTATION OF NT2N CELLS IN STROKE

The rodent model of MCA occlusion replicates many pathophysiological changes seen in clinical cerebral ischemia, allowing for investigations of treatment strategies for stroke. The potential benefits of neural transplantation of NT2N neurons to correct the neurobehavioral deficits associated with cerebral ischemia have been elucidated by the use of this model. Data has shown that ischemia-induced behavioral dysfunctions are ameliorated by NT2N neuronal grafts as early as 1 month post-transplantation (38, 39, 40). Compared with the transplantation of fetal striatal cells, which were previously shown to reverse motor abnormalities in stroke rats, NT2N neuronal grafts induced a significantly greater robust recovery. This pioneering data provides justification for the use of NT2N neurons for transplantation therapy as a means to circumvent the logistical and ethical concerns inherent with the use of fetal striatal cells.

Subsequent studies using this human neuronal cell line have revealed that pre-transplantation viability and post-transplantation survival of NT2N neurons are highly correlated with the functional recovery of transplanted stroke animals (39). These observations suggest that the positive behavioral effects seen in transplanted stroke animals can be attributed to viable and functional NT2N neuronal grafts. During the pre-transplantation period, NT2N neuron viability counts revealed a variable range of 52%–95%. Within-subject comparisons of pre-transplantation cell viability and subsequent behavioral changes in transplanted stroke animals revealed that a high cell viability just prior to transplantation surgery correlated highly with a robust and sustained functional improvement in transplant recipients. In addition, histological analysis of grafted brains revealed a positive correlation between the number of surviving NT2N neurons and the degree of functional recovery. Similar correlational data regarding fetal tissue transplantation has been reported between pre-transplantation viability or post-transplantation survival of grafted cells and behavioral outcome.

In support of the aforementioned positive correlations between motor recovery and neuronal regeneration in stroke animals, dose-dependent functional effects of NT2N neuronal grafts have also been noted (34). Stroke animals that received 40, 80, or 160 X 103 NT2N neurons dose-dependently exhibited performance improvements in both the passive avoidance and elevated body swing tests. Moreover, dose-dependent survival of NT2N neuronal grafts was observed, in that grafts of 80 or 160 X 103 NT2N neurons demonstrated a 12–15% survival of NT2N neurons in the graft, while grafts of 40 X 103 NT2N neurons demonstrated only a 5% survival. It is possible that NT2N neuronal grafts were affected by progressive stroke, suggesting again the influence of the host microenvironment. Correlational analyses revealed that ischemic animals which received 80 or 160 X 103 NT2N neurons produced a significantly better amelioration of behavioral dysfunctions as compared to those that received lower dosages of NT2N neurons. In concert with the earlier speculation that varying extent of stroke brain damage would require manipulation of the number of donor cells, this study demonstrated that transplantation of more viable NT2N neurons is required to rescue larger stroke-induced brain damage (34). Determining the optimal dosage of NT2N neurons for a given stroke case should be viewed in terms of NT2N cell viability at pre and post-transplantation, as well as the extent of stroke brain areas.

The issue of NT2N neuron procurement feasibility arises when considering devastating diseases such as stroke. Because a very narrow therapeutic window exists following a stroke episode, the immediate availability of NT2N cells must be considered. Laboratory studies have shown that NT2N neuronal grafts still produce robust functional recovery at one month post-stroke (38, 39, 40). Clinical trials also revealed that transplanted patients who had a stroke at least six months prior to NT2N neuronal transplantation displayed some trends of clinical improvement. Although such transplantation therapy could potentially reverse chronic stroke, better functional outcomes may be achieved if treatment is initiated acutely (< 3 hours) post-stroke when brain damage would presumably still be limited. Thus, a strategy incorporating immediate availability and transplantation of NT2N neurons is deemed more effective for stroke therapy.

Another important factor that needs to be closely examined in the laboratory prior to advancing NT2N cell transplantation in stroke is the severe inflammatory glial response that accompanies the early stages of the disease. Controversy exists as to whether such inflammatory response works for or against graft survival (41, 42). For example, during the early post-stroke period, cytokines and inflammatory signals are highly elevated, (43,44) which could be harmful to grafted cells. Thus, transplantation in acute stroke could be detrimental to grafted cells. However, there is

also evidence that chemoattractants may be produced by glial cells or macrophages following stroke, and these cues may guide grafted cells to the site of injury (45, 46, 47). This data suggests that an early transplantation at a stage of high glial response may aid in cell graft trafficking towards appropriate stroke target sites. Accordingly, the glial response/inflammation may produce both inhibitory and facilitatory effects during the early periods following stroke, particularly on the migration of grafted cells, and these effects warrant further investigations. In previous studies using the chronic stroke model, however, NT2N cell grafts have been shown to migrate away from the original transplant site (38, 39, 40). Stimulating NT2N cells to migrate in acute and also chronic stroke may pose as a challenge considering the large extent of brain damage inherent in the disease. It is possible that modulating the glial response/inflammation may be beneficial to NT2N cell graft migration.

Since handling freshly cultured NT2N neurons would not be altogether feasible in the clinic, cryopreserved NT2N neurons are recommended. In the laboratory, cryopreservation of NT2N neurons did not produce any significant deleterious effects on the viability of the cells prior to or after transplantation in stroke animals (39). This sustained viability of NT2N neurons following cryopreservation fares much better than fetal cells, which after cryopreservation display a significant decline in viability rendering them non-transplantable. Cryopreservation of NT2N neurons thus allows immediate transplantation of the cells in acute stroke. Whether enhanced functional outcomes can be achieved with such an acute stroke transplantation regimen remains to be examined in the laboratory.

8. NT2N CELL GRAFTS AND IMMUNOSUPPRESSION

Although the brain is perceived as an immunoprivileged site, neuronal graft rejection still persists. Indeed, the sustained motor and cognitive improvements noted in NT2N neuronal transplanted animals were only observed with systemic administration of Cyclosporin-A (CsA) immunosuppression (38, 39, 40). In contrast, the behavioral recovery in the nonimmunosuppressed animals transplanted with NT2N neurons began to diminish by about 2 months posttransplantation. Moreover, histological analysis revealed surviving NT2N cells in the brains of immunosuppressed transplanted animals but not in non-immunosuppressed transplanted animals. The near absence of visible grafts in non-immunosuppressed animals transplanted with NT2N neurons suggests host immunological rejection of the grafts as observed previously (48). Nonetheless, the magnitude of the behavioral recovery produced by NT2N cell grafts in rats that did not receive CsA was greater than that seen in animals with ischemia-induced brain injury followed by injections of rat fetal cerebellar cells or medium alone, suggesting that NT2N cell grafts promote behavioral effects at early time periods post-transplantation even in the absence of immunosuppression. However, these non-immunosuppressed animals, despite displaying significant improvements as compared to control animals at 6 months posttransplantation, still remained impaired as compared to immunosuppressed animals that received NT2N cells. Accordingly, immunosuppression with CsA enhanced the survival of grafted NT2N cells, and this finding is consistent with a previous study (48). Moreover, histological examinations revealed many surviving NT2N cells in immunosuppressed transplanted animals that exhibited a robust functional recovery for more than 6 months posttransplantation. Based on these results, the need for chronic immunosuppressive therapy as an adjunct to the transplantation of human NT2N cells in rats appears necessary in order to obtain optimal and sustained functional improvement as well as prolonged graft survival. Interestingly, no evidence from these studies or any other previous reports has been found that transplanted NT2N cells, with or without immunosuppression, have any deleterious effects on the host brain. (24, 25, 26).

In future clinical trials for neural transplantation of NT2N cells, however, chronic immunosuppression, which is normally required for successful xenografting, may not be necessary since NT2N neurons are human-derived cells. Some clinical trials of human fetal cell transplantation for PD have in fact found that the absence of immunosuppression does not deleteriously affect the survival of fetal cell grafts and their ability to produce clinical improvement (49, 50). In addition, preliminary data suggests that NT2N neurons may have immunosuppressive properties (51). Recent studies have suggested that stem cells only minimally elicit an immune response and may even secrete their own immunosuppressant factors following intracerebral transplantation (37). Similar immunosuppressant factors may also be secreted by NT2N cells, which may exert localized immunosuppression within the transplant site and allow them to circumvent host immunosurveillance. Thus, long-term systemic immunosuppression may not be necessary in humans. Nevertheless, because recent studies have indicated that immunosuppressants and their analogs exert neuroprotective effects (2, 41), perhaps adjunct immunosuppression with NT2N cell transplantation should be considered for enhanced graft survival and functional effects.

9. SPECULATIVE MECHANISMS UNDERLYING FUNCTIONAL EFFECTS OF NT2N CELL GRAFTS

The observation of acute behavioral effects in non-immunosuppressed NT2N transplanted stroke animals suggests that the neurotrophic effects of transplanted NT2N cells may have mediated functional recovery, at least for the early posttransplantation period. This finding supports neuronal rescue via neurotrophic factor therapy. In many preclinical and clinical studies of neural transplantation, the use of neurotrophic factors has been shown to significantly enhance the survival rate of grafted cells (5, 52, 53). Direct infusion of neurotrophic factors alone or their use as a transplant facilitator by either pre-treating donor cells or co-administration during and post neural transplantation therapy has been proven efficacious in CNS animal models. Thus, administration of neurotrophic factors may serve as another adjunct to neural transplantation. One of the most potent neurotrophic factors is glial-cell line derived neurotrophic factor (GDNF). Encouraging laboratory results have been reported in neural transplantation of GDNF-secreting fetal kidney cells for stroke (54, 55). Interestingly, an *in vitro* study has demonstrated that NT2N cells respond positively to putative neurotrophic factors secreted by an immortalized human fetal astrocyte cell line (56).

The observed functional improvement in stroke animals was initially ascribed to neurotrophic effects of NT2N cells to the injured area (38). However, this study did not demonstrate any direct evidence that neuroprotection was indeed a function of NT2N neuronal grafts. The first suggestion that neuroprotection by NT2N neurons could be mediated by neurotrophic factor mechanism was reported recently in a study showing that NT2N neurons are positive for GDNF mRNA (57). Because GDNF has been found to be neuroprotective for stroke animals, the indication that NT2N neurons can exert GDNF expression offers a mechanistic explanation for the observed neuroprotection by NT2N neuronal grafts in stroke. Indeed, while a similar pattern of behavioral recovery was observed in animals that received NT2N neurons and those that received fetal striatal transplants, the NT2N-transplanted animals showed a more robust recovery at 1 month post-transplantation. This effect of NT2N neurons also was evident in transplanted nonimmunosuppressed animals. Since no evidence has been reported of neural transplants replacing lost host brain tissue at this early post-transplantation period, the observed functional effects may be due to the release of trophic factors from the grafted NT2N neurons. Another indication of trophic factors mediating NT2N's action is that the effective dose of transplanted NT2N neurons needed to produce functional recovery was 10 times less than transplanted striatal cells. Therefore, at the early post-transplantation period, connectivity with or repair of the stroke brain by NT2N neurons does not account for the observed functional recovery; it is instead possible that trophic factors secreted by NT2N neurons enable stroke animals to display functional improvement.

At a later posttransplantation period, however, an alternative mechanism may underlie the observed behavioral recovery produced by NT2N neuronal grafts; NT2N cells might have replaced the degenerated host brain cells. It has been shown that after transplantation into nude mice, NT2N cells can integrate and change phenotype into neurons similar to the target neurons, such as striatal neurons (24, 25, 26, 28). NT2N neurons can potentially become striatal-like neurons and may also be capable of secreting neurochemicals or even performing functions of lost striatal cells of the host brain. Indeed, NT2N cells can be stimulated through application of neurotrophic factors such as acidic fibro-blast growth factor and activating factors such as catecholamines or forskolin to express the rate-limiting enzyme in catecholamine biosynthesis, tyrosine hydroxylase (TH) (58, 59). Of note, *in vitro* studies have shown that the percentage of TH-positive neuron-like cells in the NT2N cells treated with RA cocultured with striatal extracts exceeded by greater than 10-fold the percentage of TH-positive cells induced in sister cultures exposed to retinoic acid alone (24, 25, 26). Of note, many of the behavioral dysfunctions seen in a stroke model are dopamine-mediated behaviors (38, 39, 40). Thus, the possibility that NT2N cell grafts can be induced by the host microenvironment, particularly the remaining host striatal neurons or the whole host striatum itself, to secrete dopamine would greatly contribute to the amelioration of ischemia-induced behavioral deficits. It has also been shown that the host microenvironment of the adult mouse striatum appears to have the potential ability to induce grafted NT2 cells to differentiate progressively into fully mature, adult CNS neurons (24, 25, 26). The striatum may exert similar neuronal differentiation effects on grafted NT2N neurons. Such further neuronal differentiation of NT2N following transplantation is important, especially if it is necessary to target specific disease types that entail degeneration of different cell populations. For example, subsets of NT2N cells have been shown to express neuronal markers for dopaminergic and GABAergic neurons (60, 61), which would be appropriate cell graft source for Parkinson's disease and Huntington's disease, respectively. Because multiple cell populations are affected by stroke, the ability of NT2N cells to differentiate into many cell types will be advantageous. Furthermore, if it is possible to recreate the microenvironment characteristic of the striatum in other brain areas, such as the cortex or hippocampus, which are also damaged in stroke, then this strategy could potentially extend the efficacy of NT2N cell transplantation to a variety of stroke types and other neurological disorders. In the end, multiple mechanisms may mediate the neurobehavioral benefits produced by NT2N neuronal grafts, and these mechanisms warrant further investigations.

10. TRANSPLANTATION OF NT2N CELLS IN STROKE PATIENTS

The above preclinical studies demonstrating successful implantation of human-derived NT2N neurons into rat brains paved the way for proceeding with limited clinical trials. The target stroke patients chosen were at a chronic stage because laboratory data indicated the possibility of reversing motor symptoms associated with a stable stroke. The Food and Drug Administration approved Phase I clinical trials of transplantation of NT2N neurons to evaluate this therapy in the treatment of patients with stable stroke. NT2N cells were transplanted into patients with basal ganglia stroke and fixed motor deficits, including 12 patients aged 44 to 75 years with an infarct of 6 months to 6 years who were stable for at least 2 months (62). Serial evaluations at 12 to 18 months showed no adverse cell-related serologic or imaging-defined effects. These results suggest that transplantation of NT2N cells is feasible in patients with motor infarction.

The intracranial transplantation of certain stem cell-lines has been shown to induce tumor formation when transplants were targeted to the cortex (63). However, no evidence was found of neoplastic formation from NT2N transplants into the striatum (24). The presence of RA in the striatum may have aided in suppressing tumor formation following NT2 cell grafts and also may have facilitated further differentiation of NT2N cell grafts into neuronal lineages. Consistent with this evidence, the above report demonstrated no serious adverse events in transplanted stroke patients at one year post-transplantation. Thus, it appears that grafted NT2N cells do not exhibit neoplasticity, thereby preventing any tumor formation. However, since the intrastraital transplantation of NT2N cells has been the preclinical and clinical approach that has demonstrated consistent non-tumorgenic outcome following NT2N cell grafts, extending the transplant target sites to other brain areas outside the striatum must be approached with caution. At this time, future transplantation trials should be limited to targeting the striatum, thus focusing only on striatal stroke patients. A strategy needs to be developed to make the graft material non-responsive to tumor formation cues from the host microenvironment or to suppress

the host microenvironment from releasing such signals prior to proceeding with extrastriatal NT2N cell transplantation.

Additional challenges in the field of neural transplantation include demonstration of graft viability and functional effects. A subsequent clinical report evaluated the function of NT2N transplanted cells using PET scanning (64). Uptake of fluorodeoxyglucose (FDG) was measured at baseline and at six and twelve months after transplantation of NT2N neurons. At six months post-transplantation, 7 of 11 patients showed >10% uptake increase in FDG in the area of cell implantation, and this increase correlated with clinical improvement measured by stroke scale values. In a recent study that reported the first postmortem brain in an NT2N transplanted patient at 2 years post-transplantation (65), histological examination revealed neurofilament immunoreactive neurons resembling those seen in NT2N neurons *in vitro*. The observed NT2N cell graft survival in this patient suggests that these transplanted cells mediated functional outcome. The PET and histological data from transplanted stroke patients allow some comparisons with long-term graft survival of fetal ventral mesencephalic neurons in Parkinson's disease patients who also died from causes unrelated to the transplants. In both stroke and Parkinson's disease patients, robust graft survival was seen using PET scans and was accompanied by expression of neuronal phenotypes in grafted cells post-mortem. No overt side effects of the transplants in these patients were observed, indicating that the grafts did not exacerbate disease progression. These parallel clinical outcomes seen in stroke and Parkinson's disease patients support the use of NT2N cells as an efficacious alternative to fetal cells.

The optimal number of transplanted cells necessary to achieve improvements in functional outcome remains to be determined. In the above experiment, there was no observed difference in outcome between transplantation of 2 million or 6 million cells. Interestingly, increased uptake of FDG persisted in only 3 patients at the 12-month post-transplant evaluation (64). However, clinical improvement was maintained in 6 of the 7 patients who showed an initial increase in uptake of FDG. This finding suggests that improvement in the clinical exam may be mediated by a factor that only requires functional effects of transplanted cells during a critical time period, or even a limited number of NT2N cells may promote some degree of functional recovery. This notion of minimally required viable cell grafts for functional effects is also true for fetal cell transplantation, at least in Parkinson's disease, wherein it has been suggested that as few as 300 dopaminergic neurons could exert behavioral recovery (66). However, since recurrent stroke episodes may likely ensue following the initial stroke, transplanting more viable NT2N cells may be required for long-term improvement. Transplantation of a higher number of viable cells may also be needed for other neurological disorders, including PD, if grafted cells are affected by ongoing neurodegeneration.

One advantage of transplanting NT2N neurons into humans may be the circumvention of host immunosuppression. Whereas transplantation of human NT2N neurons appeared to be well tolerated by immunosuppressed rats, these cells, being human derived, may not require immunosuppression in stroke patients. Furthermore, since these cells are cultured cells, examination of the cells for possible infectious diseases can be performed well ahead of the scheduled transplant surgery and, therefore, a more efficient transplantation protocol can be achieved with the use of these cells as compared to using fetal cells.

While avoiding the ethical concerns on using fetal cells, transplantation of clonal cells such as NT2N neurons also allows a logistical advantage of conducting neural transplantation in a wider therapeutic window following stroke. As discussed above, since the success of treating cerebral ischemia depends highly on the timing of intervention, the ready availability of clone cells as a graft source would significantly reduce the time between the ischemic event and the therapeutic intervention. Nonetheless, the robust recovery of animals with a stable stroke following transplantation of NT2N neurons suggests the possibility of treating stroke patients even with a long delay after a stroke episode.

Many reservations must still be acknowledged regarding this pioneering clinical trial of transplantation of NT2N neurons (67). The preceding clinical trial is an open label study and was thus not designed to prove efficacy. At best, the results from this Phase I study revealed that NT2N neuronal transplantation is feasible and safe. The follow-up studies suggest no malignant tumor formation over moderate post-transplantation periods of 2 years. Continuous monitoring of the transplanted patients at longer periods of time is being performed and should reveal further safety issues associated with the transplantation therapy. In order to optimize the protocol to achieve effective and consistent improved clinical outcomes, carefully designed laboratory studies and limited clinical trials need to be considered. Subsequent clinical trials should determine the optimal number of cells needed to achieve significant clinical improvements and should develop imaging techniques that would allow characterization of grafted cells to assess viability, migration, differentiation, graft-host integration over NT2N graft maturation period.

11. RECENT ADVANCES ON THE USE OF NT2N CELLS FOR TRANSPLANTATION THERAPY

Although the transplantation of NT2N cells has great potential for theraputic efficacy in CNS disorders, concerns still remain regarding NT2N transplantation in a clinical setting. One recent study that raised such concerns found that embryonic cortical neurons and NT2N have different network properties. Neurons derived from the human NT2 cell line were found to form networks with a clustered neuritic architecture *in vitro*, while primary dissociated embryonic rat cortical neurons were found to display a more homogenous cell assembly. Also, NT2N neurons showed a mostly uncorrelated firing pattern in contrast to the primary dissociated embryonic rat cortical neurons that displayed highly synchronized bursting. These findings bring to light additional concerns that need to be considered before NT2N neurons are used clinically in central nervous system grafting (68).

While some valid concerns remain regarding NT2N cell transplantation, many recent studies have made further

progress in demonstrating the potential for therapeutic efficacy of NT2N cell transplants and for elucidating the mechanisms that underlie the effects of these grafts. One recent study has demonstrated that defined populations of genetically modified human NT2N neurons are a practical and effective platform for stable *ex vivo* gene delivery into the CNS. This study successfully displayed stable, efficient, and nontoxic gene transfer into undifferentiated NT2 cells using a pseudotyped lentiviral vector. NT2 cells were differentiated into NT2N neurons via treatment with RA and then transplanted into the striatum of adult nude mice. Transduced NT2N neurons survived and continued to express the reporter gene for long-term time points *in vivo*. Transplantation of NT2N neurons genetically modified to express nerve growth factor also significantly attenuated cognitive dysfunction following traumatic brain injury in mice (69).

Another recent study has also examined whether Lithium (Li +) treatment of NT2N neurons increases tyrosine hydroxylase (TH) expression when cells are transplanted into the striatum of hemiparkinsonian rats. Histological analysis in this study demonstrated that there was a significantly better survival of cells in the group treated briefly with lithium, thus providing an option for enhancing NT2N graft survival in future transplantation studies (70).

An additional recent study has found that NT2N neurons induced by retinoic acid express the Nurr1 receptor, which has been shown to be essential for the development, differentiation, and survival of midbrain dopamine neurons. The study also confirmed the co-expression of Nurr1 and tyrosine hydroxylase in NT2N neurons. These findings suggest that Nurr1 may be important during the development of NT2N neurons and could also be involved in their differentiation into the dopaminergic phenotype (71).

The use of NT2N cells for transplantation has also been studied recently in the laboratory in order to ameliorate spinal cord injury. The histological data from one such study revealed that graft survival in rats that received transplants was displayed in 66.7% of the surviving, grafted animals. Fiber outgrowth was also observed in both rostral and caudal directions bridging the lesion. The results of this study suggest that NT2N grafts have the potential to structurally reconnect the proximal and distal spinal cord across the region of injury, thus presenting the future possibility to extending the clinical use of these transplants to spinal cord injury as well (72).

One other such laboratory study found that transplantation of NT2N neurons could potentially be an effective means of reestablishing electrical connectivity in the injured spinal cord. In this study, rats were given a complete spinal cord contusion injury, producing a complete loss of motor evoked potentials, and then selected rats underwent transplantation with NT2N cells within the contusion site either immediately after injury or at a delayed point 2 weeks following injury. Rats receiving delayed transplants displayed a significant functional recovery as demonstrated by the return of motor evoked potentials as well as a modest improvement of motor function, again suggesting the potential for NT2N cell transplantation to restore function in spinal cord injury (73).

12. NT2N CELLS AND GENE THERAPY

Trojanowski and colleagues (1) were the first to advance the concept that NT2 cells are transfectable and capable of differentiating into postmitotic neuron-like cells (NT2N cells) following treatment with retinoic acid, thereby allowing this human neuronal cell line to serve as a "platform" for gene therapy applications for treating CNS disorders. As discussed above, NT2N neurons have been shown to engraft and mature when transplanted into the adult CNS of rodents and humans, and that their phenotypic characteristics suggest the likelihood that they are excellent platform for *ex vivo* gene therapy in the CNS; however, stable gene expression in these cells has not been optimal. A subsequent study by Trojanowski and colleagues introduced an alternative approach in circumventing the problem with NT2N cells as a gene therapy tool by initially targeting the undifferentiated NT2 cells with a pseudotyped lentiviral vector encoding the human elongation factor 1-alpha promoter and the reporter gene eGFP (69). Expression of eGFP was maintained even after the cells were differentiated into NT2N neurons and following transplantation of the cells into the striatum of adult nude mice. Expression of eGFP was maintained even after the cells were differentiated into NT2N neurons and following transplantation of the cells into the striatum of adult nude mice. Furthermore, using the same approach of lentiviral transfection in NT2 cells permits the transplantation of the genetically modified NGF-expressing NT2N cells to attenuate the cognitive decline in traumatically brain injured mice (74). Another study demonstrated that a liposome-mediated gene transfer of beta-galactosidase (beta-GAL) reporter gene obtained a high transfection rate in NT2 cells (75). Other reports have shown the efficacy of NT2 cells, as well as the NT2N cells, for other viral vector systems such as the human immunodeficiency virus type 1 (HIV-1) infection strategy (76), cationic liposomes complexed with plasmid DNA-mediated transfection vectors with the constitutive cytomegalovirus promoter or the hypoxia-inducible VEGF promoter (77), Tet-regulated herpes simplex virus vectors (78), recombinant SV40 vectors (79), and adenovirus-mediated gene delivery (80). Based on these series of studies, the undifferentiated NT2 cells and the post-mitotic NT2N cells are efficacious vehicle for delivery of exogenous proteins into the brain.

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