Yonsei Medical Journal
Vol. 41, No. 6, pp. 825-835, 2000

Transplantation of Neural Stem Cells: Cellular & Gene Therapy for Hypoxic-Ischemic Brain Injury

Kook In Park

Abstract

We have tracked the response of host and transplanted neural progenitors or stem cells to hypoxic-ischemic (HI) brain injury, and explored the therapeutic potential of neural stem cells (NSCs) injected into mice brains subjected to focal HI injury. Such cells may integrate appropriately into the degenerating central nervous system (CNS), and showed robust engraftment and foreign gene expression within the region of HI inury. They appeared to have migrated preferentially to the site of ischemia, experienced limited proliferation, and differentiated into neural cells lost to injury, trying to repopulate the damaged brain area. The transplantation of exogenous NSCs may, in fact, augment a natural self-repair process in which the damaged CNS "attempts" to mobilize its own pool of stem cells. Providing additional NSCs and trophic factors may optimize this response. Therefore, NSCs may provide a novel approach to reconstituting brains damaged by HI brain injury. Preliminary data in animal models of stroke lends support to these hypotheses.

Key Words: Neural progenitors, neural stem cells, hypoxic-ischemic brain injury, transplantation, stroke, migration, differentiation, neurons, glia

HYPOXIA-ISCHEMIA AS A PROTOTYPE OF ACQUIRED BRAIN INJURY

Stroke is one of the most common causes of death, and ranks among the most common causes of severe adults disability in developed countries.[1] Stroke accounts for a large proportion of health care costs; about 200 per 100,000 adults per year will have their first stroke. Because the incidence of stroke increases with age, the absolute number of patients with stroke is likely to increase, given that the aged population is also increasing.[2,3] However, ischemic brain injury does not only effect the adult population, but it is a major cause of mortality and severe neurodevelopmental morbidity (cerebral palsy, mental retardation, epilepsy, and learning disability) in the pediatric, especially the newborn, population.[4,5] Although the etiologies of HI brain injury in adults and children may differ, much of the pathophysiology underlying neural cell death and dysfunction is quite similar. In the case of newborn infants, despite advances in perinatal monitoring, obstetric and neonatal care, and a deeper understanding of the pathophysiology of perinatal asphyxia, the incidence of hypoxic-ischemic encephalopathy (HIE) in neonates has remained essentially unchanged over the last few decades. With the exception of thrombolysis therapy for acute stroke in the adult, current clinical management of both adult stroke and perinatal HIE has been limited to supportive measures; it is not directed toward preventing or interrupting the processes underlying brain injury or promoting regeneration.[1,4,5] Given the absence of effective therapies for stroke and perinatal HIE, it is important to derive new strategies. There has been an intense search recently for new approaches based upon the growing knowledge of the molecular mechanisms that mediate neural cell death and degeneration. Experimental therapies in animals have started, for instance, to include inhibitors of oxygen free radical generation and free radical scavengers, antagonists of excitotoxic amino acids and their receptors, calcium channel blockers, nitric oxide synthase inhibitors, trophic factors with neuroprotective actions, and anti-apoptotic agents such as caspase inhibitors.[5-7] Unfortunately, despite the recent substantial research into neuroprotection mentioned,

Received November 17, 2000
Department of Pediatrics and Pharmacology, Yonsei University College of Medicine, Seoul, Korea.
This study was partly supported by a special grant of the Dean of Yonsei University College of Medicine Research Fund of 1998.
Reprint address: request to Dr. K. I. Park, Department of Pediatrics, Yonsei University College of Medicine, Yongdong Severance Hospital, Yongdong P.O. Box 1217, Seoul 135-270, Korea. Tel: 82-2-3497-3351, Fax: 82-2-3461-9473, E-mail: kipark@yumc.yonsei.ac.kr

to date no neuroprotective agents have been shown conclusively to be clinically effective.[1,5-8]

THERAPEUTIC POTENTIAL OF NEURAL STEM CELLS AND PROGENITORS

Recently, there has been a growing interest in the therapeutic potential of NSCs and progenitors for therapy in stroke, HIE, and other CNS dysfunctions. NSCs are the primordial, multipotent, self-renewing cells that, during the earliest stages of development, are believed to give rise to the vast array of specialized cells of the nervous system. They are thought to persist all throughout life, not only in a few discrete regions but probably throughout the brain, serving homeostatic and perhaps self-repair functions. The interest in NSCs derives from the realization that these cells are not simply a substitute for fetal tissue in transplantation paradigms or simply a "better" vehicle for gene delivery. We in the field of developmental neuroscience believe that the basic biology of these cells endows them with a potential that other vehicles for gene therapy and repair may not possess.[9-11]

This biologic potential endows them with the ability actually to integrate into the neural circuitry after transplantation. This property, in turn, may allow the regulated release of various gene products, and may also allow for literal neural cell replacement. While presently available gene transfer vectors usually depend on relaying new genetic information through established neural circuits, which may, in fact, have already degenerated and become dysfunctional, NSCs may actually participate in the reconstitution of these pathways. The replacement of enzymes and cells may be targeted not only to specific, anatomically circumscribed regions of the CNS,[12-16] but also if desired, by simple modifications in technique, to large areas of the CNS in a wide-spread manner.[17-23] This ability is important because most neurologic diseases are not localized to specific sites, as is Parkinson's disease. Rather, their neuropathology is often extensive, multifocal, or even global; stroke and HIE provide ideal examples of just how broad the regions of degeneration may be. Intriguingly, NSCs may actually be uniquely responsive to neurodegenerative environments.[24-26] This type of responsiveness of NSCs may optimize cell replacement and therapeutic gene expression within the damaged CNS.

In this context, therefore, the growing interest in NSCs biology, as it might apply to HIE represents a somewhat different focus on the cellular and molecular aspects of neurodegeneration. While the above-mentioned neuroprotective strategies seek to short-circuit cell death and/or promote neuroprotection--i.e., to combat the progression of neuropathological processes, stem cell biology shines the spotlight instead on a non-pathological process--i.e., on re-invoking the developmental processes for purpose of regeneration. In other words, a putative stem cell-mediated strategy would be rooted not so much in "combating" pathology as in abetting natural self-repair processes to exist in the CNS in response to a wide range of injuries and degenerative processes.

THE RESPONSE OF NEURAL STEM CELLS TO HYPOXIC-ISCHEMIC BRAIN INJURY

Little is known about the response of NSCs to CNS injury in general, let alone HI brain injury in particular. Is it possible to repopulate an "ablated" CNS with NSCs in the way hematopoietic stem cells reconstitute lethally irradiated bone marrow? HI brain injury was initially viewed by us as an injury that is not only of importance in its own right, but might also serve as a prototype for other large, acquired brain injuries.[27] It occurred to us that to help answer this question we might be able to use one of our prototypical NSC clones, clone C17.2,[28-30] as "reporter cells." This well-characterized clone is just one of the several with stem cell features that exist in the literature multipotent, self-renewing, self-maintaining, nestin-positive, and responsive to various stem cell trophins. As one would demand of a putative stem cell, NSCs from clone C17.2 are able to participate in the development of the CNS throughout the neuraxis and across developmental periods, from fetus to adult.[17,18,23,25,26,29,30] Engrafted and integrated NSCs are visible because they have been transduced also with a reporter gene, *lacZ*, that allows the cells to stain blue when processed with Xgal histochemistry, or to appear brown or fluorescent following reaction with an antibody against E. coli β-galactosidase (βgal) in the immunoperoxidase and immunofluorescence protocols, respectively.[25,28] This ability to identify progeny of a donor NSCs is important because,

by their nature, NSCs integrate and intermingle seamlessly into the host following transplantation, do not form a discernible graft-host border, and actually come to resemble host neural cells of the same phenotype. When we refer to using clone C17.2 NSCs as "reporter" cells, we mean using well-characterized, indelibly marked cells with known ancestry, potential, and clonal relationships that are traceable, abundant, and homogenous, that intermingle imperceptibly with host cells *in vivo* and that can, therefore be used as a tool for mirroring, probing, and tracking i.e., "reporting" on the behaviors of neighboring endogenous progenitors that are otherwise invisible to such monitoring, and whose own clonal relationships and degree of homogeneity are much less certain. Such cells would also allow well-controlled experiments to proceed with minimal variability in cell population under study from experiment-to-experiment, animal-to-animal, and condition-to-condition. The type of injury in which NSCs would be investigated in these preliminary experiments would be focal HIE engendered by permanent ligation of the right common carotid artery of a week-old mouse followed by exposure of the animal to 8% ambient oxygen. This combination of ischemia and hypoxia results in extensive injury to the hemisphere ipsilateral to the carotid ligation, while leaving the contralateral hemisphere as an intact control.

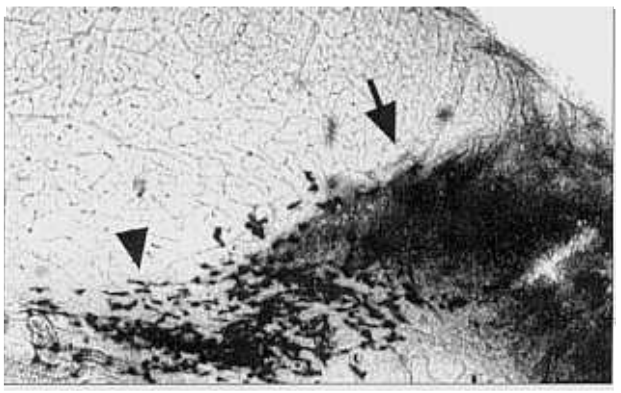

Fig. 1. Migration by transplanted "reporter" stem cells to the ischemic area of a mouse brain subjected to unilateral, focal hypoxic-ischemic brain injury. Clone C17.2 neural stem cells were injected into the left cerebral ventricle of a mouse on the day of birth (postnatal day 0 {P0}). At 1 week of age (P7), the animal was subjected to contralateral right-sided hypoxic-ischemic injury. The animal was subsequently analyzed at maturity with Xgal histochemistry to identify lacZ-expressing donor-derived cells (which stain blue). Some cells appeared to migrate along the corpus callosum (arrowhead) throughout the cerebrum toward the highly ischemic area (arrow).

In the first set of pilot experiments,[27] we wondered what might be observed if we took a normal animal in which "reporter" NSCs had become stably integrated throughout the brain during a critical period of its development (creating virtually a chimeric brain of host and reporter cells), and then exposed that animal to unilateral HI injury. The experimental paradigm, therefore, was as follows: clone C17.2 NSCs were transplanted into the cerebral ventricles of mice on the day of birth (P0), allowing the NSCs access to the subventricular germinal zone (SVZ) that lines the ventricular system running the length of the neuraxis, which results in widespread migration, stable integration, and intermixture of donor NSCs with host cells throughout the parenchyma.[17] The right hemisphere was then subjected to HI injury at 1 week of age (P7). Brains were analyzed 2−5 weeks later. The resulting picture in these preliminary studies was complex but intriguing. In contrast to the intact side, where the reporter NSCs remained widely and evenly interspersed throughout the intact parenchyma, the reporter NSCs in the HI-injured hemisphere appeared to be densely and preferentially clustered around the infarction cavity. The heavy accumulation and number of cells in this location suggested that either many NSCs had migrated to that particular area, or that the cells near this location had proliferated, or both. In addition, in the penumbra of the infarction, an increased number of donor-derived cells were identified immunocytochemically as oligodendrocytes and neurons. Neurons and oligodendrocytes are the two neural cell types that are most susceptible to HI injury and which are least likely to regenerate spontaneously in the "post-developmental" mammalian cortex. Furthermore, in the intact hemisphere, as might be expected from NSCs implanted after the completion of embryonic cortical neurogenesis, no donor-derived neurons and many fewer oligodendrocytes were observed. Therefore, following HI brain injury, NSCs appeared to evince components of altered proliferation, migration, and differentiation, which is precisely the type of behavior one might expect of a stem cell. We decided to start examining each of these components in a systematic fashion.[27]

First we wanted to answer the question, whether there was new transient proliferation by quiescent NSCs, both reporter and host cells. A transplant of

reporter NSCs was, therefore, performed at P0 into the cerebral ventricles; unilateral HI injury was induced at P7 (after the cells had stably integrated, differentiated, and become quiescent). The mice were then pulsed with bromodeoxyuridine (BrdU), a nucleotide analogue, at various post-HI injury time points. A preliminary analysis revealed that, before injury, donor-derived cells were completely quiescent, however, after HI injury, the percentage of reporter (*lacZ*+) cells that became mitotic (i.e., incorporated BrdU) increased rapidly, peaking at about 3 days after the induction of HI, and then fell back to 0 one week after HI. Host cells did precisely the same thing; their patterns of proliferation were virtually superimposable upon that of the donor cells, peaking at −3 days after HI and then returning to 0, also suggesting an induction of transient proliferation.

That so many changes peaked 3 days after injury is interesting. The literature on stroke, and on other injuries suggests that the 3−7 day interval after insult is a very metabolically, biochemically, and molecularly active temporal "window", during which a variety of mitogens, trophins, extracellular matrix molecules, and other factors are uniquely elaborated. We will return to this "window" and its impact on neural stem cell biology later in the review.

Fig. 2. Robust engraftment by transplanted neural stem cells within the ischemic region of a mouse brain subjected to unilateral focal hypoxic-ischemic injury (HI). This mouse was subjected to right hypoxic-ischemic injury on postnatal day 7 (P7). Three days later (P10), the animal receive a transplant of clone C17.2 neural stem cells within the region of infarction. The animal was analyzed at maturity with Xgal histochemistry. A representative coronal section is shown. Robust engraftment was evident within the ischemic area (arrow). Similar engraftment was evident throughout the hemisphere. Even cells that implanted outside the region of infarction appeared to migrate along the corpus callosum toward the ischemic area. The most exuberant engraftment was evident 3-7 days after HI. Immunocytochemical and ultrastructural analysis revealed that a subpopulation of donor-derive cells, especially those in the penumbra, differentiated into neurons and oligodendroglia, the two neural cell types most characteristically damaged by HI and the cell types least likely to regenerate spontaneously in the postnatal brain.

Next, we began to approach the question whether reporter NSCs (and, by extension, host NSCs), migrate to areas of neurodegeneration. NSCs (clone C17.2) were transplanted into only the left intracerebroventricular space at P0. At 1 week of age, unilateral HI injury was induced in the contralateral right hemisphere in some animals, while in other the right hemisphere was left intact. In animals with an intact right hemisphere, engrafted stem cells simply remained stably distributed and densely integrated throughout the parenchyma of only the transplanted left hemisphere. But in those animals in which the right hemisphere had been infarcted, cells at multiple levels throughout the cerebrum dramatically appeared to migrate across the corpus callosum and any available interhemispheric commissure to the infarcted region. With high magnification under light and electron microscopy, one could appreciate the leading processes of NSCs migrating along the interhemispheric connections toward the damaged areas. Even within the infarct, one could see reporter cells migrating into the heart of the necrotic area.

Therefore, there seems to be evidence that NSCs already integrated into the CNS will migrate to an area of subsequent infarction. We then addressed the question as to whether reporter NSCs implanted *after* HI injury are also drawn to areas of damage. To investigate this question, the following paradigm was followed: Unilateral (right) HI injury was induced at P7, and reporter NSCs were transplanted into the contralateral (left) cerebral ventricle 3 days later (at P10). As a control, some animals not subjected to right HI were also transplanted on the left at P10. As before, in intact animals, the NSCs remained nicely but stably integrated on the transplanted left side. However, in animals that had been infarcted on the right before transplantation on the left, reporter NSCs migrated avidly across the corpus callosum and other interhemispheric commissures to the area of infarction throughout the length of the cerebrum. Furthermore, they integrated into those infarcted

areas as if drawn or directed by a tropism for the region. When reporter NSCs were injected directly into the infarcted area on the right, they did not migrate in the other direction to the contralateral intact side, in these pilot studies.

This last manipulation, that of injecting NSCs directly into the infarct, suggests what our next set of experiments entailed. NSCs (clone C17.2) were transplanted directly into the degenerating infarcted region at various time points following the induction of unilateral HI. When implantation was performed shortly after a HI (e.g., the following day), robust engraftment was observed throughout the infarcted area. If transplantation was postponed until 5 weeks after HI, virtually no, engraftment was achieved. Engraftment was most exuberant 3−7 days after HI.

Is there, indeed, a change in differentiation fate of these reporter NSCs in the areas of degeneration compared with what might be seen in intact brain? Immunocytochemical and ultrastructural examination of the engrafted regions, particularly in the penumbra of the infarct, suggests that this is the case. Donor-derived cells (recognized by an anti-βgal antibody) were assessed for the expression of neural cell type-specific antibodies (e.g., NeuN, neurofilament, MAP-2 for neurons, CNPase for oligodendrocytes, GFAP for astrocytes, and nestin for immature undifferentiated progenitors). A subpopulation of donor NSCs in the injured postnatal neocortex differentiated into neurons (−5%) and oligodendrocytes (−4%). Other cell types were astroglial though no scarring was apparent and undifferentiated progenitors. As noted below, these numbers contrast significantly with what is found in an intact age-matched recipient neocortex. The presence of donor-derived neurons can be detected as much as 1 mm away from the heart of the infarction cavity on the side of the lesion, suggesting that a relatively large "sphere of influence" is exerted by the injured tissue. (Interestingly, occasionally we noted host-derived neurons in an otherwise severely destroyed cortex, which is consistent with our belief that some host NSCs, as do the reporter NSCs, try to shift their differentiation toward compensation for neuronal cell death, a phenomenon that we perhaps augment with our transplants). Examination of the penumbra under the electron microscope in these preliminary studies supported the immunocytochemical assessments. A significant number of donor-derived oligodendrocytes and neurons were observed. Some donor-derived pyramidal neurons receive synaptic input from the host.

The quantification of the differentiation pattern by transplanted reporters NSCs in the injured neocortex compared with that in the intact neocortex was illuminating. Whereas 5% of the engrafted NSCs on the injured side differentiate into neurons, no neuronal differentiation by NSCs was seen at all in the intact neocortex, which is consistent with both the normal absence of neurogenesis in the postnatal mammalian cortex and with our prior findings.[25,30] There was a 5-fold increase in the number of donor-derived oligodendrocytes in the injured neocortex compared with the intact neocortex. The number of astrocytes did not significantly differ between the two sides. Also, there was an upregulation of nestin in donor NSCs in response to injury (almost three times as many donor cells were nestin positive in the injured cortex compared with the intact cortex, suggesting that they may have become activated or primed to make a differentiation choice).

These preliminary quantitative data are presented to make a qualitative point. On the intact side of the infarcted animal, there was no neuronal differentiation at all; on the injured side, 5% of the donor-derived cells were neurons. The magnitude of that number is less significant than the phenomenon of qualitatively moving from consistently no neurons to neurons of any number at a stage of development when no cortical neurons should normally be born. As mentioned previously, oligodendrocytes and neurons are the two neural cell type most damaged by HI injury. It appears from these preliminary data that NSCs may be attempting to repopulate and reconstitute that area of injury, particularly within a certain temporal window, by "shifting" their normal differentiation fate to compensate for the loss of those particular cell types, especially neurons. It seems likely that, as a consequence this type of neurodegeneration, signals are elaborated to which NSCs (donor and probably host) are able to respond in a reparative fashion. Precisely what these signals are is the subject of ongoing active investigation. No doubt a complex mix of various mitogens, neurotrophins, adhesion molecules, cytokines, etc. are involved.

Although the preliminary numerical data cited above is presented principally to illustrate the "shift" toward neuronal differentiation by NSCs in response to injury, it is instructive to note that, given the vast

number of NSCs that engraft into the infarcted region, a differentiation of even 5% of such cells into neurons translates into tens-of-thousands of replacement neurons supplied to that degenerating region. We don't actually know how many neurons and how much circuitry is required to reconstruct functionally a damaged mammalian system. We do know that, fortunately, 100% restoration is not needed; older lesion data suggests that as little as 10% may be sufficient.

COMBINING CELL REPLACEMENT WITH GENE THERAPY VIA THE NSCs

Despite the fact that the neuronal differentiation of 5% of the transplanted NSCs may be sufficient to repair a HI injured region of brain, we nevertheless wondered whether that percentage could be increased. Neurotrophin-3 (NT-3) is known to play a role in inducing neuronal differentiation.[32,33] It appeared feasible that neuronal differentiation of both host and donor NSCs might be enhanced if the latter were engineered before transplantation to (over) express NT-3. A subclone of NSCs was transduced with a retrovirus encoding rat NT-3.[34] The engineered NSCs successfully produce large amounts of NT-3 *in vitro* and *in vivo*. We determined that both the parent NSCs and the NT-3-overexpressing NSC subclones, express trkC receptors (the receptor for NT-3).[35] These receptors are appropriately tyrosine-phosphorylated in response to exogenous NT-3; this phosphorylation can be blocked by K252a, an inhibitor of neurotrophin-induced tyrosine kinase activity. Therefore, it appeared that these engineered NSC clones could not only secrete excess amounts of NT-3, but that they could likely respond to NT-3 in an autocrine or paracrine fashion a very appealing scenario.

In tissue culture, these NT-3-overexpressing NSCs, like the parent NSC clone, still differentiated into all three neural cell types (neurons, astrocytes, and oligodendrocytes). However, unlike the parent clone, whose percentage of neurons falls in serum-containing medium as new cells are born, the proportion of this NT-3-expressing subclone that continues to express neuronal markers in culture for prolonged periods (> 3 weeks) remained quite high (−90%).[35]

In an experimental paradigm identical to that described previously, cells from the NT-3-expressing NSC subclone were implanted into the infarct of a unilaterally asphyxiated postnatal mouse brain 3 days after induction of HI injury.[35] The brains were analyzed 2−4 weeks later, as described above. On preliminary analysis, the percentage of donor-derived neurons was dramatically increased, to 20% in the infarction cavity and to as high as 80% in the penumbra. Many of the neurons were calbindin positive; they were also variously GABAergic, glutamatergic, or cholinergic. Donor-derived glia were rare. It appeared, therefore, that when NSCs are transplanted within regions of HI injury, a greater percentage of them engineered *ex vivo* to express NT-3, differentiate into neurons. It is likely that NT-3 acts on donor cells (as well as host cells) in an autocrine/paracrine fashion to enhance neuronal differentiation. This pilot experiment enunciates the feasibility of using NSCs for simultaneous, combined gene therapy and cell replacement in the same transplant procedure using the same clone of cells in the same transplant recipientan appealing stem cell property with implications for therapies in other degenerative conditions.

THE RESPONSE OF ENDOGENOUS, HOST NSCs IN RESPONSE TO HI BRAIN INJURY AND THEIR NEUROREGENRATIVE POTENTIAL

In the transplant studies described above, the grafted and stably integrated NSC clones, whose response to focal HI cerebral degeneration was tracked, were viewed as "reporter cells", mirroring the behavior of the brain's own NSCs which putatively alter their fate, in terms of their proliferation, migration, and differentiation, in an effort to repopulate damaged areas. Our thoughts on this issue were, that if the brain's inclinations are towards self-repair via the NSCs, then that response might be augmented. Is this truly what endogenous progenitors "attempt" to do? We launched a series of *non-transplant*-based experiments to explore whether the "reporter cells" were indeed reporting on a real phenomenon.

It has been recognized for decades[36-38] that 2 highly circumscribed regions of the mammalian cerebrum continue to generate neurons throughout life. These "privileged" areas are designated "neurogenic

regions" and exist throughout life in the olfactory bulb (OB) by way of the SVZ and in the hippocampal dentate gyrus (DG),[39-43] including in humans.[44,45] The remainder of the CNS is termed "non-neurogenic"; in other words, neuronal generation does not take place beyond fetal life, their normal period of neuron birth. Consequently, neuronal regeneration does not occur in the vast majority of the "post-developmental" CNS after injury or disease.[46] However, the fact that cerebrum does retain a capacity for neurogenesis from proliferating cells in the SVZ and DG throughout life, suggests that these neural progenitor cells may provide an endogenous population with significant neuroregenerative potential (either constitutively or following manipulation).

The findings in the previous sections postulated that, following brain injury and during phases of neurodegeneration, signals might be transiently elaborated, even in "non-neurogenic" regions, to which progenitor and stem cells can respond in a reparative fashion. Were an intrinsic capacity for producing new neural cells (including neurons) in classically non-neurogenic regions to be apparent even at low, ostensibly clinically silent levels this might attest to a degree of inherent CNS plasticity not previously appreciated. Futhermore, it might explain certain observed levels of unanticipated recovery often seen by clinicians following adult and pediatric stroke, and might lend insight into the teleological significance of persistent neurogenic zones, while offering a substrate from which better strategies for brain repair might be launched.

Following unilateral HI brain injury, in preliminary studies, the migration, and differentiation of mitotic neural progenitor cells (NPCs) in the SVZ of both.[47] First, we tracked the behavior of newly proliferative endogenous NPCs, by injecting intraperitoneally the proliferation marker BrdU, which is selectively and permanently incorporated into the nuclear genomic material of all cells entering S-phase, hence labeling dividing cells. Starting 2 hrs. after induction of unilateral HI, mice were pulsed with BrdU every 4 hrs. for the subsequent 12 hrs. As an additional independent marker of newly mitotic cells, in parallel experiments, a replication-incompetent, help virus-free retroviral vector encoding the *lacZ* reporter transgene[48] was also employed to label such cells directly. A retroviral provirus becomes permanently integrated into the genome and passes stably to the progeny of only those cells progressing through S-phase. Successful infection (as indicated by *lacZ* expression) is, therefore, another unambiguous marker of mitotic cells. In order to label proliferating SVZ cells, the *lacZ*-encoding vector was injected into both lateral ventricles of mice being subjected to unilateral HI.

HI brain injury induced a significantly increased proliferation of the SVZ progenitor population ipsilateral to the lesioned right side compared to the grossly intact contralateral left side and uninjured control group. Expansion of BrdU-positive cells was most pronounced in the dorsolateral wall of the lateral ventricles adjacent to the infarction cavity, and a relatively dense stream of "newly-born" cells oriented towards and into the injured cerebral cortex was apparent. The normal fate of most of the cells born in the SVZ (particularly the anterior portion) is to migrate rostrally along the rostral migratory stream (RMS) into the OB, where they differentiate into neurons.[39-42] Certainly that typical developmental program was evident in the intact left hemisphere. Intriguingly, although more cells were actually born in the right SVZ ipsilateral to the lesion in response to HI, significantly fewer BrdU-positive cells were present in the RMS and the number of newly-born cells that actually reached the right OB was reduced significantly as if the newly-born cells on the damaged side were "shunted" or "drawn" *away* from their normal migratory route *towards* the site of injury. Interestingly, the number of newborn-cells that reached the RMS and OB from the SVZ *contralateral* to the lesion, though certainly much greater than that ipsilateral, was also significantly reduced compared to the non-injured control group, suggesting that injury has a broad effect throughout the brain and may draw cells from even distant regions. In other words, the CNS environment appears to change radically after injury, particularly that induced by HI.

To help determine the differentiation fate *in vivo* of injury-generated BrdU-labeled cells, particularly in non-neurogenic regions, they were analyzed for their co-expression of neural cell type-specific antigens. Over a 3 week period following the final BrdU pulse, many of the cells induced to proliferate yielded new oligodendrocytes, astrocytes, and intriguingly, neurons (4.0%, 1.2%, and 1.2% at 1, 2, and 3 weeks, respectively). Interestingly, these new neurons (likely

an under-representation given the time course of the BrdU pulses) were evident not only in the compromised hemisphere, but in the contralateral hemisphere, as well, suggesting again the widespread "ripple" effect of signals emanating from even an ostensibly localized lesion. (No BrdU+ neurons were seen in the cortices of uninjured control mice.) That these newly-born neurons in non-neurogenic regions might persist permanently was suggested by their continued detection, which was essentially undiminished for at least 2 months after injury.

As a complement to BrdU labeling and to track more rigorously the fate of these newly-proliferative injury-responsive periventricular NPCs, a retroviral vector encoding *lacZ* was injected into both lateral ventricles of mice being subjected to unilateral HI. In response to HI, *lacZ*-expressing (i.e., βgal+) periventricular cells migrated into the adjacent striatum and hippocampus, into the cortex ipsilateral to the lesion, and into the cortical penumbra. Confirming the observation noted previously, that a subpopulation of the newly proliferative and migratory βgal+ cells now expressed the mature neuronal marker, NeuN, in all of these "non-neurogenic" regions, suggesting *de novo* neurogenesis. (Interestingly, even in the grossly intact contralateral hemisphere, some βgal+ periventricular cells (often in groups) also migrated into the cortex and overlying hippocampal CA1 area becoming neurons).

SPECULATIONS ON THE DYNAMIC INTERACTION BETWEEN NSCs AND HI CEREBRAL DEGENERATION

The findings in the previous section (as did the transplant studies described previously) suggest that, following CNS injury and during acute phases of resultant neurodegeneration, factors are elaborated to which donor-derived and endogenous neural progenitor and stem cells may respond in a reparative fashion, and which can promote the establishment of new neurons even within non-neurogenic regions of the "post-developmental" CNS. Neural stem and progenitor cells appear to be capable of responding to neurogenic signals not only during their normal developmental expression, but also when induced later stages during critical periods following injury. Stem cells seem to have a tropism for and a trophism within degenerating CNS regions. They seem to be able to "shift" their differentiation fate, and this phenomenon seems to be magnified at the peak of active neurodegeneration. Given these observations, we further speculate that the CNS may "attempt" to repair itself with its own endogenous pool of progenitors and stem cells, but that supply may simply be insufficient, either in number or in factors regulating mobilization, recruitment, migration, differentiation, survival, neurite extension, and synaptogenesis in the context of HI injury. Therefore, the net impact of the production of new nerve cells may be limited. If this is the case, perhaps we can augment that stem cell population with exogenous stem cells and/or exogenous trophic factors to enable more significant recovery. Such a strategy would certainly benefit from identifying those transiently expressed signals, and this identification may permit them to be supplied exogenously in order to recruit the host's own internal stem cell reservoir more effectively. In fact, donor stem cells genetically engineered *ex vivo* (as we did with the NT-3-expressing stem cells) may be one method of supplying some of those tropic and trophic factors. Under certain circumstances, one clone of the transplanted stem cells may be able to serve multiple therapeutic functions: both gene delivery and cell replacement.

Therefore, one strategy, which can take its place in the repertoire with other valuable repair strategies, may be stem cell-based: using the host's own appropriately activated reserve of stem cells augmented by an exogenous supply of stem cells introduced during or shortly after injury or neurodegeneration (apparent "windows of opportunity"). It may, in fact, be possible to treat chronic lesions by re-expressing certain "signals" (e.g., certain cytokines) that emulate the more acute phase, to which stem cells may then respond in a reparative fashion. All these speculations are absolutely predicated on exploring the dynamic processes by which multipotent stem cells make their phenotypic choices in developing and degenerating CNS.

TRANSPLANTATION OF HUMAN NEURAL STEM CELLS (hNSCs) INTO HI BRAIN INJURY

The abiding faith in "translational neuroscience" is,

of course, that the biology that endows rodent neural stem cells with their therapeutic potential is conserved in the human CNS. Progress in this regard is, gratifyingly, being made. Several neural stem cell clones and populations have been isolated from human fetal brains, and these cells appear to emulate many of the appealing properties of their rodent counterparts[22,45,49-51]; they differentiate, *in vitro* and *in vivo*, into all 3 neural cell types; they vouchsafe conservation of neurodevelopmental principles following engraftment into developing mouse brain; they express foreign genes *in vivo* in a widely disseminated manner; and they can replace missing neural cell types when grafted into various mutant mice. In order to determine whether findings with rodent NSCs in response to injury, might extend to cells from the human CNS, and to explore their therapeutic potential in the treatment of HI in infants, human NSCs (in pilot studies) were injected into the infarction cavity of mice, employing the same experimental paradigm as described above.[52] Human NSCs show robust engraftment within the ischemic region and its penumbra, migrate extensively and preferentially toward the site of injury, and differentiate into all 3 neural cell types. A subpopulation of donor-derived neurons express glutamate, GABA, tyrosine hydroxylase, and choline acetyltransferase in various CNS regions. Preliminary data suggest that human NSCs grafted into the HI-injured brain sites in mice partially restore some motor and cognitive functions, as demonstrated by rotarod performance, the step-through type passive avoidance test, and the habituation of exploratory behavior test. These findings suggest that human NSCs might be capable of replacing some neural cell populations lost to experimental HI injury in mice and could provide a rationale for ultimate stem cell-based therapy for human ischemic and other degenerative CNS diseases.

ACKNOWLEDGMENTS

Much of the work described was done in Dr. Evan Y. Snyder's Lab., Department of Pediatrics, Neurosurgery, & Neurology, Children's Hospital, Boston, Harvard Medical School, Boston, MA, USA.

REFERENCES

1. Davenport R, Dennis M. Neurological emergencies: acute stroke. J Neurol Neurosurg Psychiatry 2000;68:277-88.
2. Sudlow C, Warlow C. Comparing stroke incidence worldwide. What makes studies comparable? Stroke 1996;27: 550-8.
3. Warlow CP, Sandercock PAG, Hankey GJ, van Gijn J, Dennis MS, Barnford J, et al. Stroke. A practical guide to management. In: Warlow CP, Sandercock PAG, Hankey GJ, van Gijn J, Dennis MS, Barnford J, et al., editors. The organization of stroke services. Oxford: Blackwell; 1996. p.598-631.
4. Volpe JJ. Neurology of the Newborn. 3rd ed. Philadelphia(PA): WB Saunders Company; 1995.
5. Vannucci RC, Perlman JM. Interventions for perinatal hypoxic-ischemic encephalopathy. Pediatrics 1997;100:1,004-14.
6. du Plessis AJ, Johnston MV. Hypoxic-ischemic brain injury in the newborn. Cellular mechanisms and potential strategies for neuroprotection. Clin Perinatol 1997;24:627-54.
7. del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feuerstein GZ. Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. Brain Pathol 2000;10:95-112.
8. Tan S, Parks DA. Preserving brain function during neonatal asphyxia. Clin Perinatol 1999;26:733-47.
9. Snyder EY, Wolfe JH. CNS cell transplantation: a novel therapy for storage diseases? Curr Opin Neurol 1996;9: 126-36.
10. Snyder EY, Fisher LJ. Gene therapy for neurologic diseases. Curr Opin Pediatr 1996;8:558-68.
11. Snyder EY, Senut MC. Use of non-neuronal cells for gene delivery. Neurobiol Dis 1997;4:69-102.
12. Martinez-Serrano A, Lundberg C, Horellou P, Fischer W, Bentlage C, Campbell K, et al. CNS-derived neural progenitor cells for gene transfer to nerve growth factor to the adult rat brain: complete rescue of axotomized cholinergic neurons after implantation into the septum. J Neurosci 1995;15:5,668-80.
13. Martinez-Serrano A, Fischer W, Bjorklund, A. Reversal of age-dependent cognitive impairments and cholinergic neuronal atrophy by NGF-secreting neural progenitors grafted to the basal forebrain. Neuron 1995;15:473-84.
14. Martinez-Serrano A, Fischer W, Sderstrm S, Ebendal T, Bjrklund A. Longterm functional recovery from age-induced spatial memory impaiments by nerve growth factor gene transfer to the rat basal forebrain. Proc Natl Acad Sci USA 1996;93:6,355-60.
15. Martinez-Serrano A, Bjoklund A. Protection of the neostriatum against excitotoxic damage by neurotrophin-producing, genetically modified neural stem cells. J Neurosci 1996;16:4,604-16.
16. Martinez-Serrano A, Snyder EY. Neural stem cell lines for CNS regeneration: Basic Science and Clinical Applications. In: Tuszynski M, Kordower J, editors. CNS Regeneration. San Diego: Academic Press; 1999, p.203-50.

17. Snyder EY, Taylor RM, Wolfe JH. Neural progenitor cell engraftment corrects lysosomal storage throughout the MPS VII mouse brain. Nature 1995;374:367-70.
18. Lacorazza HD, Flax JD, Snyder EY, Jendoubi M. Expression of human β-hexosaminidase α-subunit gene (the gene defect of Tay-Sachs disease) in mouse brains upon engraftment of transduced progenitor cells. Nature Med 1996;2:424-9.
19. Lynch WP, Snyder EY, Qualtiere L, Portis JL, Sharpe, AH. Late virus replication events in microglia are required for neurovirulent retrovirus-induced spongiform neurodegeneration: evidence from neural progenitor-derived cheimeric mouse brains. J Virol 1996;70:8,896-907.
20. Lynch WP, Shapre AH, Snyder EY. Neural stem cells as engraftable packaging lines optimize viral vector-mediated gene delivery to the CNS evidence from studying retroviral env-related neurodegeneration. J Virol 1999;73:6,481-851.
21. Billinghurst LL, Taylor RM, Snyder EY. Remyelinationa: cellular and gene therapy. Semin Pediatr Neurol 1998;5: 211-28.
22. Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, et al. Engraftable human neural stem cells respond to developmental cues, replace reurons and express foreign genes. Nature Biotech 1998;16:1,033-9.
23. Yandava BD, Billinghurst LL, Snyder EY. "Global" cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. Proc Natl Acad Sci USA 1999;96:7,029-34.
24. Snyder EY, Macklis JD. Multipotent neural progenitor or stem-like cells may be uniquely suited for therapy for some neurodegenerative conditions. Clin Neurosci 1996;3: 310-6.
25. Snyder EY, Yoon C, Flax JD, Macklis JD. Multipotent neural precursors can differentiate toward replacements of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. Proc Natl Acad Sci USA 1997; 94:11,663-8.
26. Rosario CM, Yandava BD, Kosaras B, Zurakowski D, Sidman RL, Snyder EY. Differentiation of engrafted multipotent neural progenitors towards replacement of missing granule neurons in Meander tail cerebellum may help determine the locus of mutant gene action. Development 1997;124:4,213-24.
27. Park KI, Jensen FE, Snyder EY. Nerual progenitor transplantation for hypoxic-ischemic brain injury in immature mice [abstract]. Soc Neurosci 1995;21:2,027.
28. Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwieg EA, Cepko CL. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell 1992;68:33-55.
29. Snyder EY, Flax JD, Yandava BD, Park KI, Liu S, Rosario CM, et al. Transplantation and differentiation of neural "stem-like" cells: possible insights into development and therapeutic potential. In: Gage FH, Christen Y, editors. Research and Perspectives in Neurosciences: Isolation, Characterization, and Utilization of CNS stem cells. New York: Springer-Verlag; 1997. p.173-96.
30. Snyder EY. Neural stem-like cells: developmental lessons with therapeutic potential. The Neuroscientist 1998;4: 408-25.
31. Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, et al. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci USA 1995;92:11,879-83.
32. Ghosh A, Greenberg ME. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 1995;15:89-103.
33. Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RDG. Single factors direct the differentiation of stem cells form the fetal and adult central nervous system. Genes Dev 1996;10:3,129-40.
34. Liu Y, Himes BT, Solowska J, Moul J, Chow SY, Park KI, et al. Intraspinal delivery of neurotrophin-3 using neural stem cells genetically modified by recombinant retrovirus. Exp Neurol 1999;158:9-26.
35. Park KI, Jensen FE, Stieg PE, Himes T, Fischer I, Snyder EY. Transplantation of neurotrophin-3 (NT-3) expressing neural stem-like cells into hypoxic-ischemic brain injury [abstract]. Soc Neurosci 1997;23:346.
36. Sidman RL, Miale IL, Feder N. Cell proliferation and migration in the primitive ependymal zone: an autoradiographic study of histogenesis in the nervous system. Exp Neurol 1959;1:322-33.
37. Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 1965;124:319-35.
38. Altman J. Autoradiographic and histological studies of postnatal neurogenesis: IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol 1969;137:433-58.
39. Lois C, Alvarez-Buylla A. Long distance neuronal migration in the adult mammalian brain. Science 1994;264: 1,145-8.
40. Lois C, Garcia-Verdugo JM, Alvarez-Buylla A. Chain migration of neuronal precursors. Science 1996;271:978-81.
41. Goldman SA, Luskin MB. Strategies utilized by migrating neurons of the postnatal vertebrate forebrain. Trend Neurosci 1998;21:107-14.
42. Kakita A, Goldman JE. Patterns and dynamics of SVZ cell migration in the postnatal forebrain: monitoring living progenitors in slice preparation. Neuron 1999;23:461-72.
43. Wu W, Wong K, Chen J, Jiang Z, Dupuis S, Wu JY, et al. Directional guidance of neuronal migration in the olfactory system by protein Slit. Nature 1999;400:331-6.
44. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. Nature Med 1998;4:1,313-7.
45. Pincus DW, Keyoung HM, Harrison-Restelli C, Goodman RR, Fraser RA, Edgar M, et al. FGF2/BDNF-associated maturation of new neurons generated from adult human subependymal cells. Ann Neurol 1998;43:576-85.
46. Ramon y Cajal S. Degeneration and Regeneration of the Nervous System. London: Oxford University Press; 1928.
47. Park KI, Jensen FE, Stieg PE, Snyder EY. Hypoxic-

Ischemic (HI) brain injury may direct the proliferation, migration, and differentiation of endogenous neural progenitors [abstract]. Soc Neurosci 1998;24:1310.
48. Price J, Turner D, Cepko C. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci USA 1987;84:156-60.
49. Svendsen CN, Caldwell MA, Shen J, ter Borg MG, Rosser AE, Tyers P, et al. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. Exp Neurol 1997;148:135-46.
50. Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wante E, et al. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 1999;156:71-83.
51. Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Bjorklund A. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci 1999; 19:5990-6005.
52. Park KI, Snyder EY. Transplantation of human neural stem cells, propagated by either genetic or epigenetic means, into hypoxic-ischemic (HI) brain injury [abstract]. Soc Neurosci 1999;25:212.