■ REVIEW

Transplantation and Gene Therapy: Combined Approaches for Repair of Spinal Cord Injury

MARION MURRAY and ITZHAK FISCHER
Department of Neurobiology and Anatomy
MCP Hahnemann University
Philadelphia, Pennsylvania

Motor and sensory functions are lost after spinal cord injury because neurons die or atrophy and axons fail to regenerate. Until fairly recently, it was believed that damaged neurons could not be replaced and injured axons could not regenerate, and, therefore, functions dependent on injured neurons could not be recovered. We now know that damaged neurons can be rescued by providing therapeutic factors or replaced by grafting. In addition, the adult CNS contains a population of precursor cells with a potential to generate new neural cells, whose numbers and composition can be modified by extrinsic factors. The pioneering studies of Aguayo demonstrated that CNS axons could regenerate in the right environment. Subsequent studies have revealed the identity of some of the inhibitory molecules in myelin and scar tissue, and we now have a better understanding of how the CNS environment can be modified to become more permissive to regeneration. Axons that regenerate must find an appropriate target, but it may not be essential to reestablish the precise topography for some functions to be restored. There are now new and promising strategies for delivery of therapeutic genes to protect neurons and to stimulate regeneration. The ability to engineer cells by gene therapy combines the therapeutic values of cell transplantation and gene delivery. These remarkable developments from many disciplines have generated a new level of optimism in the search for a cure for CNS injury and in particular spinal cord injury. In this review, the authors summarize recent progress in these strategies and some of the challenges that remain in elucidating the most efficacious protocols for rescuing injured neurons, encouraging regeneration of their axons, and promoting recovery of function. NEUROSCIENTIST 7(1):28–41, 2001

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Peripheral Nerve Grafts and Fetal CNS Transplants

Early attempts to repair spinal cord centered on the use of peripheral nerve and fetal CNS grafts. The working hypothesis was that CNS axons fail to regenerate because the environment is inhibitory and therefore that providing a permissive environment for growing axons, such as Schwann cells or developing CNS tissue, would be conducive for regeneration of adult CNS axons. Both approaches showed promise by providing bridges into which CNS axons could regenerate, but neither was successful in eliciting long-distance regeneration in the adult CNS. Subsequent experiments using various methods to supplement transplanted peripheral nerve grafts or fetal tissue with growth factors convincingly showed that the limited axonal regeneration induced by grafts can be enhanced (Xu, Guenard, Kleitman, Aebischer, and Bunge 1995; Cheng and others 1996; Bregman and others 1997; Ye and Houle 1997). This addition of growth factors to transplants allowed the injured neurons to initiate a regeneration program. The supplemental factors were provided exogenously, which resulted in either a limited and perhaps inappropriate supply or additional invasive intervention with the danger of infection and mechanical damage. The introduction of gene transfer methods provides the alternative of grafting genetically modified cells that can act as biological minipumps to deliver these factors.

Both peripheral nerve and fetal tissue grafts have been associated with improvement of function. Multiple peripheral nerve grafts have been reported to improve function after spinal transection (Cheng and others 1996), but the complex surgical procedures combined with the application of additional factors have made this a difficult model to evaluate. Fetal tissue grafts have been used in neonatal animal models to promote functional spinal cord repair (Miya and others 1997; Diener and Bregman 1998), but the practical and ethical issues associated with the use of fetal tissue require that alternative sources be explored. Nevertheless, the first

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Address correspondence to: Marion Murray, Dept Neurobiology and Anatomy, MCP Hahnemann University, 2900 Queen Lane, Philadelphia, PA 19129 (e-mail: murray@drexel.edu).

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clinical trials are under way to examine the safety of grafting fetal spinal tissue into cysts of patients with progressive posttraumatic syringomyelia (Falci and others 1997; Reier and others 2000). These initial studies show that fetal tissue when grafted into a cyst will survive for at least several months and that the procedure so far appears to be safe.

Cell Transplantation

Transplantation of primary cells, cell lines, or genetically modified cells provides an exciting and clinically applicable strategy for spinal cord repair, which has clear advantages over either peripheral nerve or fetal tissue transplants. Cells to be transplanted into injured brain or spinal cord need ideally to have the following characteristics: They should be readily obtained, allow autologous grafting to avoid the need for immune suppression, be easily expanded and stored to produce a homogenous grafting source that can be quality controlled, permit genetic modification to introduce therapeutic products preferably by vectors that allow controllable transgene expression, be harmless to the recipient, survive and integrate when placed in the injury site, replace neurons and glia, rescue damaged neurons from cell death and atrophy, provide an environment permissive for axonal regeneration, and lead to functional recovery (Fischer 2000). Remarkably, a number of neural and nonneural cell types that exhibit some or most of these characteristics have already been used as sources for grafting into the CNS (Table 1).

Nonneural Cells

Fibroblasts, marrow stromal cells, and macrophages are examples of cells of nonneural lineage whose grafting properties have been examined in the spinal cord. These cells can be isolated from the host, genetically modified, expanded in vitro to provide sufficient stocks, and then introduced into the CNS. When used as autologous transplants, they do not require immune suppression. These grafted cells may deliver therapeutic products, provide bridges, and elicit regeneration from host axons. Because of their nonneural origin, they cannot serve as targets for regenerating axons in forming functional relays, nor can they myelinate those axons. Recent work, however, has shown the possibility of a neural potential in marrow stromal cells.

Fibroblasts

The most successful strategy for spinal cord repair so far has been the grafting of genetically modified fibroblasts. Fibroblasts can be harvested from the host skin, genetically modified with recombinant retroviral vectors, expanded, and banked. When grafted into a site of injury, they survive well without forming tumors and establish a cellular bridge around and through which axons can grow. When fibroblasts modified to produce trophic factors have been transplanted into a spinal cord injury site, they have 1) promoted regeneration, 2) rescued axotomized neurons, and 3) permitted recovery of function (Liu, Murray, Tessler, Fischer 2000).

Regeneration. Liu, Kim, and others (1999) have shown that fibroblasts engineered to produce BDNF and transplanted into the site of a lesion at the C3/4 spinal level will fill the lesion cavity, forming a continuous interface with no evidence of cyst or scar formation, and thus provide a bridge through which regenerating axons can grow. The BDNF/fibroblasts elicit axonal growth from several systems including dorsal roots and raphe-spinal axons, as well as regeneration from identified rubrospinal axons. Anterogradely labeled rubrospinal axons regenerate through and adjacent to the graft, extend distal to the transplant site in the lateral funiculus for up to 3 to 4 cm (10 segments) (Fig. 1*a*), and terminate in appropriate laminae of the spinal gray matter (Fig. 1*b*). The robust regeneration of Red Nucleus neurons suggests that BDNF provided by the modified fibroblasts has improved the intrinsic growth properties of these neurons. The ability of some of the regenerating axons to grow through the graft indicates that the production of BDNF makes the fibroblast environment permissive. Guidance of regenerating axons through the CNS white matter to appropriate targets has been thought to require neutralization of inhibitory molecules and/or re-expression of the molecular cues that are present during development. The successful regeneration of the rubrospinal axons indicates that these axons can overcome the inhibitory signals present in white matter and may recognize the location of the original tract. Retrograde labeling by fluorogold injections several segments caudal to the BDNF-producing graft indicated that about 7% of rubrospinal neurons regenerate axons that far caudally, a sevenfold increase relative to unmodified fibroblasts (Fig. 1*c*-1*f*). Preliminary grafting experiments of BDNF-producing fibroblasts into chronically injured spinal cord have shown that regenerating rubrospinal and reticulospinal axons grew in a relatively straight course through the transplant (3–4 mm), with a few fibers reaching the caudal graft-host interface (Jin and others 1999). The extent of regeneration is less than after acute grafting of BDNF/fibroblasts, but far greater than that seen after acute transplants of fetal tissue.

Fibroblasts modified to produce NT3 placed into a bilateral dorsal hemisection site, destroying corticospinal, rubrospinal, and coerulospinal tracts, will elicit regeneration adjacent and caudal to the graft (Grill, Murai, and others 1997). The extent of regeneration of the corticospinal axons was limited to 8 mm, and the axons did not grow within white matter or into the graft but were confined to gray matter. Grill, Blesch, and others (1997) also have used the same model to study the effects of implanting fibroblasts modified to produce nerve growth factor (NGF). When the graft was placed into a chronic injury site, axons from coerulospinal and primary sensory axons entered the graft, but corticospinal, raphespinal, and motor axons

Table 1.

		Tissue		Cells						
				Nonneural		Nonneuronal			Neuronal	
		Fetal CNS	Peripheral Nerve	Fibroblasts	Marrow Stromal Cells	Macrophages	Schwann Cells	Olfactory Ensheathing Cells	Immortalized Neurons	Neuronal Precursors
Properties	Readily obtained	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Autologous	No	Yes	Yes	Yes	Yes	Yes	No	No	No
	Can be expanded	No	No	Yes	Yes	Yes	Yes	?	Yes	Yes
	Genetically modified	No	No	Yes	Yes	?	Yes	?	Yes	Yes
	Neural phenotype*	Yes*	Yes	No	Yes	No	Yes	Yes	Yes*	Yes*
Effects	Rescue neurons	Yes	?	Yes*	?	?	?	?	Yes	?
	Regeneration/ sprouting	Yes*	Yes*	Yes*	Yes	Yes	Yes*	Yes*	?	Yes
	Recovery of function	Yes	Yes	Yes	?	Yes	Yes	Yes	?	?

? = not known. Neural phenotype: *includes neuronal phenotype; Regeneration/sprouting: *demonstrated regeneration.

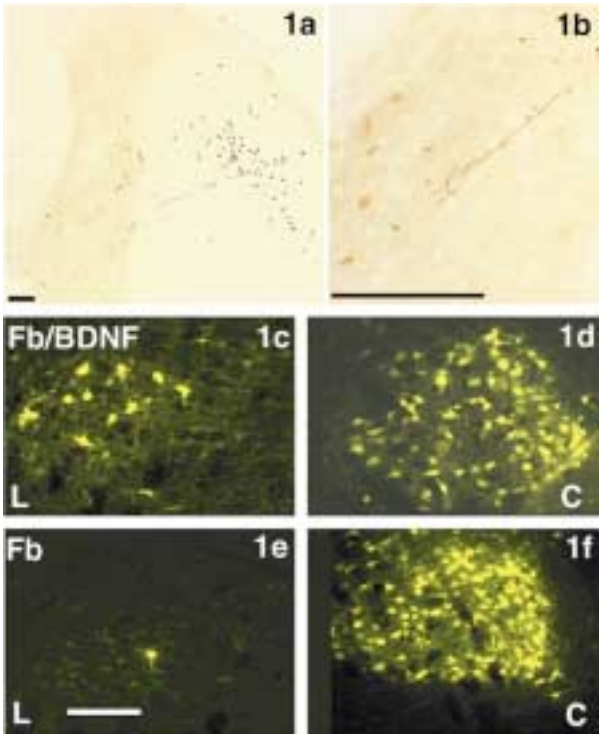

Fig. 1. Photomicrographs demonstrating regeneration of rubrospinal axons following cervical lateral funiculus lesions and transplantation of BDNF-producing fibroblasts. 1*a,* 1*b*, Cross sections of BDA-labeled rubrospinal axons in thoracic spinal cord from an animal with an Fb/BDNF transplant, 1 month postoperatively. BDA-labeled axons are present in the lateral funiculus (1*a*), although their location is more diffuse than normal. A few transversely sectioned BDA-labeled axons are also present in the gray matter entering in lamina VI-VII. 1*b* shows an axon in the gray matter with varicosities resembling terminal boutons. Scale bars: 100 μm. 1*c-f,* Photomicrographs of midbrain showing retrograde tracing of Red Nucleus neurons. Neurons were retrogradely labeled by bilateral injection of fluorogold (FG) into the spinal cord of animals grafted with BDNF-producing fibroblasts (Fb/BDNF) (1*c,* 1*d*) or unmodified fibroblasts (Fb) (1*e,* 1*f*). Animals survived for 1 month after the grafting, and the FG was injected 3 days before sacrifice. All sections were taken from the magnocellular Red Nucleus. Control (C) side (1*d,* 1*f*) and lesioned (L) sides (1*c,* 1*e*) are shown. In recipients of BDNF-producing fibroblast transplants, numerous neurons were labeled in the Red Nucleus (1*c*) contralateral to the lesion/graft, but in recipients of unmodified fibroblasts, very few neurons were labeled (1*e*). Labeling was similar in the Red Nucleus on the control sides (1*d,* 1*f*). Scale bar: 100 μm.

did not. Additional experiments grafting NT3- and BDNF-producing fibroblasts showed significantly more myelin-basic protein-positive profiles, suggesting enhanced myelination of ingrowing axons within these neurotrophin-producing grafts. BrdU labeling of dividing cells indicated that the augmented myelinogenesis was associated with increased proliferation of oligodendrocyte lineage cells (McTigue and others 1998).

The results of these experiments suggest that CNS pathways may differ intrinsically in their ability to regenerate or that they have different requirements for maximum regeneration. The protocols that will elicit the most robust regeneration from each pathway remain to be determined.

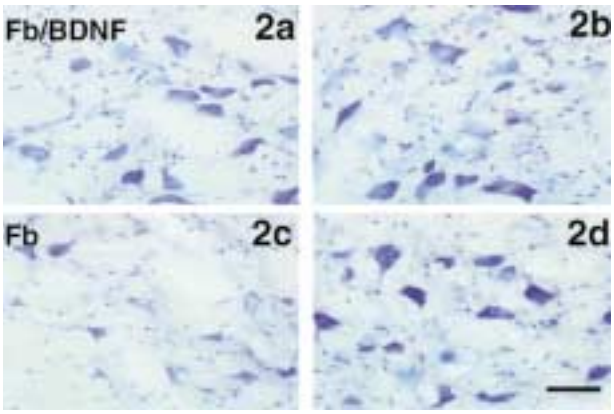

Fig. 2. Photomicrographs of cresyl violet-stained sections through the magnocellular Red Nucleus 2 months following unilateral cervical lateral funiculus injury and transplantation of BDNF-secreting fibroblasts (2*a, 2b*) or unmodified fibroblasts (2*c, 2d*). Note large neurons on control sides (2*b, 2d*) and contralateral to the BDNF-fibroblast transplant (2*a*). Fewer neurons are recognizable contralateral to the transplant of unmodified fibroblasts (2*c*), and no large neurons are present.

Rescue. The grafting of fibroblasts modified to secrete BDNF also has rescuing effects on injured neurons that die or atrophy following axotomy (Liu, Himes, Murray, Tessler, and Fischer submitted). After cervical hemisection, about 40% of neurons in the magnocellular division of the Red Nucleus undergo retrograde cell death or atrophy below the level of detection (Mori and others 1997). These cells can be rescued to varying degrees by several interventions, including grafting of fetal tissue (Mori and others 1997). Grafting of BDNF-producing fibroblasts rescued the injured Red Nucleus cells more efficiently than fetal transplants (see Figure 8). Furthermore, many of the cells that are rescued retain their normal size and morphological appearance in contrast to the cells rescued by fetal transplants, which survive in an atrophic state (Fig. 2). We do not know at present how functional these rescued neurons are, but it appears that all of the regenerating neurons labeled by fluorogold retain a morphologically normal state (Liu, Himes, Murray, Tessler, and Fischer submitted). It is therefore reasonable to suggest that these rescued neurons can also participate in surviving circuits.

Recovery of function. Rats that receive partial cervical hemisections show excellent spontaneous recovery of motor functions. More sensitive tests of forelimb usage (the cylinder test) and of locomotion in a posture-challenging task (the rope test, Fig. 3) identify persisting deficits, but rats receiving transplants of BDNF-producing fibroblasts show partial recovery of both fore- and hindlimb function (Kim and others 1999; Kim, Schallert, Liu, Browarak, Nayeri, Tessler, Fischer, and Murray submitted). This recovery is abolished after a second lesion just rostral to the ini-

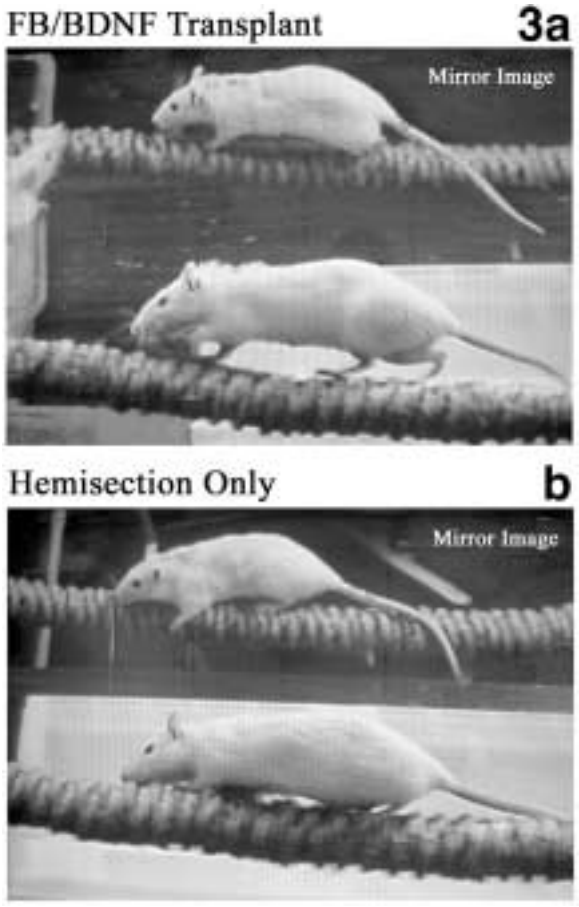

Fig. 3. Photographs of rats 1 month after partial cervical hemisection performing on the rope test. 3*a*, A rat that received a transplant of BDNF-secreting fibroblasts. Note support of hindquarters with good trunk elevation and placement of paws under the body. 3*b*, A rat that received a hemisection only. Note poor weight support and posture.

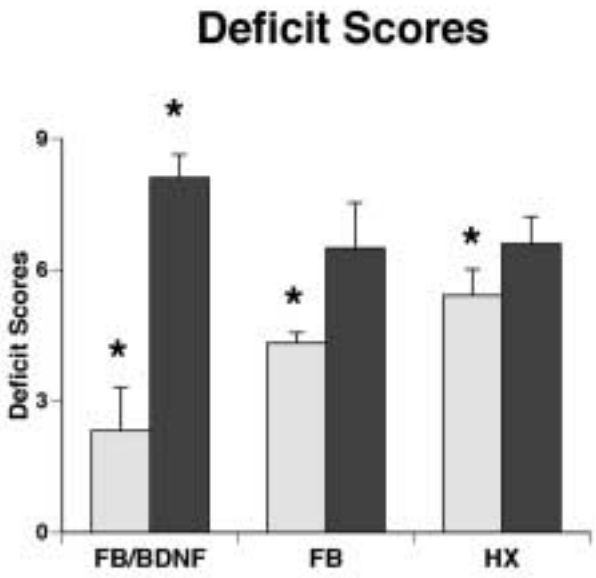

Fig. 4. Deficit scores on the rope test. The Fb/BDNF recipients showed significantly fewer deficits (more recovery) than the group that received unmodified fibroblasts, which in turn had fewer deficits than the partially hemisected group. Five weeks following a second lesion (a partial hemisection just rostral to the original lesion/transplant), the deficit scores for the Fb/BDNF group were significantly increased. The deficit scores for the FB and HX groups after the second lesion are not significantly different from their scores during the recovery period from the initial lesion/transplant. Thus the BDNF secreting transplant improved performance, and this effect was abolished by a hemisection lesion just rostral to the transplant. Light bars represent scores during recovery from the first lesion/transplant surgery, and dark bars represent scores following the second lesion.

tial transplant site, indicating that some of the recovery is correlated with the presence of the graft. In contrast, rats receiving transplants of unmodified fibroblasts show the similar deficits in forelimb and hindlimb as hemisected animals (Fig. 4). Grafts into dorsal hemisections of fibroblasts engineered to produce NT3 also promoted functional recovery (Grill, Murai, Blesch, and others 1997). Animals receiving the NT3/fibroblast grafts performed better on an integrative sensorimotor task, grid walking, than did those receiving unmodified grafts. These studies also showed that lasting functional deficits required injury to several descending motor systems, not only to the corticospinal tract.

The studies with transplants of genetically modified fibroblasts provide the most complete experimental demonstration that grafting genetically modified cells elicits regeneration of adult CNS axons that is associated with functional recovery. That axonal growth could be elicited even after delayed grafting is of obvious clinical significance for potential application to chronic injury. The challenge of improving this strategy is to develop methods to control the time and levels of transgene expression and to examine the efficacy of combination gene therapy.

Marrow Stromal Cells

Bone marrow contains, in addition to hematopoietic cells, stem-like cells for nonhematopoietic tissue that are referred to as mesenchymal stem cells or marrow stromal cells (MSCs) (Prockop 1997). MSCs share some of the favorable features for transplantation that fibroblasts offer. In addition, they have been approved for phase 1 clinical trials intended to explore the application of MSCs as a vehicle for gene therapy by autologous engraftment (Keating and others 1998). Bone marrow is harvested from a patient's iliac crest by a minimally invasive aspiration, the protocols for isolation of MSCs are relatively simple, and the cells can be cultured and stored (Pittinger and others 1999). The isolated stromal cells differentiate into cells with a variety of phenotypes. The cells can then be grown and genetically modified with recombinant adenovirus or retrovirus vectors in culture and reintroduced into the patient. In recent studies, human MSCs grafted into the rat brain without immune suppression survived for sev-

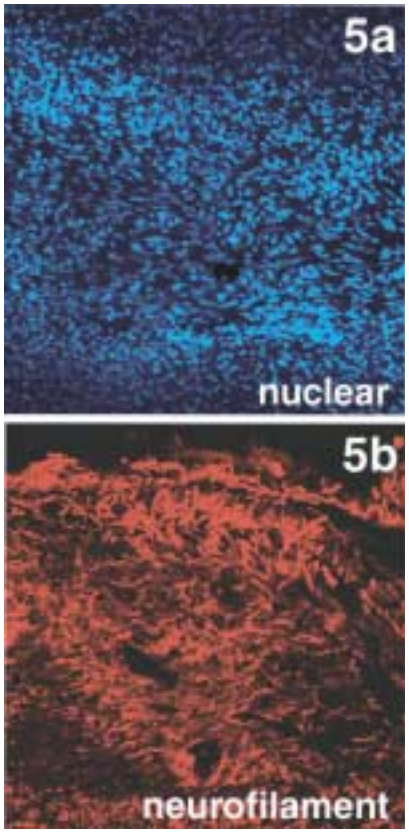

Fig. 5. 5*a*, Human marrow stromal cells labeled with the nuclear bis-benzimide dye and grafted into the hemisection cavity of the rat spinal cord at C4 level, 2 months postoperatively. 5*b*, Neurofilament-positive axons, stained with RT97 antibody, have grown throughout the graft in areas containing stromal marrow cells, shown in 5*a*.

eral months with no evidence of an inflammatory response or rejection (Azizi and others 1998). Murine MSCs injected into the neonatal mouse brain migrated throughout the forebrain and cerebellum, and some appeared to have differentiated into astrocytes (Kopen and others 1999). Grafting of MSCs transduced to produce L-dihydroxyphenylalanine (L-DOPA) in a rat model of Parkinson's disease has demonstrated the potential of these cells in treating degenerative brain disorders (Schwartz and others 1999). Preliminary experiments with MSCs grafted into spinal cord lesion sites (Fig. 5 *a*,*b*) have shown that transplants survive, integrate well, and appear to be permissive for axonal growth even without genetic modification (Himes and others 1999). These properties make MSCs a clinically attractive candidate for use in delivering therapeutic genes to injured spinal cord, providing a permissive environment for axon regeneration and the intriguing possibility that they may produce neural phenotypes (Moore 1999; Woodbury, Schwartz, Prockop, and Black 2000).

Macrophages

Activated macrophages contribute to successful regeneration in the peripheral nervous system because they remove degenerating debris and secrete factors that provide a permissive environment for regenerating axons. Although the inflammatory response to CNS injury is frequently considered to be detrimental to recovery, some elements in this complex cascade may favor regeneration. Transplantation of activated peripheral macrophages into injured CNS has recently been used to induce regeneration by CNS axons. Monocytes isolated from peripheral blood and activated by exposure to excised sciatic nerve segments were implanted into spinal transection (Rapalino and others 1998) or optic nerve transection (Lazarov-Spiegler and others 1998) sites. The macrophages, without being genetically modified, stimulated regeneration of spinal and optic axons. Improvement in hindlimb function after transplantation of activated macrophages into the spinal transection site was also reported (Rapalino and others 1998). As a result, activated macrophages have recently been approved for a phase 1 clinical trial in Israel. Recent evidence confirms a role for macrophages in stimulation of regeneration in optic axons (Yin, Leon, Hu, Irwin, and Benowit 2000).

Nonneuronal Cells

Whereas the studies with fibroblasts, marrow stromal cells, and macrophages are clearly encouraging, cells of neural lineage are obvious candidates for modification and grafting into CNS. Studies of Schwann cells have focused on their positive features; they support peripheral nerve regeneration and when transplanted into the CNS support regeneration of CNS axons. Studies of glial cells have focused on their inhibitory characteristics, astrocytes because of their contribution to the glial scar, and oligodendrocytes because of the inhibitory activities associated with CNS myelin. Glial cells are, however, normally closely associated with neurons and their processes where they act as supporting cells. The challenge is to restore or enhance the supporting properties of glial cells in a way that will lead to promising therapeutic grafting strategies. Important elements of this strategy have been the development of glial cell lines with permissive properties and the isolation and characterization of another type of supporting cell, the olfactory ensheathing glial cells. Schwann or glial cells grafted into an injury site can provide permissive bridges for axon regeneration and eliminate cyst formation that commonly accompanies spinal injury. They also have the potential to provide a more physiological environment for axons than do nonneural cells and to myelinate regenerating axons. When suitably modified, they can express and secrete therapeutic factors needed for repair. Some of these cells can be grown in the large quantities necessary for transplantation. These cells, however, cannot provide a target for synaptogenesis or replace neurons lost by the injury.

Schwann Cells

Schwann cells support axonal growth and are major contributors to successful regeneration in the peripheral nervous system. Upon injury, Schwann cells phagocytose the degenerated axons, up-regulate neurotrophin expression, and provide a pathway for regenerating axons.

They also remyelinate the regenerated axons. Methods are available now for generating large numbers of Schwann cells (Plant and others 2000). The possibility of autologous transplants derived from peripheral nerve biopsies makes them an excellent candidate for clinical application. The success of peripheral nerve grafts in promoting regeneration of CNS axons led to transplanting Schwann cells to repair spinal injury. Significant numbers of CNS axons regenerate into guidance channels seeded with Schwann cells and grafted into a midthoracic transection site (Xu, Guenard, Kleitman, and Bunge 1995), and even more axons do so when the neurotrophic factors BDNF and NT3 are added to the channels (Xu, Guenard, Kleitman, Aebischer, and Bunge 1995). When Schwann cells modified to produce BDNF were grafted into and caudal to a transection site, sensory, propriospinal, and brainstem axons regenerated toward the grafts (Menei and others 1998). Grafting of Schwann cells modified to produce NGF into uninjured (Tuszynski and others 1998) or injured (Weidner and others 1999) spinal cord stimulated the robust growth of primary nociceptive axons from the dorsolateral fasciculus and a slower growth of coerulospinal axons. The additional advantage of Schwann cell transplants is that they can myelinate newly formed axons (Keirstead and others 1999). A disadvantage may be the poor integration of peripheral Schwann cells within the CNS environment. The most promising use of Schwann cells appears to be in providing a permissive and supporting environment for regenerating axons, in combination with other interventions that permit long-distance regeneration into the host.

Astrocytes

The ability of astrocytes to support axonal growth depends on their stage of differentiation. During development, neurons migrate along the processes of radial glial cells; immature astrocytes are thus permissive for axonal elongation. In the adult, however, astrocytes form scars in response to injury that impede axonal elongation. Astrocyte cell lines with permissive and nonpermissive properties have been used to study the potential of astrocytes to support axonal regeneration (Fawcett and Asher 1999). An interesting line of astrocytes derived from C6 glioma cells, C6-R cells, has properties that resemble radial glial cells by providing a substrate for migration of grafted neurons and guidance of regenerating axons. C6-R cells injected into the brain develop a radial orientation (Friedlander and others 1998). In the injured spinal cord, the C6-R cells became oriented along the rostral-caudal axis in the white matter and growing axons appeared to track along the C6-R cell processes (Fig. 6) (Hormigo and others 2001). Powell and others (1997) studied two other cell lines, Neu7 and A7, which are, respectively, inhibitory and permissive to neurite outgrowth and can be used to define astrocytic boundaries. The grafting of the permissive A7 cell line has the potential for providing a terrain that will guide regenerating axons (unpublished results). Because these cell lines, particularly the C6-R line, may have a potential to form tumors, their application may be limited to studying the role of astrocytes and the extracellular matrix that they produce on axonal growth.

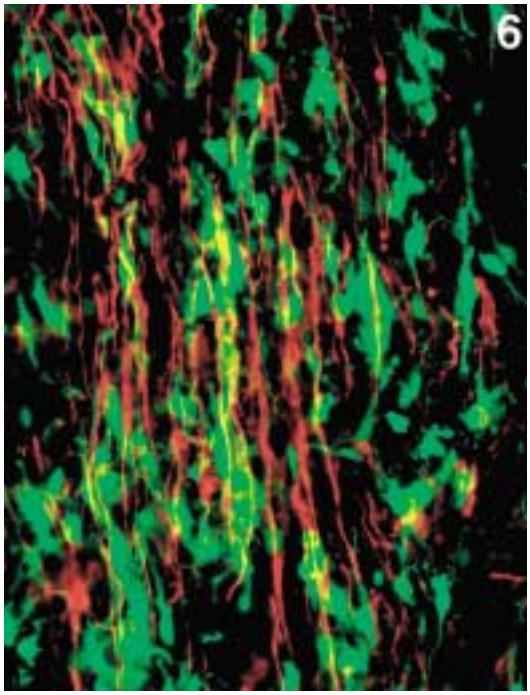

Fig. 6. Green fluorescent protein-labeled C6-R cells transplanted into spinal cord hemisection site survive, integrate, and develop a rostro-caudal orientation. TuJ1-labeled axons (red) grow into the graft in alignment with the C6-R cell processes (green) within the white matter.

Olfactory Ensheathing Cells

Both normal and transected olfactory axons have the unusual property of being able to grow into adult CNS, find their appropriate targets, and form synaptic contacts with them (Ramon-Cueto and Avila 1998). A critical element in the ability of olfactory axons to regenerate and to form specific connections is the presence of a unique supporting cell, the olfactory ensheathing cell (OEC). Together with astrocytes, oligodendrocytes, and microglia, OECs form the cellular environment of the olfactory axons. The olfactory axons are ensheathed by the OECs, which isolate them from the hostile environment of the adult CNS and thus permit axonal elongation within the CNS. OECs are also a source of trophic factors and extracellular matrix molecules, and they express adhesion molecules. These cells can also myelinate axons. In one injury model, OECs were injected into dorsal columns demyelinated by X-irradiation and ethidium bromide administration. The demyelinated axons were remyelinated, and functional studies indicated that the conduction block had been overcome (Imaizumi and others 1998). When OECs were grafted into the injured CNS, they encouraged regeneration of spinal axons. OECs injected into small lesions in the corticospinal pathway elicited regeneration of these axons into the distal spinal cord ac-

companied by ensheathing cells that migrated from the lesion site (Li and others 1998). They intermingle with reactive glia and therefore may contribute to the suppression of the inhibitory glial scar. OECs injected into the rostral and caudal stumps of transected spinal cords that had been joined by Schwann cell–filled guidance channels induced regeneration by serotonergic, propriospinal, and presumably other axons, which elongated in both gray and white matter for distances of up to 3 cm (Plant, Ramon-Cuerto, and Bunge 2000). OECs thus provide an environment similar to that provided by Schwann cells but one that is more adapted to integration and regeneration in the adult CNS. These cells permit long-distance regeneration and remyelination of the regenerated axons even in the absence of genetic modification. The methodology to produce and store large numbers of these cells is currently being developed (Plant and others 2000).

Neuronal Cells

The obvious advantage of grafting neuronal cells or cells of a neuronal lineage is that these cells can be used not only for gene delivery and formation of a permissive environment but also for replacing neurons lost by the injury. Neuronal cells can serve as targets for regenerating axons, which could contribute to the development of novel relay circuits, although we do not know how effectively these relays can be integrated into CNS circuits. Two kinds of neuronal cells have been used for transplantation into CNS: immortalized cell lines and primary progenitor cells. In particular, multipotential neural progenitor cells have emerged as the focus of one of the most promising therapeutic strategies for the repair of injured CNS (Ray and others 1999). As the understanding of the basic biology of these cells increases and the methods for isolating and manipulating them improves, the prospect of using neural progenitor cells for clinical application becomes more attractive.

Immortalized Cell Lines

Immortalized neural progenitor or stem-like cell lines are prepared by introducing oncogenes that maintain the cells in an undifferentiated state (Vescovi and Snyder 1999). Examples of such cells lines that have been used for grafting into CNS include C17.2 cells prepared from postnatal mouse cerebellum and RN33B cells prepared from embryonic rat raphe. When grafted into the adult CNS, these cells differentiate into neurons and glial cells, and when modified to express therapeutic genes, they can ameliorate a variety of experimental brain disorders and lesions. For example, C17.2 cells were used to correct the murine model of the lysosomal storage disease mucopolysaccharidosis type VII through the expression of endogenous β-glucoronidase (Vescovi and Snyder 1999). When subclones of C17.2, genetically modified to express NT3, were implanted into an ischemic mouse brain, there was a dramatic increase in their differentiation into neurons relative to unmodified cells (Park and others 1997). These results represent the first example of combination gene therapy and cell replacement where the transgene can be used both as a therapeutic factor and to influence the phenotypic fate of the grafted progenitors. When the same NT3-producing progenitors were grafted into a thoracic lesion in the spinal cord, they rescued axotomized Clarke's nucleus neurons (Himes, Liu, Solowska, Snyder, Fischer, and Tessler submitted). RN33B cells are particularly interesting because they acquire region-specific morphological properties when grafted into the cortex, hippocampus, and striatum of the neonatal and adult brain, suggesting the ability of progenitor cells to differentiate correctly even in the adult CNS (Shihabuddin and others 1995).

Studies of cell lines of human neural progenitors (Flax and others 1998; Vescovi and Snyder 1999) are important as they demonstrate that the basic principles elucidated in rodent models are also applicable for human cells. They also provide a clinically applicable alternative to fetal tissue. Neuronal cells prepared from the human NT2N embryonic carcinoma cell line that were grafted into the spinal cord showed differential axonal outgrowth (Hartley and others 1999), suggesting that these grafted human cells also respond to cues present in the adult CNS. Promising results have also been obtained from grafting neural differentiated mouse embryonic stem cells (ESs) into an impact injury model of spinal cord (McDonald and others 1999). The cells differentiated into astrocytes, oligodendrocytes, and neurons; migrated away from the lesion site; and some of the recipients showed some recovery of motor function.

Primary Progenitor Cells

Multipotential neural progenitor cells have been discovered not only in the developing CNS but also in the adult (Kuhn and Svendsen 1999). Differentiation of neural cells in the spinal cord is a process of sequential restriction in developmental potential starting with multipotential stem cells that generate lineage-restricted neuronal and glial progenitors (Rao 1999). Stem cell transplantation can be applied to CNS disorders and injury, with the choice of cells determined by the specific needs of the therapy. For example, ESs and multipotential neural progenitors can be used when it is necessary to provide multiple cell types following injury, whereas neuronal-restricted progenitors are favored to replace lost neurons in disorders such as Alzheimer's and Parkinson's.

Stem cell neurospheres isolated from the embryonic rat spinal cord in the presence of EGF and FGF remain proliferative in culture with a potential to differentiate into astrocytes, oligodendrocytes, and neurons (Chow and others 2000). Their differentiation can be directed toward neuronal phenotypes by exposure to retinoic acid (Fig. 7). Preliminary experiments show that when these multipotential stem cells are implanted into an injured spinal cord, they survive and differentiate into neurons and glial cells and, when combined with neurotrophins, show remarkable recovery of function

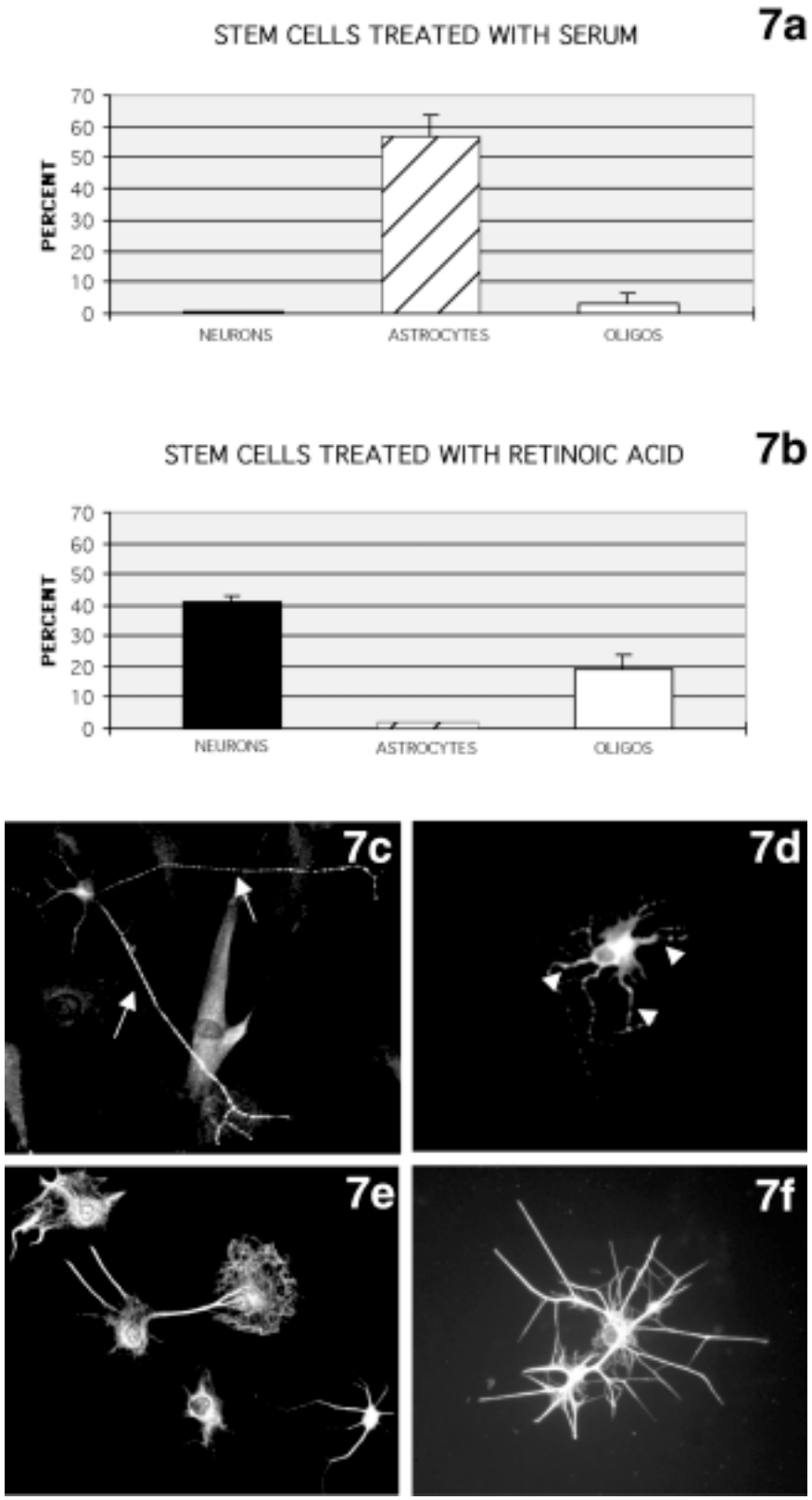

Fig. 7. Stem cells from E14 rat spinal cord were prepared as neurosphere in a medium containing EGF and bFGF. The cells were then dissociated and cultured without these growth factors but in the presence of 10% fetal calf serum (7*a*), or 10_6 M retinoic acid (7*b*) for 4 days, then fixed and immunostained for identification of neurons and glial cells. In the presence of serum, > 50% of the cells become astrocytes, with only few detectable neurons, whereas with retinoic acid, > 40% of the cells become neurons. 7*c-f*, Representative photomicrographs of neuronal and glial phenotypes derived from E14 rat spinal cord stem cells. The neuronal phenotype was identified using antibodies against MAP1B showing the presence of long axons (7*c*) and MAP2 showing several short dendrites (7d). Astrocytes were identified with an antibody against GFAP and showed cells with flat (7*e*) and stellate (7*f*) morphologies.

(Chow and others 1997). Another class of stem cells identified in the embryonic spinal cord is FGF-dependent (Kalyani and others 1997). These multipotential neuroepithelial stem cells are present relatively early in development and contribute to neurogenesis. Experiments presently under way should indicate the extent to which modified stem cells are capable of spinal cord repair.

Vectors for Gene Delivery

Gene therapy methods can have a broad range of application in the CNS that include direct injection of the recombinant vectors into the brain or spinal cord (in vivo) and gene transfer into cultured cells that are then transplanted (ex vivo). Viral vectors are most often used for gene delivery in that the viruses can efficiently infect target cells and use the host machinery to express the transgene. Viral vectors are modified viruses, constructed to become replication deficient and to contain the gene of interest, flanked by a promoter. Differences among viral vectors include their genetic material, maximum transgene size, host range of infection, virus titer, and the capacity for chromosomal integration. Viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, and lentivirus vectors. Nonviral delivery systems include delivery by plasmids, gene guns, receptor-mediated endocytosis, and antisense oligonucleotides. There has been rapid progress in developing more and better vectors (Tuszynski 1998; Fischer and Liu 2000). Here we will discuss only those that have been used in studies on spinal cord injury.

Retrovirus Vectors

Retrovirus vectors have been used to immortalize various neural cell lines with oncogenes and to introduce therapeutic genes such as NGF, NT3, BDNF, and LIF into cells for application of ex vivo gene therapy in spinal cord repair. Retrovirus integrates efficiently into the host genome and can provide long-term transgene expression, but the infection by retrovirus requires proliferative cells, and the random integration of the virus into the host DNA carries some risk of insertional mutagenesis. These properties make the retrovirus vector an excellent choice for genetic modification of mitotic cells such as fibroblasts, Schwann cells, progenitor cells, and a variety of cell lines but limit its in vivo application.

Adenovirus Vectors

Adenovirus vectors can be prepared in high titers and provide efficient gene transfer into postmitotic cells with a broad range of infectivity. Consequently, these vectors can be used for both in vivo and ex vivo gene therapy. When injected into the CNS, adenovirus is incorporated by adult neurons and glial cells and can be retrogradely transported from terminals or damaged axons to the cell body (Liu and others 1997). At high titers, adenovirus may be cytotoxic and will elicit an immune response so that experiments using this approach require careful adjustment of titer or immune suppression. Nevertheless, adenovirus has been used to introduce therapeutic genes, for example, TH, SOD, GDNF, or NT3, in animal models of neurodegenerative diseases such as Parkinson's, Alzheimer's, and motor neuron diseases (Barkats and others 1998). Adenovirus has also been used to genetically modify cells that have been used for intraspinal grafting (Liu and others 1998), making them an efficient and convenient alternative for ex vivo gene therapy in spinal cord injury. Development of new, less immunogenic viral vectors will make this a more attractive approach.

Plasmid Vectors

Genetic material can also be administered directly by injection of relatively simple plasmids that encode genes of interest. Plasmid delivery may be less efficient than delivery by viral vectors. The advantage of this method, however, is that it is less invasive than introduction of a cellular transplant and less controversial than infection of virus and therefore may have value as a clinical treatment, particularly in the subacute period. Plasmid delivery has been shown to be effective in the transfer of the anti-apoptotic Bcl-2 gene into injured spinal cord, where it was retrogradely transferred to the cell body of damaged neurons and facilitated their rescue from cell death and atrophy (Fig. 2) (Takahashi and others 1999; Saavedra and others 2000).

Gene transfer methods need to satisfy stringent safety protocols. The most critical aspects for future application of these methods to the repair of spinal cord include the ability to target and regulate the expression of the therapeutic transgene by using cell-specific promoters and regulatory elements that can turn the expression on and off.

Candidate Genes

The new and powerful methods of gene analysis by differential display and expression arrays have catalogued a large number of genes whose expression is modified after axotomy. The identity of many of these genes remains unknown, and the genetic program that is required for successful regeneration is also unknown. We do know that expression of some genes is closely associated with axonal growth and that some genes that are expressed during development are reinduced during peripheral nerve regeneration. These observations suggest that there is a partial recapitulation of the developmental program and that the expression of regeneration associated genes may be necessary, even if not sufficient, for regeneration (Nunez and Fischer 1999; Steeves and Tetzlaff 1999). Candidates for genes that improve the

intrinsic ability of CNS to regenerate include growth-associated genes such as GAP-43, whose expression is high in all instances where regeneration occurs; transcription factors such as c-jun that can regulate coordinated expression of regeneration associated genes; cytoskeletal proteins such as Tα1 tubulin and MAP1B that determine the dynamic properties of microtubules in growing axons; and adhesion molecules such as L1 and NCAM that regulate fasciculation of growing axons and modulate the environment through which axons grow. Other targets are therapeutic genes whose products can promote cell survival and regeneration. Candidates include growth factors such as neurotrophins and cytokines that can support both survival and regeneration of injured neurons; antiapoptotic and antioxidant agents that may rescue neurons from cell death and provide protection from free radicals in the toxic environment created by the injury; and matrix molecules such as laminin and selective domains of tenacin-C (Meiners and others 1999) that can improve the permissivity of the environment for regeneration. At present, experiments in which gene therapy has been shown to improve survival and regeneration have focused on two types of genes, those encoding growth factors and those encoding antiapoptotic genes.

Growth Factors

A large number of studies have reported that growth factors and cytokines can stimulate regeneration in the adult CNS. Specifically, regeneration of all classes of spinal-projecting neurons have been shown to regenerate in response to administration of neurotrophins, cytokines, and fibroblast growth factors (Tuszynski 1998; Murray 2000). We do not yet know the specific requirements for each system with respect to amount, duration, and combinations of factors that need to be administered.

Antiapoptotic Genes

Cells deprived of trophic support may undergo apoptotic cell death. This is thought to be the cause of retrograde cell death that is seen following axotomy of some central neurons. Apoptosis can be blocked by providing adequate supplementation with growth factors. Thus, supply of NT3 will rescue Clarke's nucleus and BDNF will rescue Red Nucleus neurons (Himes and Tessler 2000). The introduction of an antiapoptotic gene acts downstream of the specific growth factor requirement and therefore could have a more general protective effect. Single injections of plasmids encoding BCl2 injected adjacent to the site of a hemisection lesion in rats rescued both Clarke's (Takahashi and others 1999) and Red Nucleus neurons (Himes and Tessler 2000) with reduced atrophy (Fig. 8).

Outcome Measures

Validation of transplantation and gene therapy strategies requires use of appropriate outcome measures. These

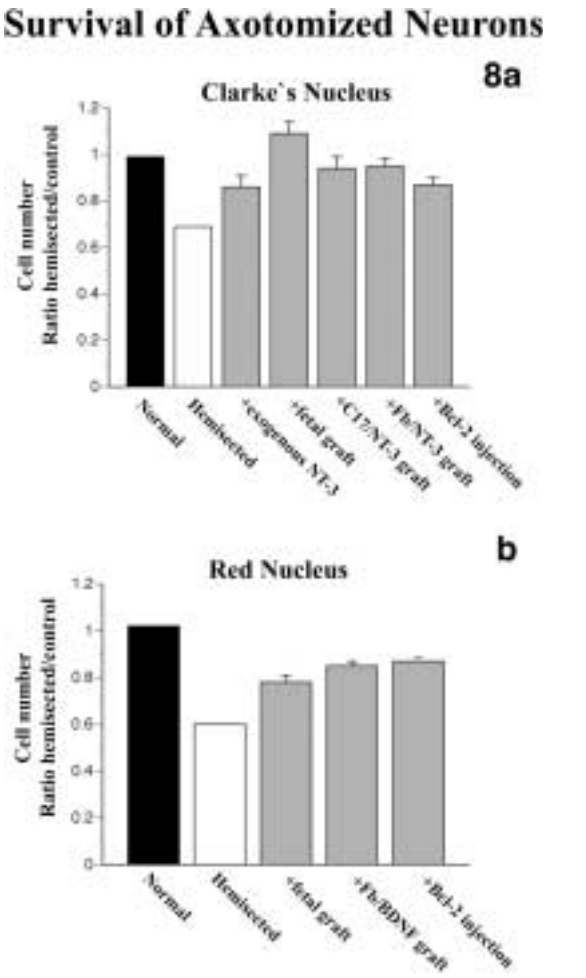

Fig. 8. Summary of studies of survival of axotomized (*a*) Clarke's and Red (*b*) Nucleus neurons after hemisection and various interventions (Murray 2000; Saavedra and others 2000). About 30% of Clarke's and 40% of Red Nucleus neurons can no longer be identified 2 months posthemisection. Those that survive are atrophic, and few or no large neurons are found. All of the listed interventions significantly increase the number of cells that survive hemisection. In addition, BCl-2 injection rescues large neurons in both nuclei, and BDNF-Fb rescues large neurons in the Red Nucleus.

measures need to address the beneficial effects for damaged neurons with respect to retrograde cell death and atrophy, unambiguous determination of regeneration and evaluation of sprouting, and appropriate tests for motor and sensory recovery of function. Unfortunately, because of the wide variety and complexity of the outcome measures, it is sometimes difficult to reconcile reports from different laboratories and thus to compare efficacy of different repair strategies.

Survival

Following spinal cord injury, axotomized neurons undergo retrograde cell death and/or atrophy. Although it is agreed that there is considerable cell death after injury in neonates, there is controversy as to whether, in the adult, axotomized Red Nucleus neurons die or

shrink beyond the level of detection. The controversy exists primarily because of preliminary data reporting that the direct application of high doses of BDNF to the Red Nucleus several months following spinal cord injury prevented cell loss and atrophy in that nucleus (Kobayashi and others 1995). It is clearly important to resolve this issue with more experimental data that include unambiguous counting and identification of neurons by specific antibodies and to determine whether neurons that can no longer be recognized histologically remain viable and potentially salvageable. Nevertheless, axotomy of adult neurons in some CNS regions results in severe retrograde changes that are likely to be incompatible with normal function. Retrograde cell loss is usually estimated by cell counting of histologically stained sections using unbiased stereological methods. There is great variability in the extent to which populations of neurons respond to axotomy. For example, 80% of retinal ganglion cells are lost after optic nerve section (Fernandes and Tetzlaff 2000). In contrast, very few corticospinal neurons are lost after corticospinal lesions unless the injury is very close to the cell bodies. Cervical interruption of vestibulospinal axons does not result in detectable cell loss (Ye and Houle 1997), but after axotomy of the rubrospinal tract in the cervical cord, 40% of Red Nucleus neurons are lost. Thirty percent of Clarke's neurons disappear after midthoracic hemisection. Neurons destined to be lost after axotomy may be rescued by appropriate and early intervention by fetal tissue grafts, grafts of cells modified to produce trophic factors (Liu, Himes, and others submitted), direct administration of trophic factors and cytokines (Ye and Houle 1997; Shibayama and others 1998; Himes and Tessler 2000), and administration of plasmids coding for antiapoptotic molecules (Takahashi and others 1999; Saavedra and others in press; Fig. 8). Red Nucleus neurons that are rescued by fibroblasts that secrete BDNF are among those that regenerate (Liu, Himes, Murray, Tessler, and Fischer submitted). It remains crucial to determined whether these rescued neurons are functional.

Regeneration

Regenerating axons are most unambiguously identified by labeling after a complete spinal cord transection. This eliminates confusion with spared and sprouting pathways. Regeneration can also be demonstrated after partial spinal lesions by retrograde labeling of cell bodies whose axons extend below the level of the lesion/transplant and anterograde labeling of specific pathways (Grill, Murai, and others 1997; Liu, Kim, and others 1999). Retrograde labeling can be used for quantitative analysis of neurons whose axons have regenerated, but the labeling site must be appropriately selected to prevent diffusion into the area of the lesion/graft and the labeling of sprouting axons. Sparing and sprouting of axons must be carefully evaluated. It is more difficult to estimate the number of regenerating axons labeled by anterograde methods, but the resolution allows tracing the trajectory of regenerating axons, determining the distance they reach, and whether they grow around and/or through the graft, in gray or white matter. Although there is often variability in the extent to which individual axons may regenerate, it is important to evaluate the maximum distance that axons regenerate because a major goal of regeneration therapies is long-distance regeneration. Questions that have not been addressed, except in lower vertebrates such as lampreys and goldfish, include whether regenerated axons make functional synaptic contacts, how precisely the original topography is restored, and whether that is important.

Recovery of Function

Appropriate tests need to be applied to demonstrate the deficit and recovery following a lesion, and recovery, or lack of recovery, following an intervention. Animals use alternate compensatory mechanisms to accomplish a task even in the absence of regeneration, and thus recovery of function, particularly after restricted lesions, often appears to be quite complete. Tests based on composite scores may be useful in indicating that some recovery occurs, but by their nature they may be insensitive to what is being recovered. Endpoint measures give information about whether a task can be completed but are not as useful as those that examine how an injured animal accomplishes a task, which can reveal more about the degree of deficit and the compensation strategy used by the animal. The BBB test (Basso, Beattie, Bresnahan, and others 1995) is a general outcome measure that has been carefully constructed, is widely used, and thus permits comparison between laboratories and provides a baseline of motor function. In most cases, this test should be supplemented by other more specific tests, chosen on the basis of pathways that are injured and that may recover. Reflex, spontaneous, and conditioned behaviors should be evaluated to provide maximum information. Behavioral tests alone give insight into the recovery process, but they may provide only limited information about how the recovery is achieved. For this, physiological and biomechanical examination may be needed.

Summary

It seems clear that a single intervention will not be sufficient to repair spinal injury. Different neuronal systems undoubtedly have specific requirements for survival and regeneration. Combinations of therapeutic agents delivered at the appropriate time will be necessary for maximum repair. One of the most promising strategies is the use of ex vivo gene therapy to combine grafting of cells with delivery of therapeutic factors.

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