

Adult Stem Cell Application in Spinal Cord Injury

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Abstract: The mechanical force incurred by spinal cord injury results in degenerative neural tissue damage beyond the site of initial injury. By nature, the central nervous system (CNS) does not regenerate itself. Cell therapy, in particular, stem cell implantation has become a possible solution for spinal cord injury. Embryonic stem cells and fetal stem cells are the forefathers of the field of stem cell therapy. Isolation and preparation of specific populations of adult stem cells have evolved to the point of stable, long-term culturing with the capability to differentiate into neural phenotypes from all three of the neural lineages: neurons, astrocytes, and oligodendrocytes. Thus, adult stem cells will transcend ethical concerns, technical difficulties, and probably immunorejection. A variety of adult stem cells have been implanted in a rat model of spinal cord injury, ranging from olfactory ensheathing cells, cultured spinal cord stem cells, bone marrow derived stem cells, dermis derived stem cells, and a few others. Although no definite decisions on which adult stem cells are most effective for this CNS injury, their ability to incorporate into the spinal cord, differentiate, and to improve locomotor recovery hold promise for a cure.

Key Words: Spinal cord injury, adult stem cells, neural stem cells, bone marrow stem cells, neurotrophic factors, endogenous stem cells, cell therapy.

INTRODUCTION

In 2002, it was estimated that there were 10,000 new cases of acute spinal cord injury per year in North America [1]. The United Kingdom had over 700 new cases of spinal cord injury in 2004 (according to the International Campaign for Cures of Spinal Cord Injury) [2]. The leading causes of spinal cord injury are vehicle accidents (41%) and violence (22%) while falls account for 21% and sports for 8% of acute spinal cord injury [3]. Although the primary mechanical injury may occur in a variety of ways, a series of molecular cascades associated with the secondary injury always occurs. Stopping or slowing this molecular cascade of reactions is the focus of clinical treatment and current research. While there is no cure for the local and distant damage sustained in spinal cord injury, current research, particularly in the field of stem cells, shows great promise.

MOLECULAR EVENTS IN SPINAL CORD INJURY

Spinal cord injury results in local and distant damage. There is an initial loss of neurons at the region of mechanical disruption but only occasionally is the inner gray matter containing cell bodies initially affected [3]. Further damage may extend the length of the entire spinal cord through a series of complex reactions and cascades (Fig. 1) [4].

The initial primary injury elicits a range of cellular disturbances, from hemostatic problems to ionic and neurotransmitter derangements (Fig. 1) [5]. There is a release and/or production of matrix metalloproteinases (MMPs), arachidonic acid, free radicals, eicosanoids, endogenous

opioids, and lipid peroxidation [6]. Abnormal accumulations of neurotransmitters, especially glutamate, result in excitotoxicity. The resultant excitotoxic damage done to the neurons during spinal cord injury causes a rapid disruption of cellular membranes [3, 7]. Then the influx of extracellular calcium through disrupted cell membranes leads to cell death [8].

The significant tissue destruction that is characteristic of the secondary injury is the cumulative effect of the primary mechanical injury. Immediately after the primary injury, the secondary injury is initiated when the site is infiltrated with immune cells, monocytes, macrophages, and the microglia of the spinal cord [9-11]. The secondary injury runs its course within the first few hours after the initial injury. Over several days the injury site enlarges longitudinally through gray matter and then through white matter. The ultimate result is the formation of a glial scar rimming a cavitation that becomes a fluid-filled cyst (Fig. 1) [12].

This regenerative failure is partially due to the replacement of neurons by the glial scar. Following primary injury, the astrocytes become reactive and upregulate the expression of the protein glial fibrillary acidic protein (GFAP) (Fig. 1) [13]. The astrocytes also strengthen their regular function and increase the flow of ions and neurotransmitter from the neurons to the extracellular fluid. The astrocytes divide and fill the vacant spaces with a glial scar, where the axons of pre-injury neurons occupied and presumably where regenerating axons would be located [14]. Therefore, limiting the damage elicited from secondary injury needs to be controlled in order to allow for possible regeneration in the spinal cord.

REGENERATIVE FAILURE

The regenerative failure of the spinal cord following injury is greatly hindered by inhibitors of nerve growth

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Spinal Cord Injury

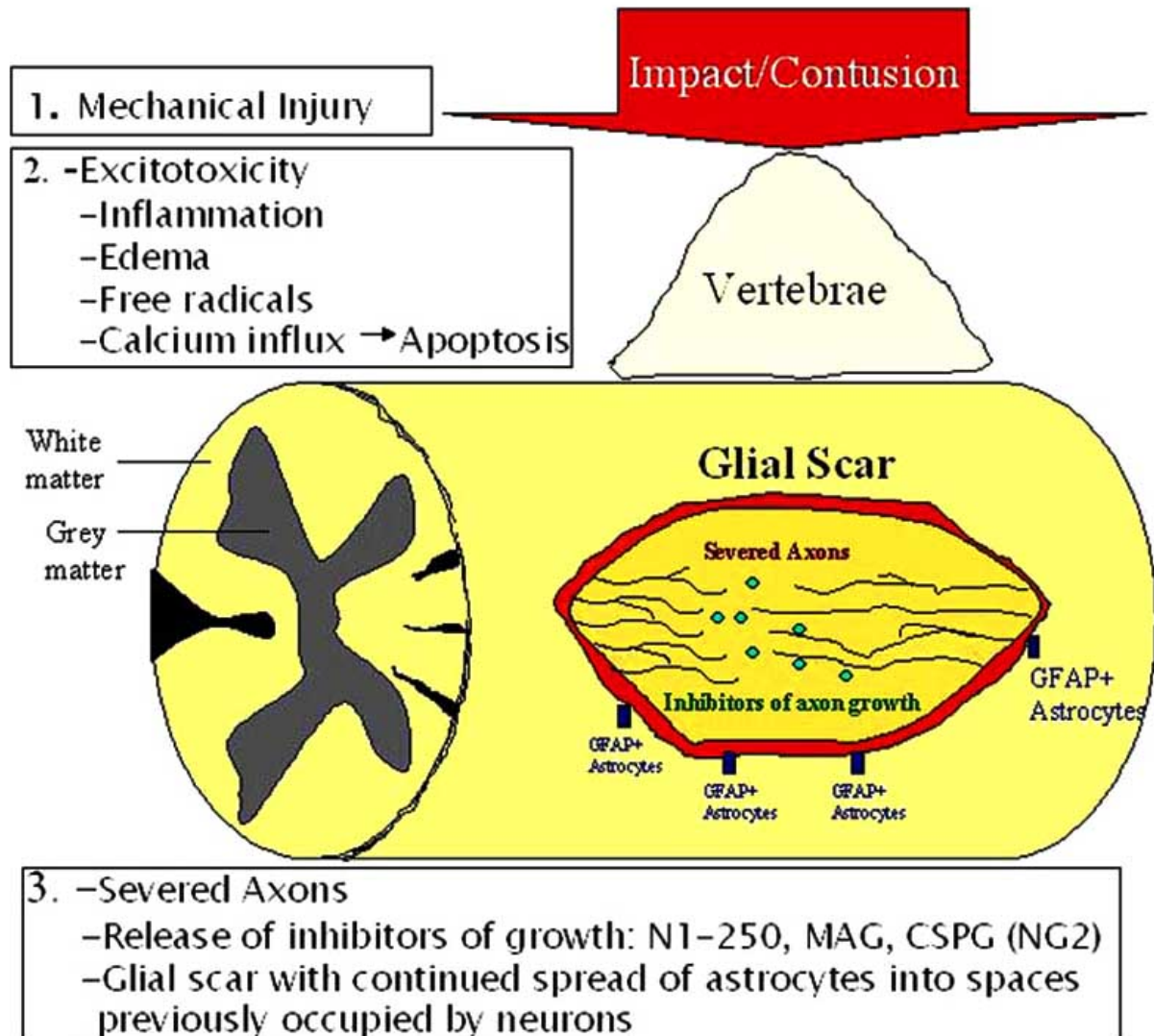


Fig. (1). Molecular events in spinal cord injury.

(Fig. 1). A variety of growth inhibitor molecules exist for maintaining homeostasis in an uninjured CNS environment. In an injured CNS environment, damaged myelin sheaths of degenerating axons release some of these myelin-associated molecules that are involved in axon regeneration inhibition. The myelin debris contains the inhibitory molecules N1-250 and myelin-associated protein (MAG) that are expressed on mature oligodendrocytes immediately after injury. MAG is also found on myelin debris and inhibits the growth of only some axons [15]. The debris may remain for several months due to the slow microglia/macrophage phagocytic activity. N1250 and MAG both mediate axon growth cone collapsing activity. Tenascin R is another inhibitory protein and is located in the extracellular matrix of the CNS, particularly in white matter. It inhibits axon growth by interacting with the axon surface molecule F3/11.

The endogenous neural cells of the spinal cord also participate in producing inhibitors of nerve growth. Chon-

droitin sulphate proteoglycans (CSPG) are extracellular matrix molecules that are neurite growth inhibitors produced by astrocytes, oligodendrocyte precursor cells, and meningeal cells. NG2 is a major CSPG that is produced by activated macrophages and oligodendrocyte progenitors. The local oligodendrocyte precursor cells also express the platelet-derived growth factor (PDGF)-receptor and accumulate in number during the injury response through proliferation and migration to reach a maximum at 7 days after the injury and then decline in number. Other CSPGs that may act as inhibitors, such as versican, brevican, and neurocan, are also upregulated by oligodendrocytes at 7 days after injury [16]. However, microglia cells are also activated in an effort to remove the myelin debris [9].

Another factor that prevents spinal cord regeneration is the lack of nerve growth promoters. It is possible that axonal growth may be supported following injury if there is differential expression of growth-initiating genes, cytokines,

cell adhesion molecules, extracellular matrix molecules, axonal structural genes, and white-matter inhibitors, all in the correct combinations. These factors allow the axons to grow over great distances and in great numbers. The strongest promoters of growth are neurotrophic factors, but there is insufficient neurotrophic factor upregulation in the gray and white matter of the spinal cord after injury to effect regeneration [16-18].

EXPLORING INDIVIDUAL FACTORS THAT MAY CONTRIBUTE TO REGENERATION

Growth factors that are found endogenous to the CNS may promote axonal development under the appropriate conditions. For example, neurotrophic factors, including nerve growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF) appear to be the strongest growth factors involved in endogenous neural differentiation [19-21]. Each growth factor has very specific populations of target cells that they effect. The delivery of neurotrophins singly or in combination reduced degeneration of injured ascending sensory and corticospinal axons in a C3 transected rat spinal cord. Delivery of BDNF with NGF and NT-3 showed that NT-3 alone was more effective in reducing the number of terminal clubs. The corticospinal neurons express the receptor TrkC that is receptive for NT-3 [22]. NT-3 may help to induce neural differentiation on a developmental level as well as the differentiation of endogenous and/ or implanted stem cells.

Changing intrinsic factors such as cAMP or inactivating Rho have been shown to promote growth within an experimental setting. Qiu *et al.* (2002) delivered the membrane permeable analogue dibutyl cAMP in an adult rat dorsal root ganglion prior to spinal column lesion [23]. This resulted in an increase in regeneration of transected sensory axons as measured by the longest GAP43-positive neurite. The Rho family of GTPases controls the actin-cytoskeleton's ability to modulate morphology, migration, proliferation, adhesion, phagocytosis, gene transcription, and in particular, neurite growth and guidance in the cytoskeletal dynamics of the growth cones [4, 24, 25].

Additionally, there is preliminary data that anti-inflammatory agents have neuroprotective effects [26, 27]. The current standard clinical treatment is to administer methylprednisolone at high doses of 30 mg/kg as a bolus within 8 hours post-injury followed by a 5.4 mg/kg/ hour infusion for 23 hours [28]. Methylprednisolone is an anti-inflammatory that limits some of the tissue damage by reducing inflammation, edema, the release of glutamate, and inhibiting the release of free radicals by local inflammatory cells [3, 29]. However, this is not a cure for spinal cord injury. An increase in the number of cells in the tissue needs to occur for functional recovery in the injured area

Replacement of the spinal cord tissue damaged in injury has been proposed as a method to increase the number of cells to maintain the integrity of the tissue. However, organ transplant does not seem to be a practical treatment for spinal cord injury. Not only would transplantation present formidable technical difficulties, but there would undoubtedly be insufficient numbers of donor cords. More than 6,000 people

in the U.S. died waiting for an organ transplant in 2001 [30, 31].

EARLY DEVELOPMENT OF CELL THERAPY

Therefore, cell therapy has become an intriguing possibility in the field of transplant medicine. This is replacing the damaged cells with cells that offer different growth factors to endogenous cells or provide a differentiation potential for incorporation with the endogenous neural cells of the spinal cord. These cells range from neural progenitor cells, to neural stem cells engineered to express an excess of growth factor, and to cells with differentiation capacities beyond that of solely the ectodermal lineage.

For example, the use of biodegradable implants, cell implants, and cells genetically engineered to over-express neural growth factors are areas currently being researched in animals to heal damaged spinal cords. This form of therapy could assuage the traumatic tissue loss associated with spinal cord injury as well as age-related diseases that are associated with cell loss, such as Parkinson's disease and Huntington's disease.

Initially, the treatment of spinal cord injury through cell therapy revolved around the application of embryonic stem cells and fetal stem cells. Embryonic stem cells, cells derived from the inner cell mass of 5-day old blastocysts, require pre-operative neural induction prior to implantation due to their predisposition to form tumors if implanted undifferentiated [32, 33]. Human fetal neural stem cells have been isolated from the midbrain or forebrain of fetuses ranging from 7- 23 weeks gestation in varying experiments [34, 35]. In addition to their capacity for neural differentiation, embryonic and fetal neural stem cells demonstrated stability, sustainability, and expandability in long-term culture systems in order for them to be considered as a possibility in human application. However, the procurement of both options may present serious ethical dilemmas. At this time, they also lack the ease of accessibility and practicality for routine clinical use. To bypass ethical issues, adult human neural stem cells have been isolated from patients undergoing surgery for the treatment of epilepsy, trauma, or tumors [36]. As culture techniques have advanced in culturing adult stem cells, the establishment of stem cell lines that can differentiate into neural phenotypes and expand in culture on a long-term basis have been developed in cells that are more easily obtainable.

ADULT STEM CELLS

In the field of cell therapy, adult stem cells are emerging as a clear alternative. Stem cells are self-renewing cells that are capable of differentiating into a cell lineage [29, 37, 38]. Adult stem cells have been isolated from a variety of different organs throughout the human body using a variety of techniques. Originally, adult stem cells were isolated from bone marrow and referred to as hematopoietic stem cells.

As scientific knowledge expanded, a population of bone marrow stem cells derived from the marrow stroma were isolated and were found to be capable of differentiating into cell phenotypes from the mesodermal lineages as well as the endodermal and ectodermal lineages, (including hepatocytes

and neurons) [29, 39-43]. Cells of the mesodermal lineage, such as the bone marrow stromal stem cells, are referred to as mesenchymal stem cells. Mesenchymal stem cells derived from cardiac muscle were also multipotent and differentiated into bone, muscle, and adipose [44]. Skeletal muscle contained stem cells that differentiated into different phenotypes, including neural lineages [20, 45]. Multipotent stem cells have also been derived from organs of non-mesenchymal origin such as brain, dermis, and liver. These can differentiate to a phenotype outside of their respective dermal lineage [46-48].

The differential capability of these populations of stem cells presented a novel opportunity for scientists to repair damaged organs with autologous stem cells that originated from healthier organs. The potential regenerative capacity, that adult stem cells hold, has been tested within the field of spinal cord injury. In the case of spinal cord injury, it is necessary for the implanted stem cell populations to be congruous with the endogenous spinal cord cells. Therefore, neural stem cells were the obvious first choice for replacing the damaged cells that were lost following spinal cord injury.

Adult neural stem cells are stem cells that are capable of differentiating into the three neural lineages: neurons, astrocytes, and oligodendrocytes. Endogenous adult neural stem cells have been detected in areas of the CNS where there is a higher turnover of cells, such as the subventricular zone of the lateral ventricles, the dentate gyrus of the hippocampus, the cortex, the fourth ventricle, and the central canal of the spinal cord [49]. The multipotent progenitor cells surrounding the central canal of the spinal cord may also migrate from the periventricular zone of the forebrain in spinal cord injury. These cells express nestin (found on neural progenitor cells) and have polysialylated neural cell adhesion molecule (N-CAM) on their surface. In addition, there may be a more primitive population in the periventricular region of the spinal cord that contributes to the other types of progenitors in the parenchyma. There still could be other, as of yet, unidentified independent pools of progenitors in the adult CNS [50]. Either way, the endogenous stem cells of the spinal cord are not able to mitigate the effects of spinal cord injury by themselves.

These endogenous neural stem cells may be cultured in the three-dimensional form of floating neurosphere aggregates derived from the adult CNS. Neurospheres may also be cultured using adult stem cells from a non-neuroectodermal lineage, such as dermis and skeletal muscle [20, 32, 51-53].

It is imperative for adult stem cells to be efficiently isolated from their tissue of origin. The field of adult stem cells has primarily revolved around their identification through CD cell surface markers in FACS analysis [54], the ability to absorb Hoescht 33342 dye [55], filtration [45, 56], and other techniques. Once the cells are properly isolated, they can be used in experimental models.

DELIVERY OF EXOGENOUS ADULT STEM CELLS INTO THE SPINAL CORD INJURY

Most models are performed on rats since the spinal cord injury response in rats is similar to that in humans. Rats are used to study the most common human spinal cord injury,

the contusion. In a contusion, the bony vertebrae that are supposed to protect the cord will instead move against the spinal cord with physical force. The spinal cord tissue beneath the vertebrae receives the damage of the blow. The most commonly used methods to create a spinal cord injury in rats and other animals is either a microsurgical transection of a specific area, crushing the spinal cord with a rod, or the use of a weight-drop to create a contusion [11]. Other experimental methods to cause spinal cord injuries in rats include excitotoxic kainic acid delivery, photochemical damage, and a compression clip [11, 18, 57]. Such an experimental model of human spinal cord injury needs to demonstrate chronic neurophysiologic and behavioral deficiency.

The majority of the rat spinal cord injury models that implant neural stem cells done to date have been using cells from the CNS fetal stem cells [34, 58], olfactory ensheathing cells [59-62], or embryonic stem cells [63-66]. With the availability of more adult stem cell lines, there is an increasing number of stem cells experiments.

NEURAL STEM CELLS

It is still important to review the pioneering work of implanted fetal stem cells in spinal cord injury. The C17.2 murine neural stem cells has been standardly used due to its multipotency, self-renewal, and responsiveness to trophins. Isolated from the neonatal mouse cerebellum, it contributes to the development of the neuroaxis and the CNS into adulthood [19]. The implantation of these neural stem cells in the injured spinal cord offer the injured environment the possibility of replacing the injured or dead endogenous cells. These cells may also produce neurotrophins to override the inhibitory mechanisms and/or bolster the growth mechanisms. Wu *et al.* (2002) placed a 20 gram rod on the exposed spinal cord dura for 90 seconds on the T8-T9 region. GFP-labeled neural stem prepared from rat hippocampal fetus were injected at 1×10^6 cells into the 4th ventricle of the brain. The cells transported through cerebral spinal fluid from the 4th ventricle to the subarachnoid space around the spinal cord. Immunohistochemical analysis at 8 months post-operatively showed that the GFP-labeled cells were positive for proteins for all three of the neural lineages, GFAP (astrocytes), RIP (oligodendrocytes), and -tubulin III (neurons) [63]. *In vivo* differentiation of the implanted neural stem cells may be part of spinal cord regeneration. However, implanting neural stem cells may repair in ways other than replacing the damaged terminally differentiated endogenous cells.

Teng *et al.* (2002) implanted 5×10^5 neural stem cells (fetal mouse clone C17.2) per poly lactic-co-glycolic acid scaffold or $10 \mu\text{l}$ of 10^7 cells/ml following a T9-T10 4-mm-long longitudinal cut along the midline of the rat spinal cord. Most of the mouse-specific antigen M2 positive cells that detected the implanted stem cells that were rarely double-labeled with GFAP but often double-labeled with nestin. NF positive cells were detected near the epicenter that were not M2 positive and therefore determined to be of host-origin. Hindlimb weight support and coordination was evaluated using a comprehensive open-field locomotor recovery scale composed of 21 operational definitions that study several aspects of the locomotion of paraplegic animals (Basso-

Beattie-Bresnahan or BBB scale). Once weekly over the course of 70 days, locomotor evaluation was performed on rats that received the scaffold with neural stem cells (stabilized mean BBB=12), that received cells alone (stabilized mean BBB=7), the scaffold alone (stabilized mean BBB=9), or just the lesion (stabilized mean BBB=6) at 56 days post-injury. Since the effect appeared to be synergistic, it is possible that the implant impeded the development of a glial scar or provided an environment that hindered the inhibitors of growth for endogenous stem cells. Meanwhile, the neural stem cells on the scaffold may have provided neurotrophins or other factors supportive to the differentiation or activation of endogenous stem cells. The experiments involving fetal stem cell clones showed promise for adult neural cell replacement and molecular support therapy in the injured spinal cord.

Vroemen *et al.* (2003) injected GFP-labeled adult neural stem cells that were cultured from the adult spinal cord at C3 to T1, directly into a transection site at C3. The neural stem cells migrated caudal and rostral to the lesion site and were positive for neural markers from all three of the neural lineages: GFAP, α -tubulin III, and GalC for oligodendrocytes (Fig. 2) [67].

In addition to the neural stem cells from spinal cord cultures, neural stem cells specifically derived from the olfactory bulb have also been used as a cell implant in spinal cord injury. The dentate gyrus of the hippocampus, the cortex, and the fourth ventricle are also recognized areas of neurogenesis but isolation of neural stem cells is more invasive and technically complex. At the juncture of the CNS and PNS, the olfactory bulb contains neural stem cells that possibly supply trophic factors and extracellular matrix molecules typical of more primitive glia as well as the ability for life-time self-renewal and multipotent neural differentiation. They have neurite-promoting factors that mediate

neuronal survival and axonal elongation. Clonal populations of olfactory ensheathing cells express mRNA for NGF, BDNF, NT-4/5, and neuroregulins [68]. The olfactory ensheathing cells that ensheath the axons of the olfactory receptor neurons from the exit point, at the basal lamina, through to the nerve fibers of the olfactory bulb [61]. During development and regeneration, they can wrap and migrate with elongating axons [69]. They may possess properties that help direct axon growth. These cells are part of both the PNS and CNS and therefore, interact with both systems.

The difference between the regeneration abilities of the PNS and the CNS may correlate with the ability the PNS has to provide trophic support and the ability to clear axon and myelin debris. Neural transmission molecules are down-regulated and signals for axon growth and elongation are upregulated in the peripheral nervous system [17, 70]. In peripheral nervous system injuries, spontaneous regeneration is supported by the ordered structure of the peripheral nerve and the supportive Schwann cells [17, 71].

Ramon-Cueto *et al.* (1998) implanted a PAN/PVC channel 6 mm in length delivered Schwann cells and a Matrigel mix following a T9 transection and removal of a 4 mm segment. 0.5 μ l of 50,000 Hoescht dye labeled olfactory ensheathing glia were injected into the 4 sites along the midline of each of the spinal cord stumps. Fig. (3) shows long-distance axonal regeneration at 6 weeks post-operation. The olfactory ensheathing glia elongated through white matter tracts, gray matter, and the glial scar with the aid of the channel [60].

In 2000, Ramon-Cueto *et al.* used olfactory ensheathing glia transplants obtained from the sciatic nerves of adult rats in a rat spinal cord transection model. The spinal cord was transected at T9 and a 4mm segment was removed and replaced with Schwann cells and Matrigel mix. The axons

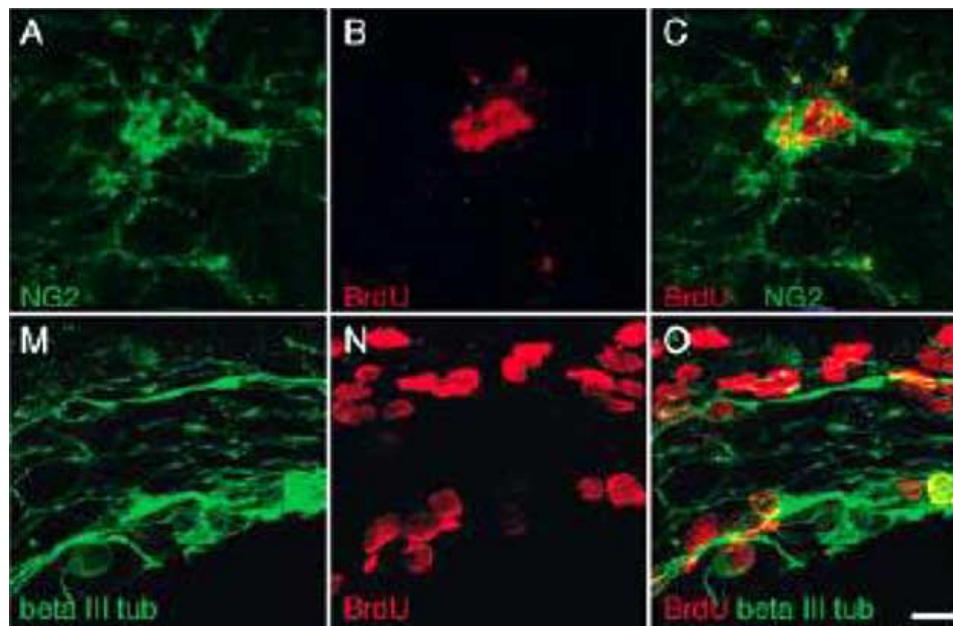


Fig. (2). 3 weeks post-implantation, the adult neural stem cells implanted in the spinal cord. A,B,C. A cell stained for NG2 and BrdU. D,E,F. A cell positive for α -tubulin III and BrdU. Many BrdU positive cells that were not positive for α -tubulin III. (Vroemen *et al.* (2003) Fig. 5, p. 748).

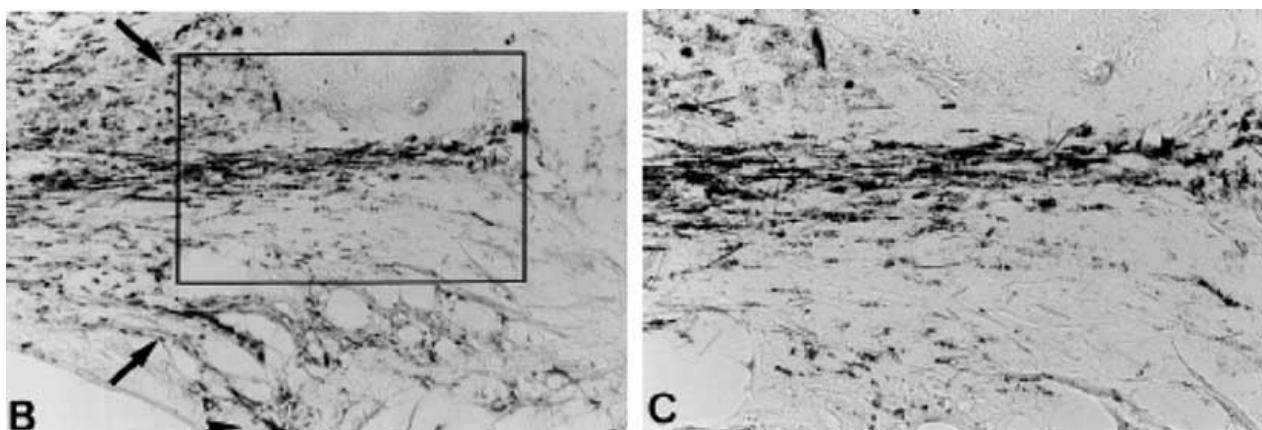


Fig. (3). Ramon-Cueto *et al.* (1998) use WGA-HRP to trace the olfactory ensheathing glia implanted in the injured spinal cord. B. Low magnification, C. Higher magnification of the respective box in B. Regenerating axons enter the caudal stump. (Ramon-Cueto *et al.* (1998) Fig. 8, p. 3811).

had long distance regeneration of 2.5 cm from the transection site in the rostral spinal cord at 8 months after surgery. The locomotor function and sensorimotor reflexes improved from 3 to 7 months post-surgery. The olfactory ensheathing glia implanted rats were also able to pass a climbing test and respond to light skin contact on their hindlimbs, demonstrating some functional recovery [59, 60].

GENETIC MODIFICATION FOR OVEREXPRESSION OF GROWTH FACTORS

Adult stem cells, whether differentiated and/or undifferentiated, may synthesize factors that stimulate endogenous differentiated neural cells or neural progenitors. The production of neurotrophic factors or undiscovered growth factors that differentiate endogenous cells may induce the endogenous stem cells to proliferate and differentiate. Alternatively, the products of the differentiated and/or undifferentiated adult stem cells could induce the differentiated endogenous cells to produce the appropriate amounts of neurotrophins which support regeneration in the spinal cord. There are a variety of scenarios in which growth factors could be integral in repairing the injured spinal cord. Presumably the *in vivo* environment would have to provide signaling factors to induce the adult stem cells to produce neurotrophic factors.

It would be ideal for neurotrophins to be provided in specific amounts, at specific times, for specific patterns of axonal growth during specific time periods post-injury similar to the neural plasticity reminiscent of neural development [18, 72]. Research groups increased the concentrations of neurotrophins at the site of injury by implanting adult stem cells genetically manipulated *ex vivo* to overproduce specific neurotrophins. Ruitenberg *et al.* (2003) transduced olfactory ensheathing glia to encode BDNF, NT-3, or LacZ. The left dorsolateral funiculus of the spinal cord was lesioned 1 mm ventral to the spinal surface and 10^5 cells were immediately injected 1 mm proximal and distal to the lesion cavity. Biotinylated dextran amine (BDA) was used to trace the rubrospinal tract of the spinal motor pathway at 4 months post-operation. There was a statistically significant difference in locomotion using a rope test between the olfactory ensheathing glia cells and the controls. The

olfactory ensheathing glia that had transduced neurotrophins had the greatest improvement at 4 weeks post-operation [73]. This is a form of combinatorial therapy in which adult neural stem cells are combined with neurotrophin delivery. The olfactory ensheathing glia cells could also provide an extracellular matrix and other types of neurotrophins to the injured neurons and neural differentiated adult stem cells.

STEM CELLS OF NON-NEURAL ORIGIN

Bone marrow-derived adult stem cells have also been implanted in spinal cord injury. Chopp *et al.* (2000) injected 2.5×10^5 BrdU-labeled bone marrow stem cells in a 4 μ l volume into the spinal cord contused site at T9, 1 week following the injury. This resulted in a 3.8 point statistically significant difference on the BBB locomotor scale between the stem cell injected rats and the control rats at 5 weeks post-operation. The stem cells migrated dorsal and ventral to the injury site and differentiated into cells positive for the proteins to nestin, NeuN (for neurons), and RIP (for oligodendrocytes) at 5 weeks.

Hofstetter *et al.* (2002) injected GFP-labeled bone marrow stem cells into a weight-drop contused spinal cord at T9 at 1 week following injury or immediately after injury. The cells were delivered in a 5 μ l volume into the injury site, 2.5 μ l 2-mm cranial and 2.5 μ l 2-mm caudal of the central injection at 30,000 cells/ μ l. This experiment also resulted in a slightly over 1 point statistically significant difference on the BBB locomotor scale between the 1 week delayed stem cell injected rats and control rats at 5 weeks post-injury. It was shown that delayed stem cells implantation in the injured spinal cord improves functional recovery. At 5 weeks, the GFP-positive cells were also positive for the protein to NeuN and neurofilaments (neurons), fibronectin, GFAP, and nestin (Fig. 4) [74].

Bone marrow-derived stem cells have also been delivered into the cerebrospinal fluid of a T8-9 weight-drop contused rat spinal cord. Ohta *et al.* (2004) injected 5×10^6 GFP-labeled marrow-derived stem cells in a volume of 50 μ l into the 4th ventricle, 2 days prior to the weight-drop contusion at the T8-T9 section of the spinal cord. Histological analysis of the spinal cords was done at 4, 7, 14, 21, 28, and 35 days

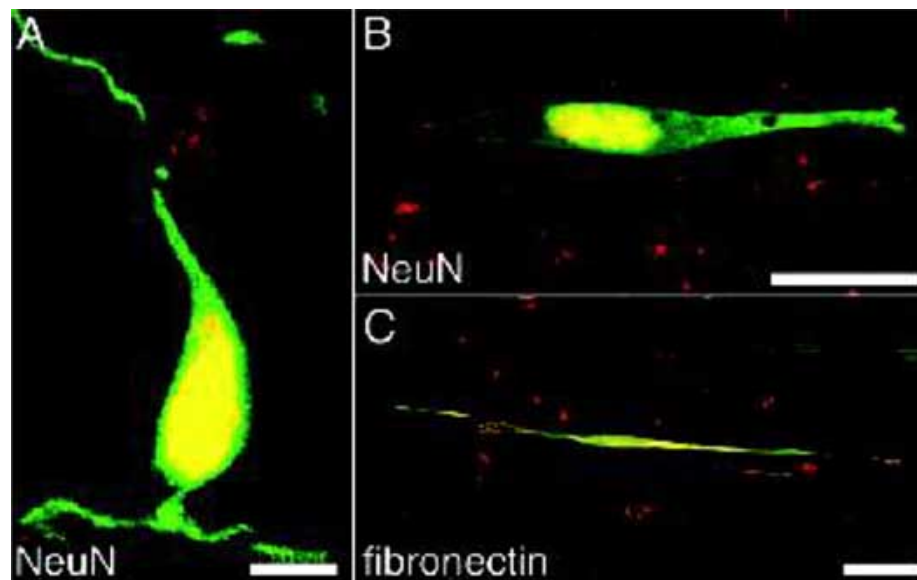


Fig. (4). Adult bone-marrow derived stem cells implanted in an injured rat spinal cord at 5 weeks. A., B. GFP and NeuN positive cell, C. GFP and fibronectin positive cell (yellow). (Hofstetter *et al.* (2002) Fig. 4, p. 2203).

prior to cell injection. The improvement in locomotor function of the stem cell injected injured spinal cords versus the control were significantly higher at all time points. There was a slightly higher than 2 point difference in score on a BBB scale at 5 weeks. The stem cells that attached on the surface (Fig. 5) and within the lesion of the spinal cord disappeared 3 weeks following implantation. The stem cells also did not differentiate to express the neural proteins to the antibodies that were used. Since a reduction in the size of the fluid filled cyst was paired with the improvement in behavioral score, it is proposed that these bone marrow derived stem cells were secreting some type of trophic substances to influence proliferation and growth of endogenous cells [75].

Lee *et al.* (2003) implanted bis-benzimide labeled bone marrow-derived stem cells in 1.5 μ l of 1.5×10^3 in T9-T13 midline-incised mice at 7 days after the insult. An injection was delivered into the injury site and an area 2 mm rostral to

the injury site. Four weeks later, the implanted stem cells survived rostral to the injury site and were positive for the GFAP antibody but no neuronal markers.

A less invasive means of isolating adult mesenchymal stem cells is from skeletal muscle. Schultz *et al.* (2005) immediately injected 300,000 adult stem cells isolated from the skeletal muscle of a ROSA26 mouse into the site of a T10 weight-drop contused rat spinal cord. There was a statistically significant difference in locomotor recovery in stem cell injected versus control spinal cord injured rats at 5 weeks, based on a BBB scale. Immunohistochemistry shows that some of the implanted stem cells were positive for the proteins to neurofilaments, oligodendrocytes, and astrocytes, all three of the neural lineages (unpublished data).

Another alternative to bone marrow-derived stem cells are stem cells from dermis. Gorio *et al.* (2004) isolated adult

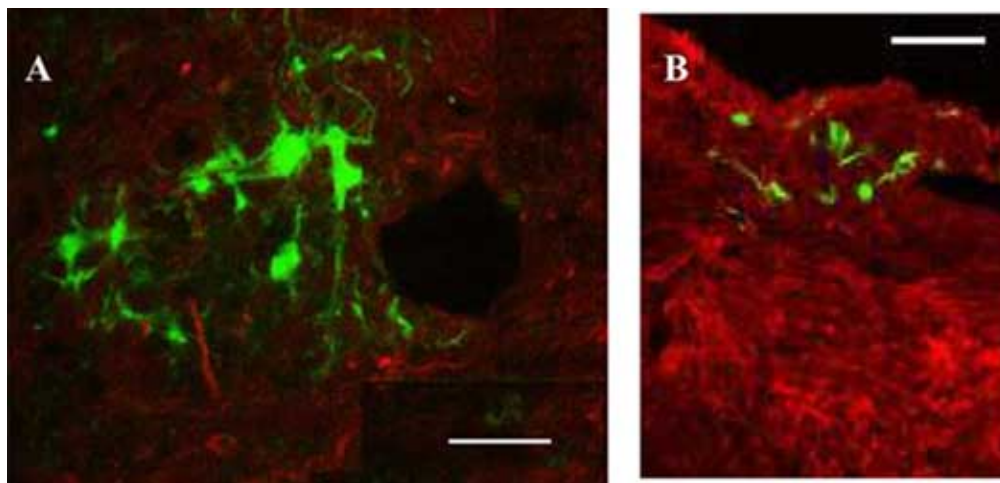


Fig. (5). The GFP-labeled adult bone marrow-derived stem cells integrated into the injured rat spinal cord. The host tissue stains for tubulin III (red). A. High magnification, B. Low magnification. (Ohta *et al.* (2004) Fig. 2, p. 269).

stem cells from skin patches from 2-day old rats and differentiated them into nestin positive neural stem cells. The cells were labeled with the cell membrane label, PKH 26, and injected at 30 minutes or 7 days post-injury, 3 mm caudal or rostral to a T8 contusion injury. The cells injected 30 minutes following injury differentiated into cells positive for the macrophage/lymphocyte markers, membrane activated complex-1 (MAC-1), cluster of differentiation-4 (CD-4) and (CD-8). Those cells eventually underwent apoptosis 60-90 days later as would macrophages/lymphocytes of that age. However, the cells injected 7 days post-injury were 65% positive for GFAP, 16% positive for neurofilament, and 2% positive to the angiogenesis associated endothelial cell adhesion marker, PECAM. None of the PKH 26 positive cells were positive for the oligodendrocyte marker, GalC [76]. These results imply that immediate delivery of adult stem cells following injury may primarily contribute to the apoptosis that takes place during the phases of spinal cord injury. The dermis-derived adult stem cells offer the least invasive method of stem cell isolation.

The above studies suggested that there are a variety of different types of stem cells that may be implanted in the injured spinal cord injury that may foster different degrees of locomotor recovery. Based on the different types of current experimental models of spinal cord injury, the type of adult stem cells most effective in promoting nerve growth or blocking inhibitors of growth may be difficult to decide. Not only is injury model a factor, but accessibility and other factors such as delivery are considered detrimental details when appropriately evaluating which adult stem cells to apply in spinal cord injury.

POSSIBLE *IN VIVO* MECHANISMS OF ADULT STEM CELLS IMPLANTED IN THE INJURED SPINAL CORD

The immunohistochemistry that most research groups have used demonstrates that the adult stem cells derived from CNS (Fig. 2), bone marrow (Fig. 4), skeletal muscle, and dermis, are capable of differentiating into neural phenotypes in the spinal cord when implanted into an injured spinal cord. Vroeman *et al.* (2003) implanted CNS derived adult stem cells that stained for neural proteins, NG2 and β -tubulin-III. Hofstetter *et al.* (2002) implanted bone marrow derived adult stem cells that stained for the neural proteins, NeuN and fibronectin. Implanted adult stem cells derived from CNS and bone marrow also had neural morphology that was elongated with processes from the main cell body. This indicates that the adult stem cells respond to endogenous cues to differentiate into the phenotypes at the site of implantation. The implanted adult stem cells that differentiate may replace the endogenous cells that died following the injury.

Attention has also been drawn to experiments where the implanted stem cells do not differentiate in the spinal cord. Vroemen *et al.* (2003) observed that some of the implanted BrdU-labeled adult neural stem cells appeared morphologically rounded and undifferentiated (Fig. 2M, N, O). Some of the implanted neural stem cells also did not stain for the antibody to NG2 or β -tubulin-III. GFP-labeled adult bone marrow-derived stem cells did not stain for the antibody to

β -tubulin-III but still contributed to the improvement of behavioral function in the case of Ohta *et al.* (2004) (Fig. 5). The cells may or may not stain with other neural antibodies. They used undifferentiated adult stem cells from a mesenchymal source. It is possible that implanted adult stem cells that did not appear to differentiate into cells expressing proteins for neural markers or develop a neural morphology, may eventually differentiate. The rate that adult stem cells differentiate within the exogenously implanted stem cell populations from different organs has not yet been established. Nor is the rate of differentiation for endogenous stem cell populations determined within individual organs when they appear to be differentiating in response to the implantation of adult stem cells. Some of the adult stem cells may stop differentiating at varying stages of multipotency and unipotency, *in vivo*. However, the adult stem cells in the spinal cord may reach a more terminal level of differentiation over longer periods of time *in vivo*.

Adult stem cells implanted in tissues may contribute to recovery in ways other than adding to the numbers of differentiated cells (Fig. 6). The CNS is an inhospitable environment for regeneration. It is possible that the adult stem cells could be contributing to a type of environmental change in the spinal cord that would encourage regeneration. Rather than contributing to regeneration, the adult stem cells could be aiding the survival of endogenous terminally differentiated and/or endogenous stem cells for the preservation of the cord. This contribution could be in the form of neurotrophins or other factors that could support axon survival. For functional recovery, the environment must allow neuronal or axonal survival, growth, and elongation [77].

Alternatively or additionally, the adult stem cells may possess extracellular matrix properties to provide a conducive environment for the attachment and growth of endogenous neural cells. The cells, or the extracellular matrix they provide, can serve in the spinal cord as a 'bridge' for axons to extend across the injured area, or cyst, to bridge the gap. Providing an extracellular matrix for neural cells is one of the regular functions of olfactory bulb cells in their endogenous environment. In some situations, the restorative properties of the adult stem cells could be bolstered by delivering them on a polymer or biodegradable implant. The use of those two treatments, with different mechanisms of action, could result in a synergistic effect. In this case, the stem cells primarily provided essential factors while the polymer provided a support system specific to neural cells of the spinal cord or CNS. However, that may only be effective if one treatment did not manually or molecularly limit the effect of the other treatment.

It may be important for the adult stem cells to be able to migrate rostral and caudal to the site of injury, unobstructed. The adult stem cells may possess an important contribution to the regeneration of long-distance axonal growth. Interestingly, there may exist some type of wave continuum of stem cells circulating through the spinal cord the way the brain has the rostral migratory stream with neural stem cells [50, 78]. It has been shown that the neuroblasts of the developing spinal cord migrate through a ventral to dorsal gradient along the spinal cord [79, 80]. This pathway may not be completely broken during injury since adult stem cell migration occurs both rostral and caudal to the site of injury [65].

Possible mechanisms: Adult stem cells in the injured spinal cord

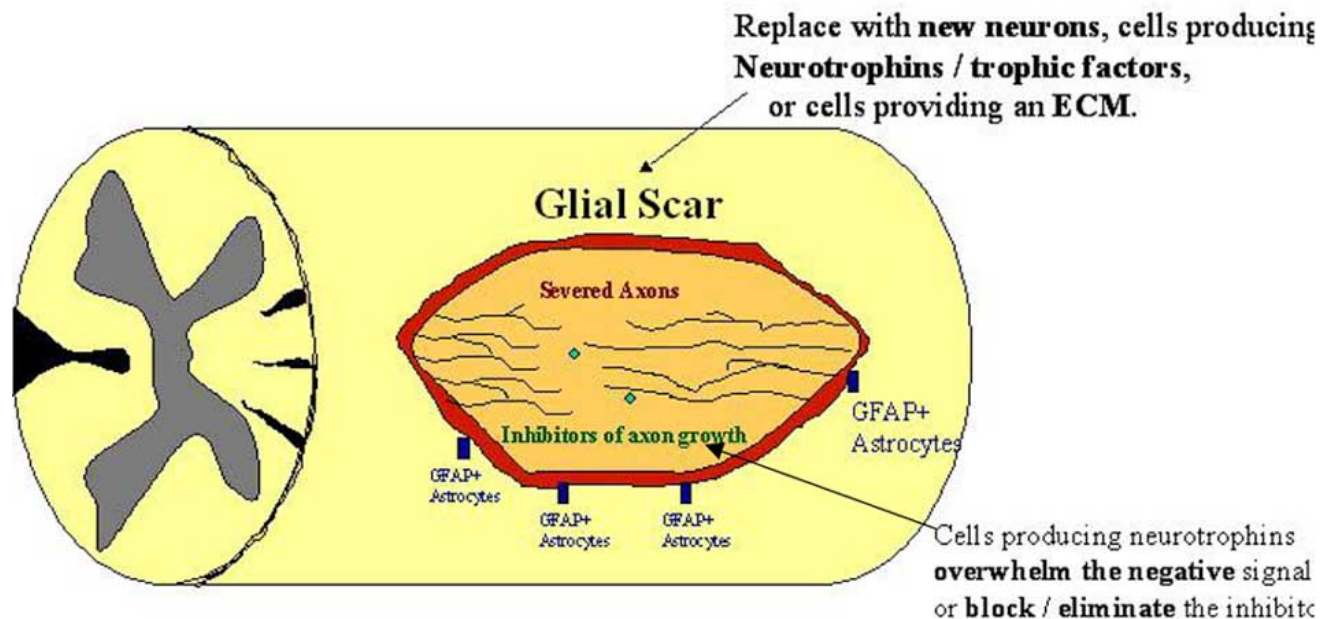


Fig. (6). Possible ways adult stem cells improve recovery in the injured spinal cord.

BRIEF MENTION OF CELL FUSION

The scientific literature has not reached a consensus on the *in vivo* fusion of injected or implanted adult stem cells and endogenous differentiated neural cells. The implanted adult stem cells may even fuse with the endogenous stem cells of the spinal cord. Some experiments have shown that bone-marrow-derived adult stem cells have the ability to fuse with a variety of cells ranging from bone marrow cells to Purkinje neurons to cardiomyocytes to hepatocytes [81, 82]. Other studies have shown that cell fusion does not exist or if it does, it is specific to the liver [83-86]. This concept should be tested within the framework of spinal cord injury in the future although the amount of relevance the existence of a cell fusion event may hold in the case of an otherwise healthy efficient therapy for restoring locomotion may be of little importance.

HUMAN ADULT STEM CELL RESEARCH IN HUMAN SPINAL CORD INJURY

A published cell therapy experiment on human spinal cord injury was conducted by Rabinovich *et al.* (2003). They implanted human neural stem cells from fetal brain and hematopoietic liver tissue (from spontaneous or therapeutic abortions at gestational age 16-22 weeks) into an injured human spinal cords 1 month to 6 years following injury. The patients, age 18-52, received one to four cell transplants in injuries that ranged from between C4 to T10. In 11 out of 15 cases, the therapy was combined with surgery to disrupt the connective tissue cyst and the implantation of olfactory ensheathing cells. Contraction of some muscles and partial recovery of sensitivity was observed in 40% of the patients, based on a Frankel scale of human function. The clinical

improvements were visualized on MRI scans amongst patients that received treatment less than 1.5 years from the date of injury. However, for the transplant of these human fetal stem cells to take place, the proliferation of macrophages and the macrophage inhibitory factor production of T lymphocytes from the recipient had to be cultured with allogeneic cell lysates *in vitro* to confirm alloantigenicity [90].

Unpublished human adult stem cell therapies for spinal cord injury are currently being conducted around the world. The Queensland Spinal Cord Regeneration Project started in July 2002. The Princess Alexandra Hospital in Brisbane, Queensland in collaboration with Griffith University is using olfactory bulb cells extracted from the spinal cord injured patient's upper nose. They inject 14 million cells into the site of injury. The Neurosurgery Clinic in Wroclaw in collaboration with the Institute of Immunology and Experimental Therapy of the Polish Academy of Medical Science is also developing methods of isolating purifying, and culturing human olfactory bulb cells for use in clinical trials of human spinal cord injury. Similar clinical studies are also ongoing in China and Portugal. <http://carecure.atintopop.com/4/OpenTopic>.

FUTURE RESEARCH

Deciding which adult stem cells are most effective in spinal cord injury is difficult to assess because many research groups generate the injury through differing techniques, delivery routes, time frames, and result analysis in terms of behavior and immunohistochemistry. There are a variety of materials available as carrier devices as well as the option of simply injecting the cells into the cord in a saline suspension. The fluid-filled cyst created by the spinal cord injury may be bypassed by building a new connection with new neurons

provided by the differentiation of the exogenous cells. The guidance of a bridge or scaffold connecting the distal and proximal end of the spinal cord injured cord may offer support or a blueprint upon which to regenerate.

A variety of factors in the recovery of the spinal cord need to be examined in devising an effective method for treating human spinal cord injury with adult stem cells. For example, the age of the animal and the type of endogenous neural cells at the specific anatomical location of injury also influence neuronal survival in spinal cord injury. Newborns may experience more neural plasticity than adults. The capacity of younger animals to recover from damage to the CNS as well as other organ systems is greater than that of older animals. The injured spinal cords of infants are more permissive for axon extension than those of adults [88-90]. There simply may be more endogenous stem cells in younger animals. This may be due to enhanced environmental cues to endogenous stem cells for repair in younger animals or decreased environmental cues in older animals [91].

Although a range of different factors are involved in the type of damage in the spinal cord injury, the destructive signal transduction pathways follow the same route each time. Preventing the multiple tissue degenerating cascades resulting from human spinal cord injury may warrant a combinatorial therapy that employs the use of adult stem cells with polymer implants, growth factors, or other technologies. Increased knowledge in the fields of adult stem cells as well as tissue engineering may provide additional venues for spinal cord injury experiments.

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