Challenges of Stem Cell Therapy for Spinal Cord Injury: Human Embryonic Stem Cells, Endogenous Neural Stem Cells, or Induced Pluripotent Stem Cells?

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ABSTRACT
Spinal cord injury (SCI) causes myelopathy, damage to white matter, and myelinated fiber tracts that carry sensation and motor signals to and from the brain. The gray matter damage causes segmental losses of interneurons and motoneurons and restricts therapeutic options. Recent advances in stem cell biology, neural injury, and repair, and the progress toward development of neuroprotective and regenerative interventions are the basis for increased optimism. This review summarizes the pathophysiological mechanisms following SCI and compares human embryonic, adult neural, and the induced pluripotent stem cell-based therapeutic strategies for SCI. STEM CELLS 2010;28:93–99

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A BRIEF SUMMARY OF PATHOPHYSIOLOGICAL EVENTS FOLLOWING SPINAL CORD INJURY (SCI)

In the last decade, many reports have demonstrated significant recovery after early medical management of SCI, although there is still no effective treatment to completely cure SCI. Understanding the pathophysiological events following SCI is essential to determine the differences of potential applications of various stem cell types for possible therapeutic applications after SCI. The SCI pathophysiology can be summarized in two complex phases [1].

Primary Injury Phase
Normally the primary injury phase has been shown to be due to either contusion (caused by shattered vertebral bones) or compression (caused by an increased pressure to the spinal cord) [2]. The cervical spine and lumbar spine are commonly affected areas of SCI. Damage to upper motoneurons results in hyperreflexia, hyperpnea, and muscle weaknesses. In contrast, the insults to lower motoneurons cause hypotonia, hypo-reflexia, and muscle atrophy [3, 4]. Many factors contribute to the events after SCI, including altered ion balance [5], lipid peroxidation [6], and glutamate release [7]. SCI may also lead to acute local ischemia, which may also contribute to secondary degeneration [8].

Secondary Injury Phase
Following the initial trauma, the secondary injury phase can be described as complex damage that occurs at the cellular level. This phase can be disclosed into several pathological events: (a) massive cell death due to the host immune system responses to the injury, (b) secondary necrosis and/or apoptosis, (c) oxidative damages after SCI, (d) excitotoxicity, and (e) axonal damages.

Immunopathological Events Following SCI
After any insult to the central nervous system (CNS), the blood-brain barrier (BBB; which normally works as a highly selective filter) is physically broken, with increased permeability allowing the infiltrated cells from the blood to invade the medullar tissue, triggering an inflammatory response (Figs. 1 and 2A). These events lead to the formation of platelet and fibrin-rich clots to decrease the bleeding [9, 10]. The neuroimmune responses and the proteins secreted by fibrin-rich clots, astrocytes, and microglia can increase the capillaries’ permeability and the expression of endothelial and junctional adhesion molecules, which facilitates leukocyte migration to the site of injury in the spinal cord and exacerbates the inflammation [11]. Microglia cells become activated following the increased level of proinflammatory cytokines, cell necrosis factors, lipopolysaccharides, and extracellular potassium [12, 13]. Proinflammatory cytokines including interleukin-1-alpha (IL1-a), interleukin-1-beta (IL1-b), and tumor necrosis factor-alpha [14] have also been shown to be capable of triggering apoptotic cell death. Microglia cells also increase the expression of monocyte chemoattractant protein-1, human macrophage inflammatory protein 1 alpha (MIP1-x), and beta (MIP1-b) and chemokines that contribute to the inflammatory process and direct leukocytes to the site of injury [15], resulting in a malfunction of oxidative metabolism in demyelinated axons [16].

Hours after SCI, astrocytes in the lesion site proliferate and increase the expression of glial fibrillary acid protein (GFAP) [2]. These astrocytes, due to their large-cell bodies
and processes, join together tightly and form astrocytic (glial) scars (Figs. 1 and 2A). These scars isolate neural tissue from inflammatory cells and decrease neuroinflammation during early phases [17]. The astrocytic (glial) scar can remain months after SCI and can be developed continuously. Glial scar formation that occurs following all injuries of CNS is beneficial for re-establishment of physical and chemical integrality of the CNS but implies an important obstacle of neuroregeneration. Inhibitory molecules secreted by glial scar-forming cells prevent functional recovery of the CNS. Therefore, several methods have been developed to suppress the formation of glial scars including administration of olomoucine (a cyclin-dependent kinase inhibitor) or rolipram (an inhibitor of phosphodiesterase 4) or the combination of such methods with cell therapy and/or triggered immune response [18–20].

Both innate and adaptive immune responses are necessary for the repair of SCI [21, 22], and autoimmune T cells are important for neurogenesis in healthy adults [23]. In SCI cases, activated microglia cells start to uptake and overexpress major histocompatibility complex class I/II proteins so that they become efficient antigen-presenting cells. When T cells pass through the BBB, they may bind to microglia cells to receive antigen. T cells also protect neurons from secondary degeneration after SCI by releasing neurotrophins, and modulating microglia or macrophage function [24, 25]. Immunostimulatory and immunosuppressive interventions result in the
prevention of further tissue damage, secondary cell death, axonal degeneration, promotion of remyelination, axonal regeneration, and facilitation of sensorimotor function recovery (reviewed in [26]).

Apoptosis, Necrosis, and Excitotoxicity Following SCI

A large population of neurons and glia located in the lesion site undergo death due to the disruption of cell membranes or as a consequence of the ischemia caused by vascular disruption, which in turn, causes hemorrhage that extends rostrally and caudally from the lesion site [27]. The massive cell death extended in the secondary phase occurs by apoptosis and necrosis, and affects all functional neurons and glial cell population, including oligodendrocytes [28]. Although the mechanism of oligodendrocyte apoptosis is not clearly known, it has been shown that Fas receptors located on the surface of oligodendrocytes can be activated by Fas ligands expressed by activated microglia [29, 30], which in turn can trigger the caspase cascade and initiate apoptotic cell death [31]. Furthermore, some serum proteins such as thrombin have a neurotoxic effect and can promote additional neural death by themselves or after activating the protease-activated receptors on the microglia [32].

The loss of ionic homeostasis and increased excitotoxicity following SCI also trigger apoptotic cell death and mitochondria dysfunction [33]. Oligodendrocytes can undergo excitotoxic cell death through their N-methyl-D-aspartate (NMDA) receptors [34], and their loss effectively initiates axon demyelination, blocking the action potential transmission.

Oxidative Damages Following SCI

The level of free radicals, which are one of the main mediators for axonal disruption in SCI, increases in the lesion site. Furthermore, during the immediate primary phase after injury a cascade of radical-mediated peroxidation starts and affects all cell membranes through oxidation of bilayer lipid membranes (Fig. 1) [35]. This event leads to the reduction in membrane permeability and disrupts the electron transport chain portion of the metabolic process.

Following SCI, the loss of neurons in the spinal cord leads to impaired motor function. The effect of neuronal death and axonal demyelination together with other inflammatory and immune responses blight the signal transduction through the spinal cord [2]. For these reasons, in addition to axonal regeneration and/or neural protection induction, replacement cell therapy could be an efficient strategy to overcome the lost function in the cases where neural loss occurs (Fig. 2B). Using cell replacement strategy, scientists and clinicians hope to bridge the lesion site by creating an environment in which remyelination, axon elongation, and formation of new circuits may occur. Therefore, in the next parts of this review we will discuss various stem cell-based therapeutic strategies for SCI especially the potential use of human embryonic stem cells (hESCs), adult neural stem cells (NSCs), and promising...
reprogrammed adult cells, that is, induced pluripotent stem cells (iPSCs).

**STEM CELL THERAPY FOR SCI**

**Human Embryonic Stem Cells**

Embryonic stem cells (ESCs) are pluripotent cells that have the capability to differentiate into nearly all cell types, including neuronal and glial fates [36]. Therefore, these cells are a promising source of differentiated oligodendrocytes and motoneurons [37, 38] and could be used to treat neurological disorders and traumas, including SCI. Following SCI, oligodendrocytes were shown to be highly vulnerable to the factors existing in inflamed tissue and may undergo cell death. This loss of myelinating cells will cause abnormal neuronal functionality but hESC-derived oligodendrocyte transplantation can restore the functional outcome [38] in animals initiated by the activation of brain-derived neurotrophic factor and IL-6 signaling pathways [39]. However, clinical applications of hESCs critically depend on their ability to differentiate toward defined and purified neural cell types in vitro. Due to often lengthy and complex differentiation protocols, current protocols for differentiation of neural progenitors from hESCs include application of undesired cell types and undefined factors (such as neural inducing stroma PA6 and MS5). Some new studies [40–42] have focused on the improvement of the methods for predifferentiation of hESCs into neural or neuronal precursors before cell transplantation to models of SCI. For instance, vitronectin in combination with retinoic acid, Sonic hedgehog, Noggin, or SB431542 drug (SMAD signaling pathway inhibitor) promote oligodendrocyte differentiation of hESCs [41], whereas the addition of insulin prevents apoptosis in ESC-derived neural precursor cells (NPCs) [42]. Noggin or SB431542 treatment improves the efficiency of neural induction, showing synergistic effects [40]. The rosette-forming cells (neuroectodermal structures capable of differentiation into neuronal cells) or neuroepithelial stem cell populations isolated from hESCs retain a broad differentiation potential [43, 44]. These cells maintain a neurodifferentiation potential even after long-term in vitro growth, retaining their ability to proliferate even after several passages. Furthermore, the growth of undifferentiated hESCs in chemically defined media without animal-derived components has been well established [45], and considerable effort is under way to induce targeted neural differentiation [38, 46, 47] of hESCs using animal- and serum-free conditions [48, 49]. Several reports [37, 49, 50], including our own [49], have demonstrated the in vitro capacity of hESCs to generate NPCs, including regionally specific neuronal subtypes. The ability to direct NPCs to regional specific neurons is a distinct property of hESC-derived NPCs, which is a considerable advantage over adult NSCs, in which developmental programming and directed differentiation have not been shown to be effective. The transplantation of hESC-derived NPCs alone may not recover demyelinated axons by a remyelinating activity but the beneficial effect of the transplanted hESC-derived NPCs could be due to a neuroprotective mechanism [51] that is provoked by an immunomodulatory [52] or a suppression effect on T cells [51, 53].

Nevertheless, there are several concerns regarding the safety of transplantation of hESCs in humans, including the controversial formation of teratomas following hESC-derived neural cell engraftment [54]. The possible reason for this conflict could be the usage of different cell lines, various differentiation protocols, and heterogeneous cell populations. Therefore, the application of prolonged differentiation of hESCs [55], fluorescent activated cell sorting or magnetic activated cell sorting [56], or inhibition of proliferation signaling pathways by genetic manipulation [40, 54] decreases the incidence of tumor formation [55] and efficiently converts hESCs into neural cells [40].

Transplantation of hESC-derived neural progenitor cells with cellular matrix protein-based synthetic three-dimensional biodegradable scaffolds such as laminin, fibronectin [56], or collagen [57] could be of advantage because these environments provide an adhesive support and may also release some growth factors such as NT-3 and PDGF [58–60]. This strategy has been confirmed after transplantation of hESC-derived neural progenitors into a rat model of SCI using collagen scaffolds [57].

With the improvement of growth and differentiation conditions, the first FDA approval (http://www.fda.gov) for the preclinical usage of differentiated hESCs for the treatment of SCI makes hESCs a very attractive source for clinical applications. However, in August 2009, the FDA put a clinical hold on hESC clinical trials because further characterization of differentiated cells and more nonclinical trials/applications of hESC-derived neural cells into animal models have been requested. Nevertheless, compared with the other sources of cell therapy, hESCs are one of the most attractive cell sources for spinal cord therapy and this strategy has been validated recently [37], as hESC-derived motoneurons can survive and integrate into the spinal cord (Fig. 2D).

**Neural Stem Cells**

Recruitment of endogenous NSCs or transplantation of NSCs would be alternative strategies for the treatment of SCI. Neural stem cells are multipotent cells with the potential to differentiate into neurons, oligodendrocytes, and astrocytes and can be efficiently propagated in vitro [61, 62]. These cells can be obtained from the spinal cord and their characteristics are different from NSCs obtained from the forebrain [63]. In the adult CNS, the tissue adjacent to ventricles and the ependymal cells directly lining the lateral ventricles are known to be rich sources of multipotent NSCs [64]. The proliferation in the central ependymal canal of the spinal cord produces many new progenitor cells that are capable of differentiating toward cells with neural and neuronal characteristics [65]. Following SCI, resident ependymal stem cells are activated and proliferate; after contusion, nearly 2 million new cells are produced at the injury site within a month, peaking at 3–7 days after injury [66] (reviewed in [67]). However, this activation is not sufficient itself to promote recovery [63]. The ependymal stem cell progeny that proliferates in response to SCI migrates to the lesion site and contributes to the glial scar. The majority of these cells show immunoreactivity for Sox9 and vimentin, with astrocyte-like morphology (but they are GFAP negative) [68], and a smaller cell population expresses Olig2, an immature oligodendrocyte phenotype.

We have recently reported a functional motor recovery after transplantation of ependymal stem progenitor cells that were derived from adult rat spinal cord suffering a traumatic lesion [66]. The cells were propagated and differentiated in vitro to obtain oligodendrocytes precursor cells (OPCs), which were engrafted after cell transplantation [66]. In general, it is believed that following SCI, endogenous or transplanted NSCs differentiate mostly into oligodendrocytes (Fig. 2C) and astrocytes, contributing to remyelination of axons and helping in the recovery [63]. Because demyelination is a progressive problem following SCI, OPC transplantation of glial-restricted...
progenitor cells (GRP) and OPCs differentiated from NSCs could be a promising strategy to treat SCI [69]. Indeed, astrocytes mediate neuroprotection, because the transplantation of lineage-restricted astrocyte precursors, also called GRPs, has been shown to be able to decrease focal motor neuron loss [70].

Mature oligodendrocytes differentiated from OPCs are expected to remyelinate the axons in white matter, and astrocytes differentiated from GRPs secrete many neurotrophic factors that could support axonal regeneration and cell survival [71, 72]. However, the glial scar formation blocks the access of OPCs to demyelinated axons, and the expression of some inhibitory molecules by astrocytes (such as Jagged1) inhibits OPC differentiation and proliferation. These two processes represent very important barriers for endogenous OPC remyelination [73].

Grown under in vitro conditions, the NSCs maintain their capacity for self-renewal after several passages and are capable of secreting neurotrophic factors [73, 74]. One disadvantage of in vitro-derived NSCs for clinical therapies is their decreased potential of differentiation after several passages [75]. Moreover, differentiation of NSCs into pure neural populations has not been reported yet [73]. Endogenous neural progenitors are inefficient in differentiation toward motoneurons because the ratio of Ngn2 to Olig2, which determines motoneuron versus oligodendrocyte differentiation, is 10-fold lower in neural progenitors than in ESCs [76]. This problem could be solved by overexpressing some genes involved in motoneuronal development including HB9, NKX6.1, and NGN2 [77] or by a coculture system with endothelial cells that enhance the motoneuron differentiation from NSCs where NSC-derived motoneuron progenitors promote functional recovery of a SCI model [78].

There are very few reports [79–82] that describe the mechanism of integration of NSCs and how they promote functional recovery after SCI. Recently, Hooshmand et al. [79] have shown that there is no change in the host microenvironment following analysis of NSC engrafted models of SCI. Lesion size, tissue sparing, glial scar, and expression of proteins such as fibronectin, NG2, versican, GFAP, and PECAM1 showed no change following cell implantation [79]. However, the source of transplanted NSCs and methods of isolation and preparation of cells prior to implantation seem to be very critical in cell survival and integration after implantation [79, 83]. For instance, monolayer cell cultures enhance the proliferation of multipotent NSCs [84], whereas neurosphere cultures result in more restricted NSC populations [79, 85]. Interestingly, monolayer and neurosphere cultures do not behave in the same manner following transplantation [86]. Additionally, the type of animal model, immunosuppression used, time of transplantation, and type of injury affect the mechanism(s) of recovery. For instance, concussion injuries result in neural and neuronal apoptosis in rostral and caudal parts of lesion sites and are accompanied by secondary inflammatory responses, whereas transection injuries result in a small zone of tissue damage but have active necrotic mechanisms [79, 87].

A part of the scientific community believes that NSCs are more preferable than hESCs for clinical applications because they are considered safer for cell therapy, as NSCs have less potential to form tumors compared with ESCs [72]. However, many critical challenges remain using NSCs for clinical applications, including the need for pure populations of differentiated cells, inefficient tracking systems, and moderate cell survival after transplantation [62, 88]. Additional obstacles of axonal regeneration and extension by cell replacement by either endogenous or exogenous NSCs remain: the formation of glial scars, the lack of neurotrophic factors, inhibitory myelin-associated molecules, and decreased levels of cAMP [73, 89].

**Future Prospective: Induced Pluripotent Stem Cells**

A potential alternative to avoid immunological rejection after nonautologous transplantation of stem cells is the use of reprogrammed adult cell (iPSC) technology, which means derivation of patient-specific and pluripotent cells derived from adult somatic cells. These cells have been generated from mouse and human somatic cells by overexpression of several defined factors [90–97]. Recently, generation of iPSCs from human NSCs with a single transcription factor, OCT4, or using direct delivery of recombinant proteins has been described [98, 99]. iPSCs have identical patterns in gene expression, chromatin methylation, and embryoid body and viable chimera formation [100] as ESCs. They are capable of differentiation toward all cell types, including neurons, glia, NPCs, and motoneurons [101, 102]. Furthermore, the derivation of iPSCs using nonviral methods [103] or by chemicals and small molecules [104] including protein iPSCs [105] makes this cell replacement strategy very attractive. Nevertheless, these cell types share similar disadvantages as other cell sources: teratoma formation, aberrant reprogramming, and the presence of transgenes in iPSC populations are the most concerning obstacles, which should be addressed before their clinical application [106].

** Conclusion**

Regeneration and replacement of neurons and glia that undergo cell death soon after injury are the main goals of all stem cell-based therapies for SCI. Although stem cell transplantation strategies have not yet been clinically approved, they are currently the most effective and efficient way to improve motor function in animal models of SCI. Successful development of stem cell-based therapies for SCI requires more intensive work to obtain a better understanding of stem cell differentiation pathways and stem cell survival upon transplantation. All stem cell replacement strategies should address these two important problems and this may be accomplished through improved differentiation protocols of hESCs/iPSCs, transplantation of OPCs and NPCs, and/or by activation of endogenous sources of neural progenitors. However, the ideal source of stem cells for efficient and safe cell replacement has remained a challenging issue that requires more investigation.

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**Disclosure of Potential Conflicts of Interest**

The authors indicate no potential conflicts of interest.

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