Transplanted neuroectodermal and induced pluripotent stem cells improve the outcome of spinal cord contusion injury: modulation of the lesion microenvironment

Ph.D. Thesis

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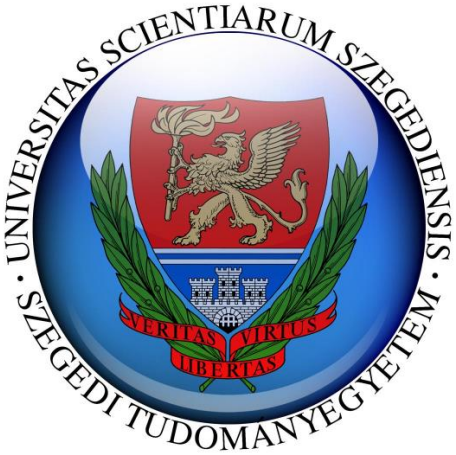

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Original papers related to this thesis

I. Pajer K, **Bellák T**, Redl H, Nógrádi A. Neuroectodermal Stem Cells Grafted into the Injured Spinal Cord Induce Both Axonal Regeneration and Morphological Restoration via Multiple Mechanisms.
J Neurotrauma. 2019 Nov 1;36(21):2977-2990.
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II. **Bellák T**, Fekécs Z, Török D, Táncos Z, Nemes C, Tézsla Z, Gál L, Polgári S, Kobolák J, Dinnyés A, Nógrádi A, Pajer K. Grafted human induced pluripotent stem cells improve the outcome of spinal cord injury: modulation of the lesion microenvironment.
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Introduction

Spinal cord injury (SCI) results in irreversible tissue damage with usually very limited functional recovery. This injury is accompanied by motor, sensory and vegetative function losses, which diminishes the quality of life of both the patient and his/her family. SCI also affects the society due to the expensive medical care, estimated to 1.5-3.0 million dollars per injured individual. The annual incidence of SCI ranges from 30 to 70 cases per million people, resulting in over half million new patients in every year globally.

SCI etiologies can be subdivided into traumatic and non-traumatic injury. Approximately 90% of the SCI cases are traumatic injury and only 10% are caused by various diseases acquired at birth or later in life including tumors, spinal disc degeneration, arthritis or loss of oxygen related to surgical maneuvers. Traumatic SCIs are mostly related to traffic or sports accidents and criminal cases, for example falling from heights or violence (gunshot, stab wound).

SCIs can also be distinguished as complete or incomplete. A complete spinal cord injury is defined with the absence of all motor and sensory functions distal to the level of injury. Incomplete injuries are characterized with some degree of remaining/recovered motor or sensory function below the lesion.

Cell transplantation is one of the most widespread and promising possibilities to induce morphological and functional improvement after SCI. Numerous multipotent or pluripotent cell types and their derivatives have been investigated in experimental models of SCI. The therapeutic potential of each varies depending on the cellular behavior, survival and proliferation, as well as the unique differentiation capacity after transplantation. Grafted cells and their derivatives are able to integrate into the host tissue or alter the lesion microenvironment rendering it permissive for regenerating axons. They are also involved in remyelination processes, trophic support or a combination thereof.

Our laboratory has provided evidence that the immortalized neuroectodermal stem cell line NE-GFP-4C, derived from the forebrain vesicle wall of p53-deficient 9-day-old mouse embryos effectively prevents the death of motoneurons destined to die after a spinal ventral root avulsion injury. It has been proven that the grafted cells produce a secretome induced by the motoneuron damage which supported motoneuron survival. In this study we hypothesized that transplantation of these cells into a contused spinal cord may lead to robust morphological regeneration and restoration of function after injury.

In our other study we used the SB5 hiPSC line that exhibited the characteristics of pluripotent stem cells, including the expression of embryonic stem cell markers and had the ability to differentiate in vitro into the three germ layers. Based on the favorable properties of the SB5 cell line we wanted to test whether grafted hiPSCs are able to lead considerable morphological regeneration and/or tissue sparing and functional recovery after injury.

Aims of the study

We intended

1. to investigate the effect of transplanted NE-GFP-4C murine neuroectodermal and undifferentiated SB5 human induced pluripotent stem cells on functional recovery.
2. to reveal the fate and differentiation capacity of transplanted cells and the changes in the microenvironment of the injured host tissue.
3. to determine the set of bioactive molecules (secretome) which are responsible for the morphological and functional improvement.

Material and methods

Spinal cord injury model

All together 90 female Sprague-Dawley rats (Animal Research Laboratories, Vienna, Austria, weighing 180-220 g body weight) and 80 female Fischer 344 rats (Biological Services, University of Szeged, Szeged, Hungary, 180–220 g body weight) were used throughout the experiments. Laminectomy was performed at the T11 vertebral level, the dura mater was exposed and the spinal cord was contused using an Infinity Horizon impactor (IH-0400, PSI Services LLC, California, USA), applying 150 kdyn force. All animals were allowed to survive 2, 3 or 8 weeks (Sprague-Dawley rats) or 2, 3, 5 or 9 weeks (Fischer 344 rats) after injury.

Transplantation of NE-GFP-4C neuroectodermal or SB5 human induced pluripotent stem cells after contusion injury

Immediately or one week after injury, NE-GFP-4C cells were transplanted intravenously or intraspinally (depending on the experimental setup) using Hamilton pipettes (Sprague-Dawley rats). Intravenously $1x10^6$ (delivered in 250 μL medium), intraspinally $5x10^5$ stem cells (delivered in 2 μL medium) were injected into the tail vein or into the lesion cavity, respectively. In a separate experimental group stem cells were grafted intraspinally mixed with human fibrin clot matrix (Baxter Healthcare Corporation, Westlake Village, California, USA, 500 I.U./mL). Control animals received medium or fibrin intraspinally (2 μL) or medium intravenously (250 μL) one week or medium intravenously (250 μL) immediately after injury.

The following experimental groups were established in this study:

1) **fibrin-1w-isp**: fibrin was injected intraspinally 1 week after injury (control)
2) **medium-1w-isp**: medium was injected intraspinally 1 week after injury (control)
3) **medium-immed-iv**: medium was injected intravenously immediately after injury (control)
4) **medium-1w-iv**: medium was injected intravenously 1 week after injury (control)
5) **NE4C-fibrin**: stem cells in fibrin were transplanted intraspinally 1 week after injury
6) **NE4C-immed-iv**: stem cells were transplanted intravenously immediately after injury
7) **NE4C-1w-iv**: stem cells were transplanted intravenously 1 week after injury
8) **NE4C-1w-isp**: stem cells were transplanted intraspinally 1 week after injury

One week after injury, SB5 hiPSCs were transplanted intravenously or intraspinally (depending on the experimental setup) into the spinal cord of Fischer 344 rats. In the case of intravenous

administration $1x10^6$ cells (delivered in 250 μL medium) were injected in the tail vein, while intraspinally $5x10^5$ cells (in 2 μL medium) were slowly deliver into the lesion cavity, through the use of Hamilton pipette. Control animals received medium only intravenously (250 μL) or intraspinally (2 μL) one week after injury.

The following experimental groups were set up in this study:

1) **medium-iv**: medium injected intravenously 1 week after injury (control)
2) **medium-isp**: medium injected intraspinally 1 week after injury (control)
3) **SB5-iv**: hiPSCs transplanted intravenously 1 week after injury
4) **SB5-isp**: hiPSCs transplanted intraspinally 1 week after injury

Retrograde labeling

Eight weeks after injury animals with long-term survival were deeply anaesthetized. Laminectomies were made at the T13–L1 vertebral level (corresponding to the L2–L4 spinal level) to expose the upper lumbosacral enlargement. The L3 spinal segment was identified and a right hemisection was performed. Fast Blue (FB) crystals (Dr. Illing Plastics GmbH, Groß-Umstadt, Germany) were placed into the gap, the dura flap was placed to the hemisection area and the wound was closed in. Rats were kept alive for seven days after the labeling, then they were re-anaesthetized and perfused transcardially.

BBB open field locomotor score

The Basso, Beattie, Bresnahan (BBB) locomotor rating scale was performed on day 3 and then every week after injury for 8 weeks. Two observers, unaware of experimental procedures tested the animals. Rats were assessed in an open field (150 × 100 cm) for 4 minutes at a similar time of the day for each testing. We randomly allocated the injured animals into experimental groups in the manner that all groups consisted of animals with comparable range of BBB scores as well as group average. This randomization ensured the presence of equivalent locomotor deficits across the groups before the beginning of treatment.

Analysis of locomotion pattern in the NE-GFP-4C study – CatWalk gait analysis

To determine and analyze the parameters of the movement pattern, the 'CatWalk' automated quantitative gait analysis system (Noldus, Wageningen, The Netherlands) was used. The following parameters were taken into account during the analysis: max area, print area, stride length, swing duration and swing speed. We measured these parameters eight weeks after the injury. Animals

had a 2-week-long training period before the contusion injury. Data were evaluated by the CatWalk® software.

Analysis of locomotion pattern in the SB5 hiPSCs study

Between the 4th and the 8th postoperative week video-based kinematic analysis was carried out. The hair of the rats was shaved off from the hind limbs and the skin was marked by a black pen above the major joints. We used a plexiglass runway equipped with a mirror system to be able to record the position of the hind limb from both lateral and rear-view aspects. Two high resolution and high-speed cameras (GoPro Hero 3+ Black Edition, GoPro; San Mateo, California, USA; DFK 22AUC03, The Imaging Source, Bremen, Germany) were used to during 3 to 4 step cycles. The animals were trained prior to the measurements to walk from one end of the runway to the other reaching a shelter and were tested every week postoperatively. By comparing specific single video frames, we measured six different parameters to get detailed information on the recovery.

Results

NE-GFP-4C neuroectodermal stem cells induce morphological and functional recovery via multiple mechanisms

General observations, functional improvement and CatWalk gait analysis

The BBB open field test was used at regular intervals to assess locomotor recovery. After the contusion injury, all animals displayed strong motor impairment. During the first week no weight supported stepping was observed in all groups. Intraspinal application of stem cells (NE4C-1w-isp) or medium (medium-1w-isp) on week 1 after the injury induced a plateau in locomotor recovery. This phenomenon is due to the minimally invasive nature of intraspinal grafting of cells or injection of medium. In contrast, systemic application of stem cells (NE4C-immed-iv, NE4C-1w-iv) or medium (medium-1w-iv) did not induce any drop in locomotor recovery. Four weeks after the injury the animals that received intraspinal grafts displayed the same BBB scores as the intravenously grafted groups. From 5 to 8 weeks after injury consistent plantar stepping with predominant parallel paw position and consistent fore- and hind limb coordination could be observed in the animals that received systemic and intraspinal grafts. Interestingly, animals whose spinal cord cavity was filled with NE-GFP-4C cells mixed with fibrin (NE4C-fibrin) had more impaired locomotion than their controls (fibrin-1w-isp). These animals showed occasional weight supported dorsal or plantar stepping without fore- and hind limb coordination.

The results of the automated gait analysis (CatWalk) confirmed our functional observations on freely moving animals. We evaluated the print and maximum area of hind limb, swing duration, swing speed and stride length 8 weeks after injury. The animals of the NE4C-fibrin group could not be processed for CatWalk® analysis as these rats displayed only occasional weight support. The functional recovery was significantly improved in the intravenously and intraspinally grafted groups (NE4C-immed-iv, NE4C-1w-iv and NE4C-1w-isp) compared with their controls.

Stem cells induce tissue sparing

Within the injured segment a large, centrally located cystic cavity was formed containing cellular debris. The morphological analysis involved measuring the rostrocaudal distribution of spared gray and white matter in the injured segments 8 weeks after the injury. Both the intravenous and intraspinal application of stem cells resulted in significantly improved tissue sparing relative to the controls. The highest percentage of spared tissue was detected in those animals that received intraspinal grafts (NE4C-1w-isp). The intravenously treated animals (NE4C-immed-iv and NE4C-1w-iv) also displayed high, but non-significantly lower amount of spared tissue compared with the intraspinally treated animals. The greatest amount of white matter localized to the ventral and ventrolateral parts of the spinal cord in these groups. In contrast, in animals treated with stem cells mixed with fibrin (NE4C-fibrin), the spared white and gray matter was smaller than in all the other groups.

The basic parameters of the cavity such as lesion area at the epicenter (cystic area) and length of the cavity on week 8 following the injury revealed that both the intravenously and intraspinally treated animals (NE4C-immed-iv, NE4C-1w-iv, NE4C-1w-isp) had significantly smaller cavity length and lesion area than their controls. No significant difference was found in animals that received their stem cells embedded in fibrin (NE4C-fibrin) compared with their control group.

Retrograde neuronal tracing analysis, preservation of serotonergic innervation following intraspinal grafting

Next, we evaluated whether axonal regeneration/sparing was promoted by the grafted stem cells. Retrograde labeling with the fluorescent dye Fast Blue (FB) from the right L3 segment revealed that significantly higher numbers of FB-labeled propriospinal neurons were found in the Th5, Th2, C6 and C2 spinal segments in animals treated with stem cells (NE4C-immed-iv, NE4C-1w-iv and

NE4C-1w-isp) than in their controls. The number of retrogradely labeled neurons in the NE4C-fibrin group was not statistically different from the control groups.

Both the total number of FB cells in the brain stem and their numbers in the selected brain stem nuclei (reticular formation, lateral vestibular nucleus and inferior olivary nucleus) projecting distally to the lesion site were significantly higher compared with their controls. This was also the case for layer V neurons in the motor cortex whose axons rarely were able to project into the distal spinal cord. Only the intraspinally grafted animals (NE4C-1w-isp) showed an exception as these rats displayed significantly greater numbers of retrogradely labeled pyramidal cells compared with the controls and the intravenously grafted animals (NE4C-immed-iv, NE4C-1w-iv).

In contrast, the raphe nuclei of animals that received intraspinal grafts (NE4C-1w-isp) displayed significantly more FB-labeled neurons than those of intravenously grafted animals (NE4C-immed-iv, NE4C-1w-iv) or their controls.

Serotonergic fibers reportedly exert strong supraspinal influence on locomotion. Sprouting of the remaining 5-HT+ fibers caudally to a contusion injury is found to be limited by successful therapeutic approaches. Accordingly, descending serotonergic fiber density was measured caudal to the injury in the ventral horn of the L2 spinal segment of intravenously and intraspinally grafted animals (NE4C-immed-iv, NE4C-1w-iv, NE4C-1w-isp) and in the control group (medium-1w-isp). We found a 5-to-5.7 fold increase of 5-HT+ fiber density in the control and in the intravenously grafted groups. In contrast, a small, 1.8 fold increase of density was observed in the intraspinally grafted animals compared to the uninjured value.

Modulation of the lesion environment

Next, we investigated whether the grafted cells have the potential to alter the microenvironment of the lesion rendering it permissive for regenerating axons. Therefore, we examined the densities of astrocytes and microglia/macrophages, the expression of Eph-A4 and ehprin-B2 molecules and the deposition of chondroitin sulfate proteoglycan (CSPG) 2 and 3 weeks after the injury around the lesion cavity.

Both intraspinal or intravenous applications of stem cells induced significant reduction of astrocytosis at both time points indicated by significantly lower GFAP densities in these cords compared with their controls. Decreased GFAP densities were accompanied in these animals by limited CS-56+ depositions. However, strong astrocytic reaction was detected in animals that

received stem cells in fibrin glue (NE4C-fibrin). Accordingly, CS-56 densities in these animals were as high as in their controls. Moreover, microglia/macrophage densities paralleled that of the astrocytes indicating a close link between the proliferation of these cell types.

Earlier studies have shown that following spinal cord injury, EphA receptor subtypes and their ephrin ligands are upregulated and these interactions may create an environment that is unfavorable for neurite outgrowth and functional regeneration. The EphA4-ephrinB2 binding reportedly induces collapse of neural growth cones in the presence of reactive astrocytes. While EphA4 expression did not change significantly in the systemic and intraspinal treatment groups, ephrinB2 expression was found significantly lower in the intraspinally grafted animals 2 and 3 weeks after injury. The same effect was noted in the systemic treatment group only on week 3.

Differentiation of the grafted cells in the injured cord

To determine the differentiation potential of the grafted stem cells in the injured cord, we analyzed the expression of murine markers that are able to uniquely identify grafted mouse cells in a rat environment.

One week following intravenous transplantation (NE4C-immed-iv, NE4C-1w-iv) only M2-positive astrocytes could be seen in the injured cord and all these cells disappeared by the end of the investigation period. Stem cell-derived astrocytes settled down mainly in the wall of the cavity and expressed the glial marker GFAP.

In contrast, the intraspinally grafted stem cells (NE4C-1w-isp) differentiated into neurons (M6+) and astrocytes (M2+) one week after transplantation. Stem cell-derived oligodendrocytes could not be detected at this stage. The majority of these stem cell-derived neurons and astrocytes also expressed beta(III)-tubulin/TUBB3 and GFAP, respectively. The grafted cells filled the cystic cavity without migrating out of it to the surrounding spinal cord tissue. At the end of the survival period stem cell-derived neurons, astrocytes and oligodendrocytes (MOG+) were found in the wall of the cavity. Neurofilament 200kD+ and MOG+ profiles were observed in close relationship to each other indicating that the stem cell-derived oligodendrocytes might have contributed to the remyelination of naked axons.

The eGFP expression of the grafted cells had decreased or ceased, suggesting that the eGFP expression pattern diminishes with differentiation confirmed by our earlier study, too.

Factors produced by grafted stem cells induce functional recovery in the intraspinally grafted animals

In case of intraspinal grafting the transplanted cells are opposed to the wall of the cavity. The fact that only limited numbers of graft-derived cells settled in the wall of the cavity suggested that there is a paracrine secretory mechanism acting on the host spinal cord. To determine the factors secreted by the grafted cells, we analyzed the expression of 10 factors (BDNF, GDNF, IL-1 alpha, IL-6, IL-10, TNF-alpha, MIP-1 alpha, NT-4/5, VEGF, PDGF-A), thought to exert beneficial effects on the damaged cord. Four out of the ten factors were found to be expressed in the graft (GDNF, IL-6, IL-10 and MIP-1 alpha). GDNF showed strong expression patterns both in the host cord and in the graft. Strong IL-6 expression was confined to the grafted cells accompanied by weaker staining in the host spinal cord. IL-10 and MIP-1 alpha expressions showed similar distribution pattern to that of GDNF, but the expression of these two factors within the host cord was restricted to the tissue located ventral to the cavity. Interestingly, the control group animals (medium-1w-isp) that did not receive any graft showed no expression of the various factors. Comparison of immunoreactivity of all four factors across the control (medium-1w-isp) and grafted (NE4C-1w-isp) experimental spinal cords revealed a robust increase in GDNF, IL-6, IL-10 and MIP-1 alpha expression in the host tissue of grafted group.

Grafted SB5 human induced pluripotent stem cells improve the outcome of spinal cord injury

Hind limb locomotor pattern has been improved following intraspinal transplantation of hiPSCs

During the first week, injured rats showed no weight supported stepping. The animals showed frequent to consistent weight supported plantar steps and occasional consistent weight supported plantar steps at week 5 in all groups. At weeks 6 and 7 the stem cell-treated animals (SB5-iv, SB5-isp groups) showed a slight increase in BBB score compared to their controls. From 1 to 8 weeks after injury, we found a statistically significant interaction only between the SB5-isp and their controls at weeks 6–8 after injury. These rats showed consistent weight supported plantar steps and consistent fore- and hind limb coordination.

The kinematic analysis of transplanted and control rats was evaluated for metatarsus surface angle, tibia-surface angle, lateral placing, knee flexion, tarsus off angle and ankle flexion. During pre-

injury training, all rats accurately accomplished the test. After SCI, all the four groups (medium-iv, medium-isp, SB5-iv and SB5-isp animals) demonstrated deficits in hindlimb placements. Consistent with the BBB results, all groups showed a non-significantly improvement up to week 5. From week 6 onwards the rats that received hiPSCs treatment intraspinally (SB5-isp) progressively improved until week 8. The kinematic analysis assessments also revealed that the SB5-isp animals displayed a consistent improvement in the metatarsus surface angle, tibia-surface angle, lateral placing knee flexion, tarsus off angle and ankle flexion in hindlimb placement in contrast to control animals that displayed slight recovery after SCI. Only the intraspinally grafted animals (SB5-isp) were able to approach the intact pre-training values and showed statistically significant improvement compared with their controls.

Intraspinally grafted hiPSCs enhance tissue sparing

Histomorphometric analysis was performed to quantify spinal cord tissue changes 9 weeks after SCI. Within the injured segment a large, centrally located cystic cavity was formed containing cellular debris or trabecula. The greatest amount of intact-looking white matter localized to the ventral and ventrolateral parts of the spinal cord in the experimental groups. Reduced cystic tissue was observed in the epicenter and 0.5 mm rostrally and caudally to the injury epicenter in grafted animals. Significantly decreased lesion area was identified at 1 mm and 2 mm rostrally and at 1.5 mm and 2 mm caudally to injury in the spinal cord of animals in the SB5-isp group (SB5-isp vs. medium-isp group). Quantification of spared tissue in injured spinal cords based on cresyl-violet staining indicated that a significantly greater amount of tissue was preserved in the animals of the intraspinally grafted group compared with its controls (SB5-isp vs. medium-isp group).

hiPSCs treatment leads to improved connections as revealed by retrograde tracing

Next, we evaluated whether axonal regeneration/sparing was promoted by the grafted hiPSCs. Labeling of propriospinal and supraspinal neurons in the spinal cord and brain was evaluated by placing the retrograde tracer (Fast Blue) caudally to the injury into the right L3 segment, the numbers of retrogradely labeled neuronal somata in the spinal cord, brain stem and brain were determined. These data refer to the number of neurons above the injury site which axons take up the tracer in the caudal spinal cord segment. Significantly higher numbers of FB-labeled propriospinal neurons were found in the Th5, Th1, C6 and C2 spinal segments in animals treated

with hiPSCs (both SB5-iv and SB5-isp) than in their controls. It should be noted that the number of retrogradely traced neurons decreased with the distance from the labeled segment. We also identified retrogradely labeled neurons in the brain stem (particularly in the reticular formation, raphe nuclei) and in the somatomotor cortex. Following intraspinal implantation of hiPSCs significantly higher number of retrogradely labeled neuronal somata were found in the different brain regions. In the case of systemic (iv) delivery of hiPSCs (SB5-iv), non-significantly higher number of FB+ neurons were found in these supraspinal locations compared with their controls.

hiPSC treatment influences the astroglia and microglia/macrophage reaction

Next, we investigated whether the hiPSC treatment has the potential to alter the microenvironment of the lesion rendering it permissive for regenerating axons. Therefore, we examined and quantified the densities of astrocytes and microglia/macrophages and the deposition of chondroitin sulphate proteoglycan (CSPG) 9 weeks after the injury around the lesion cavity. Analysis of the immunostained sections showed differences in the amount of GFAP, CS-56 and GSA-B4 expression among the groups. Both intraspinal and intravenous application of hiPSCs induced significant reduction of astrocytosis indicated by significantly lower GFAP densities in these cords compared with controls (SB5-isp, SB5-iv *vs* their controls). While hiPSC treatment altered the GFAP immunointensity, marked reduction of CS-56 immunoreactivity could be observed compared to controls (SB5-isp, SB5-iv vs. their controls, $p < 0.01$). Similarly, microglia/macrophages in the lesion sites of grafted animals showed significantly reduced microgliosis (GSA-B4 lectin histochemistry) compared with the control groups (SB5-isp, SB5-iv vs. their controls, $p< 0.01$). However, grafting of hiPSCs induced significantly decreased microglia/macrophage densities at week 8 after grafting compared with the intravenous hiPSC treatment (SB5-isp *vs* SB5-iv, $p < 0.01$).

Intraspinally grafted iPSCs differentiate preferentially along a neuronal lineage

To examine the feasibility of hiPSCs transplantation as a therapeutic tool for SCI, we grafted the cells into an injury cavity 1 week after SCI. One, two, four and eight weeks after transplantation, immunohistochemical analyses were performed to examine the survival, migration, proliferation and differentiation patterns of the grafted hiPSCs in the injured spinal cords. hiPSCs were mapped for expression of human SC-121 (reacts with a human cytoplasmic protein) and SC-101 (reacts

with any human cell nucleus) in adjacent sections. At these early survival times, the hiPSCs did not migrate yet away from the graft.

The engrafted hiPSCs survived remarkably, expressed SSEA-4, proliferated within the injured spinal cord and formed clusters or showed disperse distribution patterns. The intensity of SC-121 immunostaining was stronger within the clusters formed by grafted cells than in their vicinity. The proliferation capacity of the clusters was evaluated with Ki-67 immunostaining and appeared to be low. In contrast, around the clusters more Ki-67+ cells were displayed at both survival time points. Quantitative analysis showed that 1 and 2 weeks after transplantation, 26-29% of the SC-121-positive and 29-33% of the SC-101-positive grafted cells were Ki-67 positive.

Using cellular markers, we investigated the differentiation pattern of hiPSCs in the SB5-isp group at these early survival time points. Our findings demonstrated that the majority of the transplanted hiPSCs differentiated along a neuronal lineage as demonstrated by TUBB3/SC-121 and TUBB3/SC-101 co-expression at both experimental time points (TUBB3/SC-121: ~ 66% at 1 week and ~73% at 2 weeks; TUBB3/SC-101: ~62% at 1 week and ~74% at 2 weeks). These findings suggest that grafted hiPSCs promote preferential differentiation toward the neuronal lineage. We did not observe any hiPSC-derived cells expressing GFAP 1 week after transplantation.

At 4 weeks after hiPSC transplantation, sporadically observable SC-121-positive or SC-101-positive profiles were present in the grafted area. At 8 weeks after grafting, phagocytosed SC-121+ cellular fragments were readily observed in GSA-B4-positive macrophages.

Protein expression pattern of neurotrophic factors and cytokines in the grafted cells 1 week after transplantation

To determine the protein production (secretome) of SB5 cells in vitro, first we analyzed the expression of 10 factors (BDNF, GDNF, IL-1 alpha, IL-6, IL-10, TNF-alpha, MIP-1 alpha, NT-4/5, VEGF, PDGFA), a selection based on our earlier results. Strong immunoreactivity of GDNF, TNF-alpha and VEGF was found in vitro in undifferentiated SB5 cells.

Three out of the ten factors were found to be expressed in the graft (GDNF, IL-10 and MIP-1 alpha). None of these factors was found to be expressed in the host spinal cords. The expression of the factors was characterized by granular appearance. IL-10 showed similar distribution pattern to that of GDNF while weak MIP-1 alpha expression was confined to the grafted. Interestingly,

controls or SB5-iv group showed no expression of the various factors within injured cords 2 weeks after the injury.

Discussion

Transplanted NE-GFP-4C and SB5 cells induce tissue sparing and improved functional recovery

Both intraspinal and intravenous application of NE-GFP-4C neuroectodermal stem cells and SB5 undifferentiated hiPSCs decreased the extent of secondary damage in the injured cords. The significant amount of spared tissue made it possible to maintain the temporarily compromised connections between the intact regions above and below the injury site.

The considerable white matter sparing in the NE-GFP-4C treated animals was further supported by the fact that the lumbar cord segments of grafted animals displayed limited sprouting of 5-HT fibers as compared with control cords. The intraspinal grafting of SB5 cells also preserved serotonergic fibers. These phenomena are in agreement with previous works that have reported enhanced plasticity of serotonergic fibers leading to improved recovery of locomotion. The descending serotonergic tracts directly modulate the locomotor function. Disruption of the serotonergic pathways following SCI prevents the activation of locomotor central pattern generator and results in a subsequent depletion in 5-HT.

The tissue sparing yielded higher number of retrogradely labeled neurons in the spinal cord and the various brain regions of grafted animals compared with the controls. In our both studies higher number of retrogradely labeled brain stem neurons could be detected in stem cell grafted groups compared with controls. Regeneration of the axons of brain stem neurons is essential for locomotor recovery in the rat following SCI. Thus regeneration/sparing of these neurons has the potential to improve functional recovery if they reach or retain their connection with the targets distal to the lesion.

Grafted NE-GFP-4C and SB5 cells modulate the lesion microenvironment from inhibitory to permissive

Inhibitory factors upregulated after spinal cord injury and this leads to the inhibition of axon growth. In addition, external barriers set limitations to regeneration. Therefore, we investigated

whether the grafted cells have the potential to alter the microenvironment of the lesion rendering it permissive for regenerating axons.

Grafting of NE-GFP-4C neuroectodermal stem cells induced significantly lower astrocyte/microglia reaction than found in the control spinal cords. This resulted in lower amounts of CS-56 and ephrin-B2 in the microenvironment of the lesion and these favorable changes rendered the glial scar permissive for axonal regeneration. It appears feasible that these changes (astrocyte/microglia downregulation along associated with low densities of CS-56 and ephrin-B2) all induced an altered lesion microenvironment which promoted the regeneration of injured axons.

The hiPSC treatment also had the potential to alter the microenvironment of the lesion rendering it permissive for regenerating axons. Analysis of the immunostained sections showed differences in the amount of GFAP, CS-56 and GSA-B4 expression among the groups. Both intraspinal and intravenous application of hiPSCs induced significant reduction of astrocytosis indicated by significantly lower GFAP densities in these cords compared with controls. While hiPSC treatment altered the GFAP immunointensity, marked reduction of CS-56 immunoreactivity could be observed compared with controls. Similarly, microglia/macrophages in the lesion sites of grafted animals displayed significantly reduced microgliosis (GSA-B4, lectin histochemistry) compared with the control groups.

Intraspinally grafted NE-GFP-4C and SB5 cells have different fate and differentiation capacity

In vivo experiments from other laboratories have shown that the survival of transplanted cells is greater in the injured spinal cord than in the intact environment. However, the inhibitory factors of the injured cord make the environment unfavorable for the grafted cells to generate new neurons or myelinating oligodendrocytes. The cellular density of grafted cells also influences the cell fate and differentiation capacities.

To determine the differentiation potential of grafted NE-GFP-4C neuroectodermal stem cells we used murine markers that were able to uniquely identify grafted mouse cells in a rat environment. The intraspinally grafted stem cells (NE4C-1w-isp) differentiated into neurons (M6+) and astrocytes (M2+) from one week after transplantation. Stem cell-derived oligodendrocytes could not be detected at this stage. The majority of these stem cell-derived neurons and astrocytes also expressed beta(III)-tubulin/TUBB3 and GFAP, respectively. The grafted cells and their derivatives

filled the cystic cavity without migrating out of it to the surrounding spinal cord nervous tissue. At the end of the survival period stem cell-derived neurons, astrocytes and MOG+ oligodendrocytes were found in the wall of the cavity. NF200kD+ and MOG+ profiles were observed in close relationship to each other indicating that the stem cell-derived oligodendrocytes might have contributed to the remyelination of naked axons. The eGFP expression of the grafted cells had decreased or ceased, suggesting that the eGFP expression pattern diminishes with differentiation confirmed by our earlier study, too.

The iPSC-derived neural stem/progenitor cells are characterized by low expression level of immune-related proteins and immunosuppressive effects. The grafted SB5 cells were located in the lesion area and did not migrate away from the graft. The transplanted cells formed clusters of living cells within the graft area and approximately 27% of the grafted cells were positive for Ki-67. The low proliferative rate in the cell clusters combined with vigorous proliferation in the periphery of the graft suggested that the lack of cell migration and cluster formation may have induced extensive differentiation. Further analysis with neuronal markers (beta(III)-tubulin/TUBB3) showed that approximately 75% of the grafted cells differentiated into a neuronal lineage. In contrast to other studies no glial differentiation was found among the grafted human iPSCs. Although we clearly observed the close association of transplanted cells with endogenous astroglial processes at the graft-host border, no GFAP-positive grafted cells were detected. Our results suggest that the transplanted hiPSC failed to differentiate into mature myelin-forming oligodendrocytes, too. These observations suggest that the microenvironment of the injured spinal cord restricts the differentiation capacities of engrafted iPSCs and the cell source is crucial for the fate of the transplanted cells.

Intraspinally grafted NE-GFP-4C and SB5 cells produce a set of bioactive molecules

Increasing number of studies suggests that the grafted stem cells are able to secrete neurotrophic factors and cytokines which modify the lesion microenvironment via paracrine mechanisms.

Our results suggest that the grafted NE-GFP-4C stem cells may contribute to the improved functional recovery following contusion injury by a dual mechanism. The mechanism of action is based on the major paracrine immunomodulatory and neurotrophic effect and, to a much lesser extent on a cellular replacement mechanism. It appears evident that the grafted NE-GFP-4C cells produce a set of cytokines (IL-6, IL-10 and MIP-1 alpha) and GDNF. As reported by our laboratory

earlier, NE-GFP-4C cells did not produce any of these factors in culture. Accordingly, the grafted cells start factor production after a short period of time following grafting and this suggests the presence of a strong communicative interaction between the injured host tissue and the grafted cells.

Similarly to the results of NE-GFP-4C stem cell transplantation the intraspinally grafted hiPSCs produced bioactive molecules including cytokines (IL-10 and MIP-1 alpha) and the neurotrophic factor GDNF. The expression of these factors by grafted hiPSCs indicates a strong signaling and modulatory process in the injured spinal cord.

Intravenously transplanted NE-GFP-4C and SB5 cells

Previous studies have shown that intravenous cell treatment is a promising strategy to induce both morphological and functional recovery following subacute SCI. Intravenous infusion of stem cells for the treatment of SCI had a protective effect on blood vessels, reduced the area of spinal cord cavitation and promoted the restoration of motor function.

The intravenously transplanted NE-GFP-4C cells differentiated only into astrocytes and settled down mainly in the wall of the cavity. With the use of this transplantation paradigm only IL-10 was expressed around the lesion and no stem cell derivatives were found at the end of the survival period. The intravenous application of NE-GFP-4C cells was able to reduce the microglia/macrophage reactions in the affected spinal cord segments. These positive changes were associated with higher number of FB+ neurons. This could lead to improved functional recovery.

In the case of intravenously transplanted SB5 cells we did not find graft-derived cells in the host cord. Similarly to the NE-GFP-4C study the intravenous application of hiPSCs was also able to reduce the microglia/macrophage reactions and deposition of chondroitin-sulphate, but no significant tissue sparing was observed in the affected segment. Interestingly, intravenous hiPSC application uniquely increased the number of retrogradely labeled neurons in the spinal cord. Despite the higher number of retrogradely labeled neurons and decrease of glia reaction and deposits of CSPGs in the injured spinal cords, we could detect only moderate locomotor recovery in these animals. Our results also confirmed the rapid clearance of the intravenously administered hiPSCs.

These results suggest that intravenously applied NE-GFP-4C and SB5 cells may exert their primary effects outside the spinal cord perhaps by altering the immune-mediated secondary

pathological events after spinal cord injury. This phenomenon requires further studies to elucidate the exact mechanism of action.

Conclusions

In summary we can state that grafted NE-GFP-4C neuroectodermal stem cells and the undifferentiated SB5 hiPSCs are able to induce tissue sparing, morphological and functional recovery after spinal cord contusion injury. This effect is mainly due to paracrine secretion mechanism of the transplanted cells producing neurotrophic factor and cytokines, including GDNF, IL-6 and IL-10 and MIP-1 alpha. The secreted factors appeared to have rendered the microenvironment axonal growth-permissive and prevented the death of damaged neurons and glial cells otherwise destined to die. These results have supported the functional multipotency feature of stem cells that allows them to adapt to the lesion environment, which suggests the presence of a strong communicative interaction between the injured host tissue and the grafted cells, leading to the release of a “lesion-induced secretome” by the grafted cells.