Bone Marrow Stromal Cell - Mediated Neuroprotection for Spinal Cord Repair

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Voor mijn ouders

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PREFACE AND THESIS OUTLINE

Traumatic spinal cord injury affects an estimated 500.000 people in the United States and Europe alone and between fifteen per million (Europe) and forty per million (North America) new cases are reported each year. Mechanical force at the time of injury results in immediate neural cell death, severing of axons, rupturing of blood vessels, and overall loss of tissue integrity. Injury-induced events including inflammatory responses and release of cytotoxic substances result in progressive neuronal and oligodendrocyte death, demyelination, and axonal damage. Secondary injury contributes significantly to the pathology of spinal cord injury. The formation of scar tissue and, ultimately, a fluid-filled cyst, and the presence of axonal growth-inhibitory factors, create a chronically hostile environment for repair at and around the injury epicenter. As a result, spontaneous repair of anatomical damage and functional improvement after spinal cord injury is poor. Therapeutic interventions so far have had limited success resulting in a steadily growing group of people with paralysis due to spinal cord injury. The lifetime costs for care for a spinal cord injured person vary between one and three million dollars, making it one of the most expensive conditions to treat. The personal and societal consequences drive the research for treatments for spinal cord injury.

Current research on spinal cord repair-promoting approaches uses animal models and focuses on neuroprotection, to limit secondary tissue loss; axonal regeneration, to promote growth and synaptogenesis of damaged axons; and plasticity, to recruit newly grown and/or spared axons into axonal circuitries involved in motor and sensory function. Over the last decades, the potential of cell transplantation as a reparative approach for the injured spinal cord has become evident in animal models. Transplanted cells may secrete trophic factors that exert paracrine effects that limit secondary injury, promote axonal regeneration and/or plasticity, or serve as a substrate for regenerating axons. Alternatively, cell transplants may be a source for replacement of lost and damaged neural cells. The mesenchymal stem cell-like bone marrow stromal cell (BMSC) is among the cell types that have been explored for spinal cord repair. BMSCs were shown to elicit anatomical repair accompanied by improved, but still partial, recovery of function. At

present, our understanding of the extent of the repair potential of intraspinal BMSC transplants and their underlying mechanisms is incomplete. This thesis has two main goals: (1) to expand our knowledge of BMSC therapy for spinal cord repair, and (2) to investigate approaches to enhance the therapeutic efficacy of intraspinal BMSC transplants. In the studies an adult rat model of spinal cord contusion was employed because a contusion is the most prevalent mechanism of spinal cord injury in humans.

A comprehensive overview of the repair potential of stem cells for central nervous system repair and of BMSCs for spinal cord repair is provided in **Chapter 1**. In part A of chapter 1, stem cell terminology and experimental and clinical studies on stem cells for central nervous system repair will be discussed. In part B of Chapter 1, the advantages and disadvantages of using BMSCs as cell therapy for spinal cord repair are discussed. In addition, the spinal cord contusion model will be described in detail. To expand our knowledge of BMSC therapy for spinal cord repair, we focused on functional recovery and anatomical correlates thereof (**Chapter 2)** and the role of brain-derived neurotrophic factor (BDNF) in BMSC-mediated anatomical repair (**Chapter 3**). Comprehensive behavioral analyses were used to measure the effects of BMSC transplants on motor, sensorimotor, and sensory function recovery after BMSC transplantation into the adult rat contused spinal cord. Gene therapy was used to manipulate BDNF production in transplanted BMSCs to determine its involvement in BMSC-mediated repair. To enhance the therapeutic efficacy of BMSC transplants, we focused on BMSC survival in the injured spinal cord. The effect of reduced macrophage presence at the injury (transplant) site on BMSC survival was investigated by using clinically relevant anti-inflammatory drugs (**Chapter 4**). Whether increased survival enhances the effect of BMSC transplants on repair of the contused spinal cord is described in **Chapter 5**. A reverse thermal gel, poly(ethylene glycol)poly(serinol hexamethylene urethane) or ESHU with anti-oxidant properties was used to increase BMSC transplant survival and so prolong their presence in the injured spinal cord. In **chapter 6** the findings of our studies and future directions will be discussed. **Chapter 7** and **8** provide a summary of this thesis in English and Dutch, respectively.

Chapter 1

Introduction

Introduction Part A

Stem Cells for Central Nervous System Repair and Rehabilitation

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ABSTRACT

The central nervous system (CNS) has limited capacity for self-repair. Current treatments are often incapable of reversing the debilitating effects of CNS diseases that result in permanent and/or progressive physical and cognitive impairments. One promising repair strategy is transplantation of stem cells, which can potentially replace lost neurons and/or glia or promote repair through secretion of trophic factors. Various types of stem cells exist, each with their own advantages and disadvantages. Although no consensus exists regarding the optimal cell type to use, moderate functional improvements have been shown in animal models of CNS diseases using different types of stem cells. However, the precise mechanism of action behind their beneficial effects remains unknown. In addition, many barriers to clinical use still need to be resolved before transplantation of stem cells can be used as effective biologics. These barriers include—depending on the stem cell type—possible tumor formation, difficulty with harvest, limited in vivo differentiation and integration, and ethical issues regarding use.

INTRODUCTION

Central nervous system (CNS) diseases are often characterized by complex immune-mediated cytotoxic and apoptotic processes that result in the loss of function and permanent loss of neural cells[1]. Although many CNS diseases result from a loss of viable cells, a therapeutic approach must consider the type of cell lost to have a beneficial outcome. For example, Parkinson disease requires replacement of lost dopaminergic substantia nigra neurons, whereas multiple sclerosis requires reconstitution of functional oligodendrocytes. Stem cells have the potential to address this demand for specific cells for specific diseases because of their multipotency, and thus stem cell therapy is a promising biologic therapy to consider for persons with CNS diseases.

In the past decade an explosive amount of stem cell research has been conducted, resulting in an insightful scope of knowledge on stem cell biology. Continuing research will be essential before effective bedside treatments for CNS diseases may be developed. This review aims to provide a critical overview of stem cell use for repair of CNS diseases relevant for rehabilitation medicine.

DEFINITION, ORIGIN, AND VARIOUS TYPES OF STEM CELLS

By definition, a stem cell is capable of self-renewal and of differentiating into at least one other cell type. The zygote is referred to as a totipotent stem cell. The blastocyst contains an inner cell mass consisting of self-replicating cells that can become all but trophoblast cells (the outer layer blastocyst cells that later become the placenta); these cells are known as pluripotent stem cells (Figure 1). When these cells enter into 1 of the 3 primary germ layers—ectoderm, mesoderm, or endoderm—they are referred to as multipotent stem cells. These cells then can become precursor cells, which are unipotent cells that differentiate into the final cell types within differentiated tissues (Figure 1).

When stem cells are harvested from embryonic tissue, they are considered embryonic stem cells (ESC). When stem cells are taken from tissues from the adult body, they are

referred to as adult stem cells (ASC [note: adipose tissue-derived stem cells are sometimes also referred to as ASC]) or somatic stem cells. The existence of ASC was first demonstrated within the adult hematopoietic system, which throughout natural life gives rise to new blood cells[2]. After this discovery, ASC were demonstrated within numerous other adult tissues such as neural stem cells (NSC) in the brain, epidermal neural crest stem cells (EPI-NCSC) in hair follicles, muscle-derived (mesenchymal) stem cells in muscles, and bone marrow stromal cells (BMSC) in bone marrow. The functions of ASC are poorly understood, but one rational possibility would be that ASC support repair of the tissues in which they reside. At present, however, this theory has not been confirmed unequivocally, and it certainly does not appear to be the case in the CNS, where endogenous restoration is poor and disease or trauma typically elicits permanent damage.

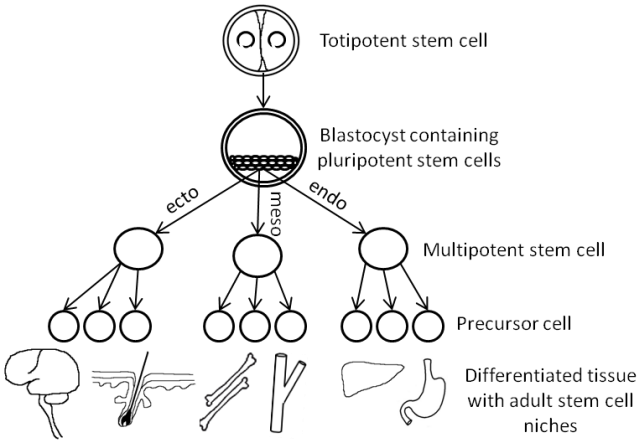

Fig. 1. Hierarchy of stem cells. Totipotent cells can develop into all cell types of the body, pluripotent cells can become all but trophoblast cells, multipotent cells can give rise to all cells within 1 of the 3 germ layers, and precursor cells are unipotent cells that will become terminally differentiated cells of specialized tissue. Ecto = ectoderm germ layer; meso = mesoderm germ layer; endo = endoderm germ layer.

Recently, a third type of stem cell has emerged—the induced pluripotent stem (iPS) cell, discovered by Takahashi and Yamanaka in 2006[3]. The iPS cell is generated from an adult somatic cell by introducing transcriptional factors whose ectopic expression reprograms the cell into a pluripotent cell. The groundbreaking discovery 'that mature cells can be reprogrammed to become pluripotent' has earned Yamanaka the Nobel Prize in

Physiology or Medicine 2012. The prize was shared with John B. Gurdon who in 1962 used an enucleated oocyte into which the nucleus of an adult cell was transferred to create a stem cell capable of forming a blastula and eventually a tadpole[4]. In June 2013 the somatic cell nuclear transfer method was for the first time successfully used for human embryonic stem cell generation[5]. The discovery of generated stem cells is opening exciting new avenues in the field of regenerative medicine (for review, see Bellin et al.[6]).

UTILITY OF NEURAL AND NON-NEURAL STEM CELLS

NSC can contribute in different ways to repair of the brain and spinal cord. They can potentially differentiate into neurons and/or glial cells and replace those that were lost as a result of the disease or trauma. Alternatively, NSC can serve as vectors for growth factors that could support cell survival, cell proliferation, axon regeneration, and blood vessel formation, which can all positively influence CNS repair. It is also possible that stem cells serve as a substrate for regenerating axons and thus contribute to repair. Thus far, numerous studies have demonstrated the potential of stem cells for CNS repair. Interestingly, the mechanisms underlying their benefits remain elusive.

Embryonic NSC have a robust capacity to differentiate into neural cells and are therefore suitable for repair strategies based on cell replacement. However, their impressive differentiation capacity comes with uninhibited proliferation, which could result in tumor formation after transplantation. This factor, together with ethical concerns surrounding their harvest, has limited the application of ESC for CNS repair. Adult NSC also are capable, albeit less so than ESC, of differentiating into neural cells, and in contrast to ESC, they are not known for causing tumors after transplantation. Thus adult NSC are good candidate cells for neural replacement approaches. A disadvantage of adult NSC is that they are difficult to obtain because they need to be harvested from the adult brain or spinal cord.

Compared with embryonic and adult NSC, non-neural stem cells are more readily obtainable. For instance, BMSC reside in bone marrow, EPI-NCSC in hair follicles, and muscle-derived (mesenchymal) stem cells in muscles, and all these tissues are relatively

easy to harvest from adults. Some of these non-neural stem cells offer additional advantages such as the low expression of major histocompatibility complex I molecules by BMSC that would help evade immunologic rejection. Importantly, it was reported that several types of non-neural stem cells could (trans)differentiate into neural cells[7], which has opened new avenues for CNS repair. However, at present, this potential to become a neuron, astrocyte, or oligodendroglial cell has not been unambiguously proven and is in fact a subject of controversy. If this ability to transdifferentiate into neural cells is low or absent, their benefits in replacement strategies would be poor. On the other hand, non-neural stem cells may offer effective means to repair the CNS through their ability to secrete repair-supporting molecules such as growth factors. Moreover, in accordance with their decreased differentiation capacity, these cells are less inclined to unrestrained proliferation and are therefore less tumorigenic. Table 1 provides an overview of the relative advantages and disadvantages of different types of stem cells. The current controversies and challenges within the field of regenerative medicine are best illustrated with the following example. For a mesodermally derived BMSC to be suitable for CNS repair based on cell replacement, it will need to transdifferentiate into a neuron or neural glial cell. For this transdifferentiation to occur, the BMSC will first need to revert into a pluripotent cell, subsequently differentiate into an ectodermal precursor cell, and

Table 1. Relative advantages and disadvantages of embryonic and adult stem cells for human use in CNS disease

	ESC	ASC		iPS
		NSC	MSC	
Differentiation/cell replacement potential	Very good	Good	Poor	Good
Harvest	Controversial	Difficult	Easy	Easy
Tumorigenicity	High	Low	Low	High

Abbreviations: ESC, embryonic stem cell; ASC, adult stem cell; NSC, neural stem cell; MSC, mesenchymal stem cell; iPS, induced pluripotent stem cell

then differentiate into a neuron, astrocyte, or oligodendrocyte. Several studies have shown that BMSC can be induced in vitro to express neuronal markers and even to have some electrical neuronal properties, but true transdifferentiation into a fully functioning neuron is strongly debated[8]. Similarly, a few in vivo studies[7,9] have shown expression of neuronal markers and/or anatomic integration after transplantation of BMSC, but neuronal functionality (ie, synapse formation, firing of action potentials, and release of neurotransmitters) or glial functionality has not been shown unequivocally. For example, Kopen and colleagues (1999)[7] reported the expression of glial fibrillary acidic protein, a marker for astrocytes, in BMSC after transplantation into mice brain ventricles and concluded that they had transdifferentiated into mature astrocytes. The expression of specific neural markers is an important first step toward applying BMSC for CNS cell replacement, but it appears to be a rare event, and it is not a demonstration that the cell has become a functional component of the nervous system.

ANATOMIC AND FUNCTIONAL REPAIR AFTER STEM CELL TRANSPLANTATION IN ANIMAL MODELS OF CNS DISEASE

In the past decade a number of studies showed that transplantation of NSC can result in histologic and/or functional improvements in rodent models of various CNS diseases. Cummings and colleagues[10] demonstrated remyelination of axons and functional improvements after transplantation of NSC into a mouse spinal cord injury model. Improved motor function was observed in hemiplegic mice after implantation of monkey ESC[11]. Table 2 provides a selected overview of studies that have transplanted neural and non-neural stem cells in different CNS disease models and reported repair. Typically, in ESC transplantation paradigms, some degree of differentiation into neurons and glia is shown. From the results it appears that NSC preferentially differentiate into astrocytes. Some may also differentiate into oligodendrocytes, but very few differentiate into neurons. Whether these newly generated neural cells then integrate within the host CNS tissue is not always clearly demonstrated. Despite the alleged in vitro ability, it is not often reported that non-neural stem cells become neural cells after transplantation into the CNS. Nevertheless, anatomic and/or functional repair has been demonstrated. Sieber-

Blum[12] showed improvements in sensory connectivity and in touch perception after transplantation of EPI-NCSC in a mouse spinal cord injury model. In this study it was proposed that the neural crest cell–derived EPI-NCSC have the advantages of ESC and ASC because they are able to differentiate into oligodendrocytes and neuroblasts without being tumorigenic and are easily obtained from the bulge of hair follicles.

Table 2. Selected overview of studies that have implanted stem cells in rodent models of spinal cord injury, stroke or Parkinson's disease

Type of stem cell	Disease Model	Differentiation	Functional Outcome	Reference
ESC	SCI, rat	Astrocytes, oligo's, neurons	↑BBB	McDonald et al.[36]
Fetal NPC	SCI, rat	Neurons	↑Pellet retrieval	Ogawa et al.[37]
BMSC	SCI, rat	-	↑BBB	Hofstetter et al. 2002[21]
NSC	SCI, mouse	Neurons, oligo's	↑BBB	Cummings et al.[10]
EPI-NCSC	SCI, mouse	Neurons, oligo's	↑Touch perception	Sieber-Blum[12]
iPS	SCI, mouse	Neurons, oligo's, astrocytes	↑BMS	Tsuji et al.[13]
ESC	Stroke, mouse	Neurons	↑Beam walking, ↑rotarod	Ikeda et al.[11]
BMSC	Stroke, rat	Oligo's, astrocytes	↑body swing test, ↑footprint analysis	Chen et al.[9]
NSC	Stroke, rat	Neurons, astrocytes	-	Kelly et al.[38]
BMSC	Parkinson, rat	-	↑Rotational behavior	Wu et al.[15]

Abbreviations: ESC, embryonic stem cell; SCI, spinal cord injury; oligo's, oligodendrocytes; BBB, Basso, Beattie, and Brasnahan-scale; NPC, neural progenitor cell; BMSC, bone marrow stromal cell; NSC, neural stem cell; EPI-NCSC, epidermal neural crest stem cells; iPS, induced pluripotent stem cell.

Another cell type that has putative neural differentiation capacity without being tumorigenic is the iPS cell. The therapeutic potential of iPS cells was nicely demonstrated in a mouse spinal cord injury model, which revealed that transplanted iPS cell–derived neurospheres differentiated into all 3 neural cell types, participated in remyelination, promoted axonal outgrowth, and improved locomotor function[13]. Additionally, this study circumvented tumor formation by pre-evaluation and selection of the neurospheres for tumorigenicity[13]. This study was partially confirmed by Nutt and colleagues, who showed successful transplantation and integration of iPS cell-derive NPCs into an early chronic spinal cord injury model, however, without evidence of functional improvement.[14]

IMPROVING THE OUTCOME AFTER STEM CELL TRANSPLANTATION INTO THE DAMAGED CNS

Notwithstanding recent reports that transplanted stem cells can become neural cells, the key mechanism for functional improvements observed after ASC transplantation in the CNS is thought to be neuroprotection, that is, limiting the loss of tissue. Neuroprotection can be accomplished through the secretion of growth factors, such as brain-derived neurotrophic factor, glial cell line–derived neurotrophic factor, and nerve growth factor. A cell transplant provides long-term delivery of growth factors, which is an important advantage over direct injection, because growth factors dilute rapidly and typically have short half-lives. For this reason, recent studies have genetically engineered stem cells to overexpress such growth factors, with the aim of enhancing their neuroprotective capacity and, as a result, their repair-supporting potential. Wu and colleagues[15] showed a neuroprotective effect of glial cell line–derived neurotrophic factor-overexpressing BMSC in a Parkinson model. Axonal regeneration and enhanced functional recovery was found after transplantation of brain-derived neurotrophic factor-overexpressing BMSC in a spinal cord injury model[16].

The beneficial effects of stem cells can also be increased by pre-differentiating the cells in vitro prior to transplantation. This pre-differentiation can be achieved by growing the cells according to a particular induction protocol that pushes the cells into a desired lineage.

Davies and colleagues[17] showed improved axon regeneration and locomotor function in rats with spinal cord injuries after transplantation of astrocytes differentiated from embryonic glial-restricted precursors, but not undifferentiated glial-restricted precursors. Hofstetter and co-workers[18] transduced NSC with neurogenin-2 to suppress astrocytic differentiation prior to transplantation into rats with spinal cord injuries and demonstrated prevention of graft-induced sprouting, decreased allodynia, and improved functional recovery. Although these 2 studies achieved some degree of restoration with use of opposite differentiation protocols, it is clear that both demonstrated that the repair-supporting abilities of stem cells can be positively influenced prior to transplantation.

Another way to improve the outcome is by combining stem cell transplantation with putative additive or synergistic treatments. Even though combinatorial strategies are thought to be essential to achieve biologically significant repair, exploration of these strategies has been sparse. Combining BMSC transplantation with an inhibitor of Rho-kinase[19], a molecule known to prevent neurite outgrowth, or with olfactory ensheathing cells[20], another adult cell type that has been shown to benefit anatomic and functional CNS repair, did not improve functional outcomes in rats with spinal cord injuries more than BMSC transplantation alone. In a similar model, the combination of BMSC with physical exercise did not improve function compared with control subjects[21]. On the other hand, in both a spinal cord injury[22] and a stroke[23] model, the outcome after transplantation of brain-derived adult NSC with olfactory ensheathing cells was improved compared with transplantation of each of the cell types alone. In a rat model of cerebral ischemia, the combination of BMSC with erythropoietin showed a synergistic effect on neurogenesis and memory performance but not on locomotor function[24]. Because of our limited current knowledge about mechanisms underlying single treatments, it is difficult to select the appropriate combinations in which the single interventions would exert additive or synergistic effects. Future elucidation of mechanisms will allow more rationally targeted combinatorial repair strategies.

It is clear that, despite some promising results, stem cell–based repair of the damaged CNS still has major challenges to overcome before it can be successfully applied in a clinical setting. These challenges vary between tumorigenic and ethical concerns with ESC[25], differentiation issues with ASC[26,27], and survival of transplanted stem cells in general[28]. Poor survival of stem cells after injection into damaged CNS tissue can be due to poor vascularization of the transplantation site or a result of inflammation with accompanying secretion of cytotoxic molecules and rejection by activated immune cells. To optimally benefit from transplanted stem cells, it will be necessary to develop strategies to improve survival of the cells after transplantation. Although concomitant treatment with immunosuppressive drugs[27], improving the timing of cell transplantation[28], and transplantation of cells within a scaffold all have been shown to benefit cell survival, the majority of cells still die within weeks of transplantation. Clearly, further studies that focus on optimizing stem cell survival after transplantation into the damaged CNS are imperative.

CLINICAL TRIALS

Despite our incomplete knowledge, several clinical trials are currently being conducted in which stem cells are being transplanted in patients with Parkinson disease, stroke and other neurological disorders, with variable results thus far. After transplantation of human fetal mesencephalic tissue in patients with Parkinson disease, grafted neurons have been reported to survive and integrate, with improvements in several outcome parameters. These improved parameters included a 37% reduction in Levodopa dose, a 40% improvement in 18F-Flurodopa uptake (a measurement of dopaminergic activity in the putamen), a 44% improvement in the Unified Parkinson Disease Rating Scale Motor Score (while being off medication), a 39% decrease in “off medication” time, and a 49% decrease in “on medication” time with dyskinesia, according to a meta-analysis[29].

Impressively, up to sixteen years after transplantation, dopaminergic innervations in basal ganglia could be restored to normal levels and was associated with relief of motor symptoms.[30] Variability across studies, however, is high. The differences between the observations in different clinical trials are likely due to variances in recipient

characteristics (eg, younger patients seem to show better recovery after neural grafting), the use of different surgical techniques, and/or (lack of) immunosuppressive drug administration, resulting in decreased graft survival.

The first clinical trial using human ESC for spinal cord injury was approved in 2009 by the United States Food and Drug Administration (FDA). In this Phase I trial, oligodendrocyte progenitor cells derived from human ESCs were safely transplanted into five severe spinal cord injury patients. In November 2011 however, the trial was discontinued for financial reasons.[31] Another Phase I/II trial by StemCells Inc using human CNS stem cells for spinal cord injury is currently underway in Switzerland and Canada and has recently (October 2013) been approved by the FDA[32]. Small clinical trials in other neurologic diseases, including stroke[33] and Huntington disease[34], seem to support the potential use of stem cells, because moderate functional improvements are being achieved in some patients. For example, after transplantation of neuronal cells in 12 patients who have sustained a basal ganglia stroke, 6 patients showed improvements on the European stroke scale (a gain of 3 to 10 points) 12 to 18 months after transplantation[33]. Transplantation of fetal neural tissue in 5 patients with Huntington disease resulted in cognitive improvements in 3 patients the first 2 years after surgery, which then faded after 4-6 years, as measured by the Unified Huntington Disease Rating Scale and neuropsychological tests. Safety was shown up to ten years postoperatively[35]. Several other clinical trials are in or have completed phase I/II of safety, but large trials of efficacy of stem cells for neurological disorders are still lacking.

CONCLUSION

Transplantation of stem cells can potentially be used for treatment of various CNS diseases. Progress is being made in the laboratory, and in various animal models of CNS disease/disorders, moderate functional improvements are being reported. The underlying mechanisms are still mostly unclear. In addition, determination of which stem cell type would be best for a particular CNS disease/disorder is still largely unresolved. Clearly many issues need to be elucidated before safe and effective stem cell-based therapies can be

designed for bedside treatments of neurologic disorders. All these issues warrant further investigations before stem cells can live up to their potential as effective biologic treatments for CNS disease.

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Introduction Part B

Bone Marrow Stromal Cells for Repair of the Injured Spinal Cord

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INTRODUCTION

In 1927 Harvey Cushing described the outcome for soldiers with spinal cord injury (SCI) sustained during World War I: "Fully 80 percent died in the first few weeks in consequence of infection from bedsores and catheterization. Only those cases survived in which the spinal cord lesion was a partial one" [1]. Nowadays, this has been reversed. In well-organized systems of care for trauma and SCI and due to improved critical-care medicine most patients survive the initial hospitalization. At present, there is no treatment available that effectively re-establishes disrupted axonal circuitries that are necessary to restore injury-induced functional deficits. Due to the lack of a cure and the improved health care, the number of wheelchair bound people increases steadily each year. Currently, in the United States there are an estimated 400,000 people with SCI, with an annual incidence of 11,000 (The National Spinal Cord Injury Statistical Center, Birmingham, AL). In Western European countries similar leading causes of SCI are obtained as in the United States, with vehicular crashes and falls as leading causes of SCI and predominantly young males affected[2,3]. In contrast to the developed countries, in the less developed countries a shift of etiology can be observed towards falls[4] and violence[5-7].

Following the first medical care in a hospital, continuing medical care is necessary to maintain the SCI patient's health and quality of life. This does not lead to functional repair. Repair-promoting pharmaceutical and/or surgical interventions will be necessary to significantly change the functional outcome after SCI. Transplantation of repair-supporting cells is considered a candidate repair approach. A bone marrow stromal cell (BMSC) transplant has shown great promise for spinal cord repair. This chapter will give an overview of the pathophysiology, clinical consequences, assessments, and treatments of SCI and will then focus on BMSC as a possible therapy for SCI. In addition, the SCI model system used in subsequent chapters will be explained.

PATHOPHYSIOLOGY AND CLINICAL CONSEQENCES

A direct force to the vertebral column can cause damage to bony and soft tissue structures. Torn ligaments or fractures can cause instability of the vertebral column with

potential risk of additional damage. Fracture dislocation and hematomas can directly compress the spinal cord and cause immediate neural cell death, axon damage and demyelination, resulting in instant loss of motor and sensory function. After the first destructive events, a sequence of molecular and cellular pathophysiological events, including an aggressive inflammatory response within the damaged tissue, leads to additional tissue loss at the injury epicenter and at distant sites (secondary injury)[8,9]. The functional consequences of SCI are highly variable and depend on the degree of tissue damage, which in turn depends on the impact severity. In patients with SCI with a relatively small amount of tissue damage, some endogenous recovery of function can be observed, which is most likely resulting from plasticity of the spinal nervous tissue[10,11]. In people with SCI with extensive tissue damage the neurological deficits are generally major and permanent. There are very few reports of people with a large injury that regain motor function to a degree that independence can be achieved.

Over 95% of SCI patients survive their initial hospitalization. The relatively young age when SCI occurs, improved medical care, and lack of effective therapies are responsible for the continually increasing number of paralyzed people with SCI. This puts a high financial burden on the patient, his/her family, and society[3,12]. The psychological consequences of SCI should not be underestimated and appropriate guidance of patient and family should have an important place in the management of SCI[13-15]. Patients need time to accept their deficits. One can expect an initial period of denial and/or inability to fully comprehend the consequences of the paralysis caused by the injury. After the patient realizes his/her fate to the fullest extent, a period of acceptance will have to run its course[13]. After that, the patient needs to learn to live with his/her disabilities, and this may be accompanied by bouts of depression. The mental state of the patient can have its effect on his/her medical treatments[14].

SCI is the second most expensive condition to treat in the United States after respiratory distress syndrome in infants and is ranked third in medical conditions requiring the longest stay in hospitals[16]. The costs of lifetime care for a SCI patient varies between 1 and 3

million dollars. The Center for Disease Control in the United States estimated that about 10 billion dollars are spent yearly on SCI treatment excluding the management of pressure ulcers, a common adverse effect of SCI, which adds another billion dollars per year[17].

TREATMENT

An acute and a chronic phase can be distinguished after SCI. Since SCI is often a consequence of severe accidents, initial treatment is generally focused on stabilization of the patient. There is insufficient evidence that would support standards of care during the acute phase of SCI. It is advised to maintain patients in an intensive care unit for close monitoring of respiratory and hemodynamic complications. For adequate spinal perfusion, which can be at risk due to injury-induced edema, a mean arterial pressure of 85-90 mmHg should be maintained[18]. Depending on the type of injury, surgical interventions should be considered to decompress the spinal cord and or stabilize the spinal column[19,20]. Decompression surgeries may accelerate functional improvements and result in shorter hospitalization and rehabilitation periods[17,21] . However, it does not result in an improved functional outcome[22]. A lack of consensus of care during the acute phase of SCI is in part due to the large variability among injuries and makes its early management complicated. If bone fragments continue to compress the spinal cord, early surgery may be vital to prevent exacerbation of spinal cord tissue destruction. However, in cases without a clear sign of such urgency there is no consensus on whether and what type of early surgical/clinical interventions must be implemented. The type of surgical intervention should be considered on a case-to-case basis, which makes it complicated to study the efficacy of intervention in the acute phase after SCI in randomized and controlled clinical trials.

Besides surgical interventions, pharmacological treatments to limit the secondary injury after SCI are often considered. The best-known treatment is a high dose of the glucocorticosteroid, methylprednisolone sodium succinate (MPSS) within 8 hours after the injury[23-25]. Experimentally it was demonstrated that a high dose of MPSS reduces the inflammatory response and limit tissue loss after damage to the spinal cord. The effects of

MPSS in patients with SCI were investigated in 3 consecutive National Acute Spinal Cord Injury Studies (NASCIS)[23-25]. The results demonstrated that MPSS treatment in the acute phase of SCI resulted in neurological improvements up to 6 months after injury. After a thorough review of the results from the NASCIS studies and a more comprehensive assessment of the benefits and risks involved in high dose MPSS treatment, the therapeutic benefits are now disputed[26-28]. Especially in patients with complete SCI high dose steroid treatment can lead to adverse effects such as myopathy and wound infection that may negatively influence functional outcome and in some cases may be life-threatening[28,29]. Currently, many SCI clinics worldwide have discontinued the 'standard' acute administration of MPSS after SCI.

Treatment paradigms in the chronic stage after SCI are multidisciplinary and intensive. Different complications may occur that each demands specific interventions. For instance, SCI can lead to pain[14,15], decreased fertility[30], and autonomic dysreflexia with loss of bladder and bowel control[31]. It has to be taken into consideration that many SCI patients get accustomed to the specific injury-related pain they experience and as a result reveal their distress to their physician often at a late stage[32,33]. For some SCI-related conditions, such as decreased fertility, it is the patient's personal desire that should guide the physician's actions. Other common problems that arise after SCI are septicemia, respiratory insufficiency, and pneumonia due to muscle atrophy. These complications may cause clinical deterioration and could eventually result in death. They often occur without typical symptoms. It is imperative that SCI patients receive annual screenings and long-term follow-ups to prevent these secondary complications. It is advised to treat patients on a regular basis with pneumococcal and influenza vaccine to prevent opportunistic infections. Monitoring the skin and urinary tract and implementing aggressive treatments against pressure ulcers and urinary tract infections is needed to reduce the risk of septicemia. Appropriate nutrition and exercise should also be incorporated in the (new) lifestyle. Rehabilitation programs should be implemented to reduce the risk of cardiovascular disease[34].

BONE MARROW STROMAL CELL THERAPY

Mesenchymal stem cells from bone marrow (here referred to as bone marrow stromal cells (BMSCs)) have therapeutic potential for the injured spinal cord[35]. BMSC were shown to differentiate into bone, fat, tendon and cartilage cells[36]. Although still debated, it has been reported that BMSC can transdifferentiate in vitro into liver cells[37], skeletal cells[38], cardiac muscle cells[39], and neural cells[37,40]. Besides this ability, BMSC are also known to produce different types of growth factors that could potentially influence nervous tissue repair positively. Together, these abilities make BMSC interesting for repair strategies for the injured spinal cord.

Several other aspects make BMSC interesting candidates for cell-based approaches for central nervous system repair. Firstly, BMSC are relatively easy to obtain from a fairly routine bone marrow extraction followed by a quick centrifuge and culture procedure to remove the hematopoietic cells. Secondly, BMSC are easy to culture as they do not need complicated growth media or special culture circumstances. Basic cell culture equipment is sufficient to successfully culture millions of BMSC. Thirdly, BMSC are easy to transduce with viral vectors which, if necessary, may be helpful to boost the overall reparative abilities of the cells. The use of viral vectors to genetically modify cells prior to transplantation has not yet become mainstream as there are some biological and ethical issues that need to be resolved. Finally, BMSC do not have the ethical concerns that embryonic or fetal stem cells have, and therefore circumvent public rejection as a possible treatment for neural and non-neural trauma and disorders.

At this time, there is no irrefutable evidence that BMSC transplanted into the damaged nervous tissue differentiate into neural cells that successfully replace lost cells. Also, there is no convincing evidence that neural cells derived from grafted BMSC contributed to functional improvements after transplantation. As long as the potential of BMSC for differentiation into neural cells is in debate, the ability to produce and secrete different types of growth-promoting molecules, which include several neurotrophins and cytokines, is the more interesting and more likely characteristic of BMSC that makes these cells

important candidates for spinal cord repair approaches. By releasing these molecules, BMSC can positively influence the consequences of spinal cord injury and support anatomical and functional repair (Figure 1).

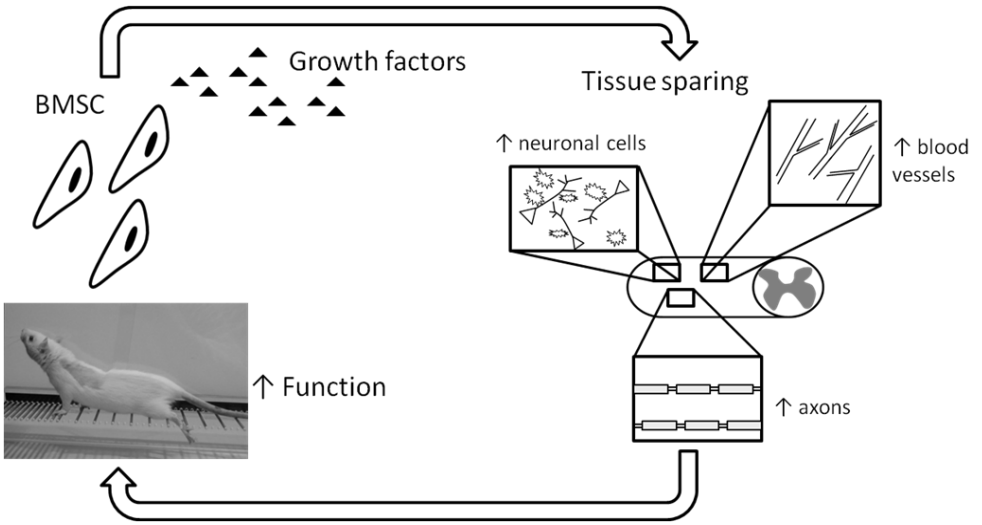

Fig. 1. BMSC secrete various growth factors, including BDNF, VEGF, NGF and NT-3. These factors are thought to limit the loss of tissue in the injured spinal cord, contributing to the increased functional outcomes after BMSC transplantation.

RAT MODEL SYSTEM

Promising therapies for spinal cord injury are typically tested in rodent models, and mostly in rats. Similar as in humans, a SCI in the rat results in progressive loss of the grey and white matter creating large fluid filled cysts. Proliferation and activation of astrocytes result in formation of scar tissue, which acts as a barrier for axonal regeneration. Importantly, as in humans, there is no spontaneous regeneration in the injured spinal cord in rodents. The histological similarity between human and rat spinal cord injury has made the rat an extensively studied model for experimental therapeutic strategies, including BMSC transplantation.

The most widely used model of spinal cord injury involves a spinal cord contusion inflicted by an impactor device. A contusion is clinically the most frequently occurring type of spinal

cord injury; approximately 75% of all human injuries are contusions. The consequences of a contusive injury in rats are similar as the known consequences in the contused human spinal cord. Figure 1 shows the rat model system for spinal cord contusive injury.

An alternative model for a contusion-like spinal cord injury is the clip compression model. The main difference between the impactor-inflicted contusion and the clip-inflicted compression is time. With an impactor the spinal cord is compressed for a brief moment of time while with a clip the spinal cord is compressed for a longer, regulatable, time. The clip model is clinically more relevant as most spinal cord injuries are inflicted by a lasting compression rather than a brief one.

There are a number of other, non-contusive, spinal cord injury models employed in laboratories around the world to test treatment paradigms. These are valuable in their own right to investigate the underlying mechanisms and/or validity of certain approaches. Partial transections of specific regions in the spinal cord are used especially to study the effects of treatments that aim to promote axonal regeneration; specific descending or ascending pathways can be damaged with relatively small local knife cuts and the regeneration response quantified at later time points. The involvement of specific axonal pathways in locomotor function can also be investigated using partial transections. The main disadvantages of partial transections are the low clinical relevance and the possible misinterpretation of results due to compensatory sprouting, i.e., other previously non-involved axonal pathways become involved in particular functions. Another model that has been used is the complete transection of the spinal cord. Although this is not often seen in the clinic, complete transections are particularly advantageous to study cell types for their ability to promote regeneration of damaged axons without contaminating sprouting of undamaged pathways and to serve as bridging material between spinal cord stumps. This model is also suitable to study the efficacy of synthetic or natural biomaterials for their efficacy to serve as carrier of cells or drugs. A disadvantage besides the low clinical relevance is that rats with a completely transected spinal cord are more laborious to maintain.

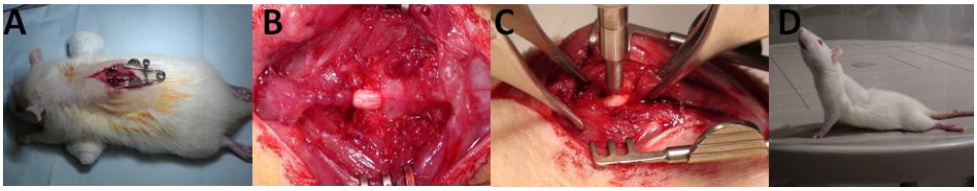

Fig. 2. Rat spinal cord contusion model. A. A laminectomy is performed exposing the underlying spinal cord. B. Enlarged view of the exposed spinal cord segment. C. A computerized impactor is used to contuse the spinal cord. The piston is attached to a sensor to record velocity, force and displacement to ensure consistency. D. A moderate contusion results in loss of function at and below the level of injury and loss of bladder function.

BMSC INJECTION

It is difficult to provide standard guidelines for cell preparation because every cell type requires special conditions and circumstances for optimal isolation and culturing. Cell injection procedures may vary but are essentially similar. The standard procedures to harvest, culture and genetically modify BMSC with lentiviral vectors encoding for green fluorescent protein (GFP) to enable easy identification in vivo, as well as to inject BMSC as used in our laboratory are depicted in Figure 5. The length of the culture (preparation) time for BMSC depends on how many cells are needed to fill the damaged area. Thus, the number of BMSC necessary depends on the overall loss of tissue which, in turn, depends on the severity of the initial insult and on the time between insult and transplantation. Imaging techniques may provide the necessary information to guide the decisions on damaged tissue volumes and number of cells.

There are a number of studies that have explored injection paradigms other than straight acute injections into the injury site. BMSC have been infused systemically or into the 4th ventricle[41], or transplanted acutely into the cervical[42] or thoracic spinal cord[43,44] or into the chronically injured cord[45].

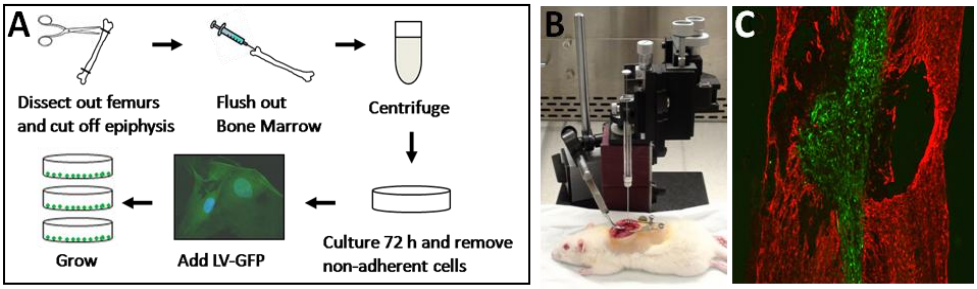

Fig. 3. Transplantation of BMSC. A. BMSC are isolated from femurs of rats by cutting off the epiphyses and flushing out the bone marrow. Cells are plated onto plastic culture dishes. Non-adherent hematopoietic stem cells are removed and the plastic-adherent BMSC are infected with LV-GFP. B. Cells are injected into the spinal cord contusion epicenter using a Hamilton syringe with a pulled glass needle attached, held within a micromanipulator. C. Appearance of transplanted BMSC (green) in the contused rat spinal cord seven days post transplantation (20 µm thick section at 2.5 x magnification). The red color represents immunohistochemically stained glial fibrillary acidic protein (GFAP), a commonly used marker for astrocytes.

TIMING OF TRANSPLANTATION

In an experiment by Nandoe Tewarie and colleagues[46] , BMSC were transplanted into a moderately contused adult rat spinal cord at 15 min, and at 3, 7, and 21 day post-injury and BMSC survival was closely assessed both during the transplantation procedure and up to four weeks after transplantation. In addition, the effect of the timing of BMSC transplantation on tissue sparing was determined. BMSC were collected from culture dishes, kept on ice, and passed through a glass pulled needle for injection into the contusion site. This procedure resulted in a majority (67 %) of the BMSC intended to be transplanted being present in the contusion at 15 min after transplantation. Thereafter, BMSC numbers rapidly decreased. The rate at which cell death occurs is different when transplanting acutely or delayed. In an acute transplantation paradigm (15 min post-contusion) and sub-acute transplantation paradigm (3 days post-injury) BMSC survival is better than in a delayed transplantation paradigm (7 days or 21 days post-injury). The percentages of BMSC in the contusion at seven days after transplantation are 32% and 52% for acute and sub-acute transplantation, respectively, and 9% for delayed transplantation. Four weeks after transplantation, almost no BMSC can be found in either paradigm (see figure 4). Interestingly, the presence of BMSC for this short period of time is sufficient to elicit tissue sparing. Acute and subacute transplantation, but not delayed

transplantation results in neuroprotection, and tissue volumes in these paradigms are strongly correlated with the number of BMSC present[46]. These results indicate that timing of BMSC transplantation is important for optimal survival and neuroprotective effect, with acute and subacute transplantation being superior to delayed transplantation. However, because of the clinical relevance of delayed treatment, it seems imperative to find strategies to improve BMSC survival in delayed paradigms.

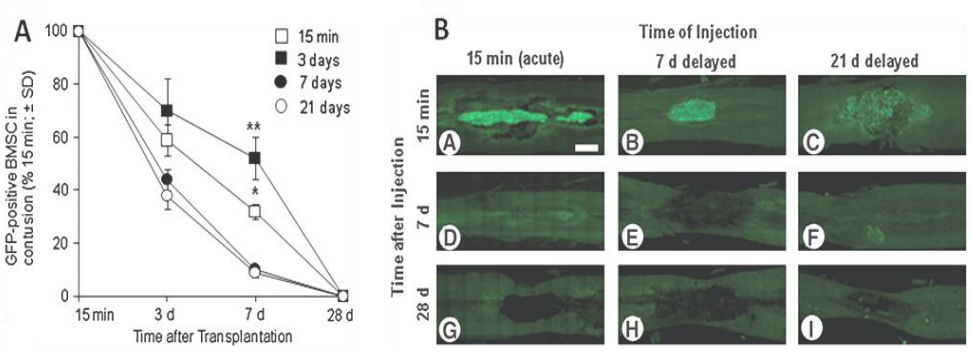

Fig. 4. A. BMSC numbers within a moderate contusion in the adult rat thoracic spinal cord decrease during 28 days post-injection. The rate at which cell death occurs is higher when BMSC are transplanted 7 or 21 days post-contusion, compared to BMSC transplantation 15 min or 3 days after contusion. B. The decreasing transplant is shown at 15 min (A–C), 7 days (D–F), and 28 days (G–I) after an injection at 15 min (acute), 7 days, and 21 days, respectively, post-injury. All microphotographs are from horizontal cryostat sections. (A) Scale bar, 600 mm in A–I.

Previously, using a rat contusion injury model, Hofstetter and colleagues[43] showed that more BMSCs survived when transplanted one week after injury compared to immediately after injury. The surviving cells were located within trabeculae that span the injury site. These data are in disagreement with those from the Nandoe Tewarie study[46] although long-term results were in agreement with only 1% of the cells (about 3000 total) surviving at 4 weeks after grafting.. The difference in early survival between the two studies may be that Hofstetter and co-workers injected the BMSC not only into the contusion but also rostral and caudal thereof into the spinal cord nervous tissue. Possibly, the surviving cells were located nearby but not in the contusion epicenter. Most studies have reported a poor survival of BMSC. Nandoe Tewarie and colleagues[46] demonstrated that the contusion milieu is less detrimental during the first week after injury than the second and fourth

week after injury. What factors are important for BMSC survival *in vivo*? BMSCs are cultured in medium containing 10-20% serum. Factors other than present in serum are not essential for their survival and proliferation within the culture dish. In fact, addition of growth factors such as BDNF, FGF-2, or NT-3 instigates differentiation of the BMSCs into neural-like cells rather than affect survival. To date, the factors that may promote BMSC survival in vivo are unknown and further investigations are necessary to reveal them.

CONCLUSION

Stem cells have gained attraction over the last years in the field of neuroscience. In vitro it has been shown, although still disputed, that Bone Marrow Stromal Cells can transdifferentiate into cells of neural lineage. This has made this adult stem cell type interesting for neural transplantation paradigms. After transplantation of BMSC in the injured spinal cord most cells die. Nevertheless, especially in early transplantation, cells have a neuroprotective effect on the host tissue. This effect may well be the result of secretion of growth factors. Further studies are needed to investigate the true potential of BMSC.

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Chapter 2

Bone Marrow Stromal Cell-Mediated Tissue Sparing Enhances Functional Repair after Spinal Cord Contusion in Adult Rats

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ABSTRACT

Bone marrow stromal cell (BMSC) transplantation has shown promise for repair of the spinal cord. We showed earlier that a BMSC transplant limits the loss of spinal nervous tissue after a contusive injury. Here, we addressed the premise that BMSC-mediated tissue sparing underlies functional recovery in adult rats after a contusion of the thoracic spinal cord. Our results reveal that after two months BMSCs had elicited a significant increase in spared tissue volumes and in blood vessel density in the contusion epicenter. A strong functional relationship existed between spared tissue volumes and blood vessel density. BMSC-transplanted rats exhibited significant improvements in motor, sensorimotor, and sensory function which were strongly correlated with spared tissue volumes. Retrograde tracing revealed that rats with BMSCs had twice as many descending brainstem neurons with an axon projecting beyond the contused spinal cord segment and these correlated strongly with the improved motor/sensorimotor functions but not sensory functions. Together, our data indicate that tissue sparing greatly contributes to BMSC-mediated functional repair after spinal cord contusion. The preservation/formation of blood vessels and sparing/regeneration of descending brainstem axons may be important mediators of the BMSC-mediated anatomical and functional improvements.

INTRODUCTION

Contusion of the adult rat thoracic spinal cord causes immediate locomotor and sensory impairments of the hindlimbs[e.g.,1,2]. Spontaneous recovery in locomotor ability reaches plateau levels a few weeks after a contusion[3-6]. Sensory function remains impaired[7,8] with only small improvements months after trauma[9].

Transplantation of repair-promoting cells into the contused spinal cord has been explored as an intervention to restore function over what is spontaneously observed[e.g.,2,10-15]. Bone marrow stromal cells (BMSCs) are among the candidate cell types for spinal cord repair[14,16]. BMSCs are relatively easy to obtain which warrants their promise for clinical application[17-19].

BMSC transplantation into the contused rat spinal cord improves overground walking[20-29]. However, this particular gain in motor function was not observed in several other studies[30-33]. Little is known about the effects of BMSC transplants on contusion-induced sensory impairments. Himes and colleagues[23] showed that BMSC transplantation into the contused adult rat spinal cord does not affect thermal hyperalgesia.

The mechanisms underlying BMSC-mediated repair of the contused spinal cord remain elusive. Cell replacement is doubtful as grafted BMSCs survive poorly[e.g.,16,24,34] and their differentiation into neural cells is moot[35-38]. Another possible repair mechanism is neuroprotection resulting in tissue sparing in the contused segment. Larger spared tissue volumes are often[e.g.,2,39-41] but not always[42,43] accompanied by improved functional outcome.

In the present study, we tested the premise that BMSC-mediated tissue sparing underlies functional recovery in adult rats with a contused spinal cord. Previously, we showed that BMSC transplantation into the three-day old contused adult rat spinal cord elicits tissue sparing[34]. We employed this paradigm and investigated the contribution of tissue sparing in functional outcomes after two months. Moreover, blood vessel formation and descending brainstem axon sparing/regeneration were explored for their role in tissue sparing and functional restoration.

MATERIALS AND METHODS

Ethics and surgical approval

Rats used in this study were housed pre- and post-surgery according to the National Institutes of Health and the United States Department of Agriculture guidelines. Air in the cages was continuously refreshed and water and food were available ad libitum. At all times during the experiment, rats were kept within a double-barrier facility. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University and the University of Pittsburgh.

BMSC culture and lentiviral transduction

BMSCs were harvested from the marrow of femurs of adult female Sprague-Dawley rats (n = 6, 200-230 g; Harlan, Indianapolis, IN, USA) according to previously described methods[44,45]. Lentiviral vectors (LV) encoding for green fluorescent protein (GFP) were used at an MOI of 150 to transfect BMSC at passage 0 to enable identification after transplantation[34]. BMSCs from the third passage were used for transplantation and of these 63 % expressed GFP as determined by FACScan/FACSorter (Becton Dickinson Immunocytometry Systems Biosciences, San Jose, CA, USA). The cells used for transplantation expressed the typical BMSC markers, CD90 and CD105 but not blood cell markers, CD34 and CD45 or the immune cell marker, HLA-DR[45].

Spinal cord contusion

Adult female Sprague-Dawley rats (n = 24, 200-230 g; Harlan) were sedated with intraperitoneal injections of 60 mg/kg Ketamine HCl (Phoenix Pharmaceuticals, St. Joseph, MD, USA) and 0.4 mg/kg medetomidine (Domitor®, an alpha-2-adrenergic agonist; Orion Corporation, Espoo, Finland). The back was shaved and cleaned with Betadine and 70 % alcohol, and Lacrilube ointment was applied to the eyes. The lower thoracic (T) spinal column was exposed, the T9 lamina was removed, and the underlying T10 spinal cord was contused using the Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC, Lexington, Kentucky, USA) at 200 kdyn[45,46]. Two rats with recorded force and displacement that deviated over 5 % from the intended values were excluded. Two rats died before

receiving the contusive impact. The wound site was rinsed with phosphate-buffered saline (PBS) with 0.1 % gentamicin (Abbot Laboratories, North Chicago, IL, USA), the muscles were closed in layers using 5.0 sutures, and the skin was closed with Michel wound clips. Rats received a subcutaneous injection of 1.5 mg/kg atipamezole hydrochloride (antisedan®; an alpha-2-adrenergic antagonist; Pfizer Inc., New York, NY, USA) to reverse the sedative and analgesic effects of medetomidine. Ten ml lactated Ringer's solution was injected subcutaneously and 6 mg/kg gentamicin (Abbott Laboratories) intramuscularly. The rats were kept at 37 °C until fully awake and were then returned to their cages. Between contusion and transplantation three days later, rats received daily 5 ml Ringer's solution (subcutaneously), 6 mg/kg gentamicin (Abbot Laboratories; intramuscularly), and 0.03 mg/kg Buprenorphin (Buprenex; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA; subcutaneously) and the bladder was emptied manually twice per day.

BMSC transplantation

Three days post-contusion, rats were tested using the Basso-Beattie-Bresnahan rating scale (BBB test)[3,47]; three rats were removed from the study as their BBB score was higher than 5[4,34]. The remaining rats were sedated with intraperitoneal injections of 60 mg/kg Ketamine HCl (Phoenix Pharmaceuticals) and 0.4 mg/kg medetomidine (Orion Corporation). The T10 spinal cord segment was re-exposed and 1 x 106 BMSCs in DMEM (n = 9) or DMEM alone (control group; n = 8) (total volume was 5 µl in both cases) was injected into the contusion epicenter using a Hamilton syringe with a pulled glass needle attached (tip diameter: 150 µm) fixed within a micromanipulator[2]. The cells were injected over a 5 min period using an electronic micro-pump and the needle was kept in place for an additional 3 min. The wound site was closed in layers. The rats were kept at 37 °C until fully awake and then returned to their cages. The following three days, rats received daily 5 ml Ringer's solution (subcutaneously), 6 mg/kg gentamicin (Abbot Laboratories; intramuscularly), and 0.03 mg/kg Buprenorphin (Buprenex®; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA; subcutaneously). The bladder was emptied manually twice per day until reflex voiding occurred (± two weeks). Throughout the remainder of

the experiment rats were monitored daily; all rats survived without a need for treatment for pain or distress.

Testing of locomotor function

Overground locomotor recovery of automated hindlimb movements was assessed using the BBB test[3,47] once a week for eight weeks post-injection over a 4 min period by two testers unaware of the treatments. Rats were familiarized with the open field and baseline values were confirmed.

Higher motor functions were assessed using the BBB sub-score[48]. In the predominant paw we determined position (internal/external at initial contact (IC) and liftoff (0 points), parallel at IC and internal/external at liftoff or vice versa (1), parallel at IC and liftoff (2)), toe clearance (none (0), occasional (1), frequent (2), consistent (3)), tail position (down (0), up (1)), and trunk instability (yes (0), no (1)). Scores were summed for a possible maximum score of 7[48].

Locomotion pattern was assessed using footprint analysis (modified from de Medinaceli et al.[49]). Before (baseline) and at four and eight weeks post-injection, the forepaws were dipped in red ink and the hindpaws in blue ink and footprints were recorded during walking across a paper track (100 cm long, 7 cm wide) and used to measure angle of hindpaw rotation, hindlimb base of support, and hindlimb stride length. Average values per paw were calculated from at least five sequential steps. Values for both hindpaws were averaged. Hindpaw rotation was defined by the angle formed by the intersection of the line through the prints of the third digit and the metatarsophalangeal joint and the line through the central pad parallel to the walking direction. Hindlimb base of support was determined by measuring the core to core distance of the hindpaws central pads. Hindlimb stride length was defined by the distance between the central pads of two consecutive prints. At the time of footprint testing all rats exhibited weight support.

Sensorimotor function of the hindlimbs was assessed before (baseline) and at four and eight weeks post-injection using horizontal ladder walking[50]. Rats walked across a 100 cm long horizontal ladder three times each test. The passages were videotaped and played back in slow-motion for accurate evaluation. Only the middle 60 cm of the ladder was

used for measurements. Small (foot or part of foot), medium (foot and part of lower leg), and large (full leg) slips were counted and individually and collectively expressed as a percentage of the total number of steps.

Testing of sensory function

Thermal hyperalgesia (i.e., an increased response to a nocuous stimulus) was measured at four and eight weeks post-injection by assessing the withdrawal response time to a heat source applied using a Hargreaves' heat source[51]. Rats were acclimated for 5 min before measurements in a Plexiglas test box with an elevated mesh floor. The radiant heat source with constant intensity was aimed at the mid-plantar area of each hindpaw and the time (in sec) until limb withdrawal was recorded. The heat source automatically shuts down at 15 sec to prevent injury. The resting interval between measurements was 20 min. All rats withdrew their limb before the cut-off time was reached. Baseline measurements were taken before contusion. Three middle measurements out of five of each paw were averaged and expressed as a percentage of the baseline.

Mechanical allodynia (i.e., a pain-related response to a normally innocuous stimulus) was measured at four and eight weeks post-injection by assessing the force to the hindpaw resulting in limb withdrawal. Rats were acclimated for 5 min before measurements in a Plexiglass test box with an elevated mesh floor. An electronic von Frey anesthesiometer[52] was applied perpendicularly to the mid-plantar area of each hindpaw and the pressure increased until limb withdrawal. The force (in g) at withdrawal was recorded. Before contusion baseline measurements were taken. Three middle measurements out of five of each paw were averaged and expressed as a percentage of the baseline.

Retrograde neuronal tracing

After the last functional tests were completed, rats were injected with 1.2 µl fast blue (FB) at 7 mm caudal to the contusion epicenter (between vertebrae T10 and T11) to retrogradely label neurons with an axon projecting caudal to the contusion/transplant. The tracer was injected in 6 deposits of 0.2 µl using a glass needle (approx. diameter: 150 µm) attached to a 5 µl Hamilton syringe held within a micromanipulator[2,53].

Histological procedures

One week after retrograde tracer injections, rats were anesthetized with an intraperitoneal injection of 60 mg/kg Ketamine HCl (Phoenix Pharmaceuticals) and 0.4 mg/kg medetomidine (Domitor®, Orion Corporation). After deep sedation was confirmed, the heart was exposed and 0.1 ml Heparine (500 IU; Henry Schein, Melville, NY, USA) was injected into the left ventricle. Next, 300 ml saline followed by 500 ml 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) was pumped through the cardiovascular system. The spinal cord and brain were removed and post-fixed overnight in the same fixative at 4 °C. The brainstem, motor cortex, and a ten mm long spinal cord segment centered at the contusion site were transferred to phosphate-buffered 30% sucrose. These tissue blocks were embedded in Shandon M-1 Embedding Matrix (Thermo Electron Corporation, Pittsburgh, PA) and cut on a Leica CM 1950 cryostat. The spinal cord segment was cut into ten series of 20 µm-thick horizontal sections and the brainstem and motor cortex were cut into ten series of 40 µm-thick transversal sections. The sections were mounted onto glass slides and stored at −20 °C until further processing. One series of sections was stained with cresyl violet to demonstrate the cytoarchitecture of the contused segment. Another series was stained with luxol fast blue to demonstrate presence of myelin.

Immunohistochemistry

Four series of cryostat sections were permeabilized with 0.3% Triton X-100 in PB (0.1 M, pH 7.4), immune-blocked with 5% normal goat serum in PB at room temperature for 30 min, and then immunostained[53]. The following primary antibodies were used: polyclonal rabbit antibodies against glial fibrillary acidic protein (GFAP, 1:200; Incstar Corp., Stillwater, MN), and monoclonal mouse antibodies directed against rat endothelial cells (RECA-1 (20), 1:100; AbD Serotec, Raleigh, NC). Then, the sections were washed with PBS and incubated for 2 h at room temperature with goat-anti-mouse IgG or goat-anti-rabbit IgG coupled to Alexa Fluor® 594 (1 : 200; Molecular Probes). Staining specificity was confirmed by omitting the primary antibody. Stained sections were covered with glass

slips with Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA) and kept refrigerated until analysis.

Measurement of spinal cord tissue sparing

Cresyl violet-stained sections were used to determine in a blinded fashion the volume of spared tissue in each spinal cord using the Cavalieri estimator function of Stereo Investigator® (MicroBrightField Inc.). The Gundersen Coefficient of Error was < 0.05 for all measurements. In each tenth section the volume of spared tissue in a 3.4 mm long segment centered at the contusion epicenter was determined. Within the analyzed segment, tissue was considered spared if it lacked cavities, areas with densely packed small cells resembling infiltrated neutrophils and lymphocytes, and neurons with darkly stained cytoplasmic Nissl bodies[2,34,43]. The volume of spared tissue was expressed as a percentage of the average volume of a comparable uninjured spinal cord segment.

Measurement of blood vessel density

One series of sections was stained with RECA-1 antibodies[54] and used to determine the blood vessel density (RECA-1-positive blood vessels per tissue surface area) using Stereo Investigator® (MicroBrightField Inc.) in a blinded fashion. In each tenth section, at 20 x magnification, three areas were analyzed; the epicenter, rostral to the epicenter, caudal to the epicenter. Within each area, three samples were taken (320 x 420 µm each) in which all RECA-1-positive structures were outlined. In most cases, the measuring area of the three contiguous samples was larger than the width of the spinal cord in the epicenter but smaller than the width just rostral and caudal to the epicenter. Therefore, we normalized the blood vessel surface area for the measured tissue surface area to get the blood vessel density (blood vessel area per 0.1 mm^2). The values per level in each section were averaged to give the blood vessel density per level per rat.

Quantification of GFP-positive BMSCs and FB-positive neurons

One series of spinal cord cryostat sections was covered with a glass slip with Vectashield with DAPI (Vector Laboratories, Inc.) and used to determine the number of GFP-positive

BMSCs in the contusion using Stereoinvestigator (MicroBrightField Inc., Colchester, Virginia, USA). In each tenth section with GFP-positive cells, the transplanted area was outlined manually at 4 x magnification and covered with a 250×250 µm grid. At 60 x magnification with oil immersion, GFP-positive cells were marked using the optical fractionator with a 60×60 µm counting frame. BMSC numbers were corrected for the 63 % transduction rate[34].

One series of brainstem and motor cortex sections was covered with a glass slip with Vectashield (Vector Laboratories, Inc.). FB-positive cells in the red nuclei, reticular formation, vestibular nuclei, raphe nuclei, and motor cortex (hindlimb area) were counted by an investigator unaware of the treatments. FB-labeled neurons counted in the gigantocellular reticular formation and raphe nuclei were summed because these nuclei are difficult to distinguish from each other. All numbers were multiplied by 10 to obtain total numbers of FB-positive cells per nucleus[2].

Statistical analysis

Student T-test and, in case of multiple time points, repeated measures ANOVA followed by Tukey's post hoc test were used to determine statistical differences between groups. Differences were accepted as statistically significant at $P < 0.05$. Correlation analysis was used to determine the relationship between variables and expressed by the coefficient of determination (r^2) and the significance level (p). Relationships were considered strong when $r^2 > 0.25$ and accepted when $p < 0.05$.

RESULTS

BMSC-transplants elicited tissue sparing

Spared tissue volume in a 3.4 mm long segment centered at the contusion (Fig. 1A) was determined in BMSC-transplanted rats (Fig. 1B) and control rats (Fig. 1C) at eight weeks post-injection. With a BMSC transplant the volume of spared tissue relative to that of a similar spinal cord segment in an uninjured/untreated rat was 24.8 ± 1.4 % (SEM), which represented a significant 66 % increase ($p < 0.01$) in tissue sparing compared to controls (14.9 ± 1.6 %; Fig. 1D). The tissue that was spared in BMSC-transplanted rats consisted

mostly of white matter (97.03 ± 2.6 %) and little of gray matter (2.97 ± 2.6 %; Fig. 1E). In three out of nine BMSC-transplanted rats, GFP-positive BMSCs were present in the contusion at eight weeks post-injection (Fig. 1F). Some of these BMSCs were round and other elongated (Fig. 1G). The total numbers of BMSCs in these three rats were 3937 (0.4 % of injected number of cells), 11460 (1.1 %), and 13429 (1.3 %). In all rats, the contusion epicenter was surrounded by GFAP immunostaining, which was used to identify the contusion epicenters (Fig. 1H, 1I).

Rats with BMSCs demonstrated improved motor and sensorimotor functions

Overground walking ability (Fig. 2A) was not different (repeated measures ANOVA) between treatment groups. Both groups had a final score of about 11 (Fig. 2B) indicating frequent to consistent weight-supported plantar stepping without forelimb-hindlimb coordination. Higher locomotor functions were significantly different (repeated measures ANOVA) between groups ($F(1, 126) = 240.6$; $p < 0.0001$). The final score was 4.0 ± 0.3 (SEM) in rats with BMSC and 1.8 ± 0.3 in controls (Fig. 2C).

Footprint analysis (Fig. 2D) revealed a significant difference between groups in angle of hindpaw rotation (repeated measures ANOVA: $F(1, 20) = 28.5$; $p < 0.0001$; Fig. 2E) and hindlimb base of support ($F(1, 20) = 15.6$; $p < 0.001$; Fig. 2F) but not hindlimb stride length ($P = 0.29$; Fig. 2G). The angle of rotation of rats with BMSCs

was 38.6 % smaller (M = 17.8; 95% Cl [15.8, 19.8]) at four weeks and 25.7 % smaller (M = 19.8; 95% Cl [18.8, 20.8]) at eight weeks post-injection compared to controls (M = 29.0; 95% Cl [26.3, 31.7] and M = 26.5; 95% Cl [26.0, 27.0], respectively; Fig. 2E). At eight weeks post-injection, external rotation of the hindpaws was increased in BMSC-transplanted rats compared to baseline (Fig. 2E). The base of support of BMSC-transplanted rats was 17.0 % smaller (M = 39.0; 95% Cl [36.0, 42.0]) at four weeks post-injection compared to controls (M = 47.0; 95% Cl [45.0, 49.0]) and similar at eight weeks post-injection (Fig. 2F). At the later time point, the base of support in transplanted rats was 12.3 % larger than baseline (Fig. 2F).Horizontal ladder walking (Fig. 3A) was significantly different between groups (repeated measures ANOVA: $F(1, 24) = 24.8$; $p < 0.0001$). The numbers of slips was expressed as a percentage of the total number of steps needed to cross the measuring

area (average of both groups: 14.4 ± 0.2 (SEM) at four and 15.0 ± 0.3 at eight weeks). For total slips, the improvement in rats with BMSCs was 66.0 % at four weeks (M = 32.1; 95% Cl [28.9, 35.3]; Fig. 3B) and 69.6 % at eight weeks (M = 21.4; 95% Cl [18.8, 24.0]; Fig. 3C) compared to controls. and 1545 ± 220 in white matter only. The blood vessel density in epicenter white matter in BMSC-transplanted rats was significantly ($p < 0.01$) 4.5-fold higher than in controls and 2.3-fold higher than in uninjured rats (Fig. 5E).

BMSC-transplanted rats had improved sensory functions

Thermal hyperalgesia (Fig. 4A) was significantly decreased in rats with BMSCs compared to controls (repeated measures ANOVA: F (1, 0.41) = 53.5; $p < 0.0001$; Fig. 4B). The improvement was 43.7 % (M = 10.2; 95% Cl [9.9, 10.5]) at four weeks and 24.6 % (M = 7.6; 95% Cl [7.3, 7.9]) at eight weeks post-injection (M = 7.1; 95% Cl [6.7, 7.5] and M = 6.1; 95% Cl [5.8, 6.4], respectively; Fig. 4B).

Mechanical allodynia (Fig. 4C) was significantly decreased in rats with BMSCs compared to controls (repeated measures ANOVA: F (1, 24) = 26.9; $p < 0.0001$; Fig. 4D). The improvement was 34.7 % at four weeks post-injection (M = 96.9; 95% Cl [94.2, 99.6]) and 51.7 % (M = 88.0; 95% Cl [85.8, 90.2]) at eight weeks post-injection (M = 72.3; 95% Cl [70.0, 74.6] and M = 58.2; 95% Cl [56.5, 59.9], respectively; Fig. 4D). Table 1 provides an overview of the functional test results.

Rats with BMSCs had higher blood vessel density in the contusion epicenter

The density of blood vessels was assessed in the contusion epicenter, the white matter in the epicenter, and outside of the epicenter (Fig. 5A). In the epicenter, blood vessels were mostly larger and with fewer branches (Fig. 5B) than outside of

the contused area (Fig. 5C). The blood vessel density in the epicenter was significantly ($p < 0.001$) 6.5-fold higher in BMSC-transplanted rats than in controls (Fig. 5D). Outside of the epicenter there were no significant differences between groups (Fig. 5D), which was in agreement with previous reports[55]. The latter value was similar as that found in the epicenter in BMSC-transplanted rats and in the spinal cord of uninjured rat (Fig. 5D). In uninjured rats the average blood vessel density was 4922 ± 397(SEM) in the spinal cord,

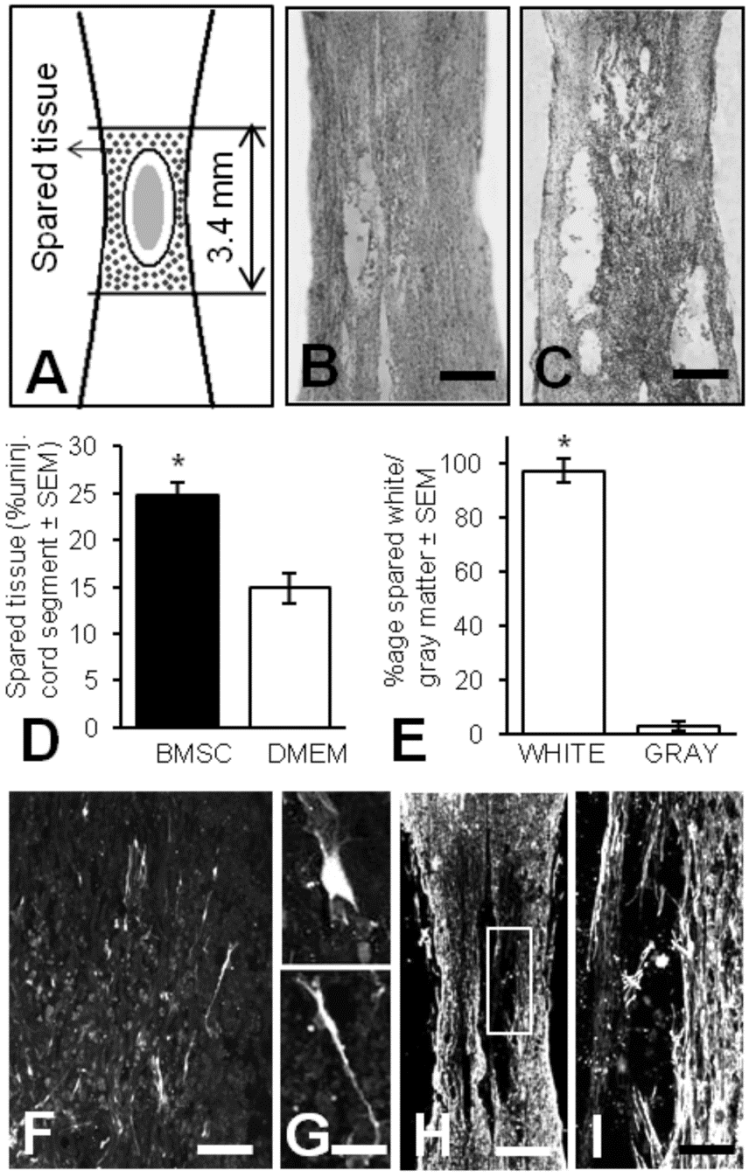

Fig. 1. BMSCs increased the amount of spared tissue in the contusion epicenter. Spared tissue volumes were determined at eight weeks post-transplantation within a 3.4 mm long spinal cord segment centered at the contusion (A) in BMSC-transplanted rats (B) and controls (C). BMSCs elicited a neuroprotective effect as the spared tissue volume in transplanted rats was significantly ($p < 0.01$) 66 % larger than in controls (D). In BMSC-transplanted rats, 97 ± 2.6 % of the spared tissue consisted of white matter (E). Three out of nine transplanted rats had GFP-positive BMSCs in the contusion (F), which exhibited largely variable morphologies (G). GFAP-positive astrocytes were present in the contusion epicenter in all rats (H). In most rats, GFAP-positive astrocytes were found within tissue strands in the epicenter (I). Note panel I is outlined in panel H. Bar in B is 500 µm. Bar in C is 500 µm. Bar in F is 250 µm. Bar in G is 50 µm. Bar in H is 500 µm. Bar in I is 250 µm.

8299 ± 645 in gray matter only, and 1545 ± 220 in white matter only. The blood vessel density in epicenter white matter in BMSC-transplanted rats was significantly ($p < 0.01$) 4.5-fold higher than in controls and 2.3-fold higher than in uninjured rats (Fig. 5E).

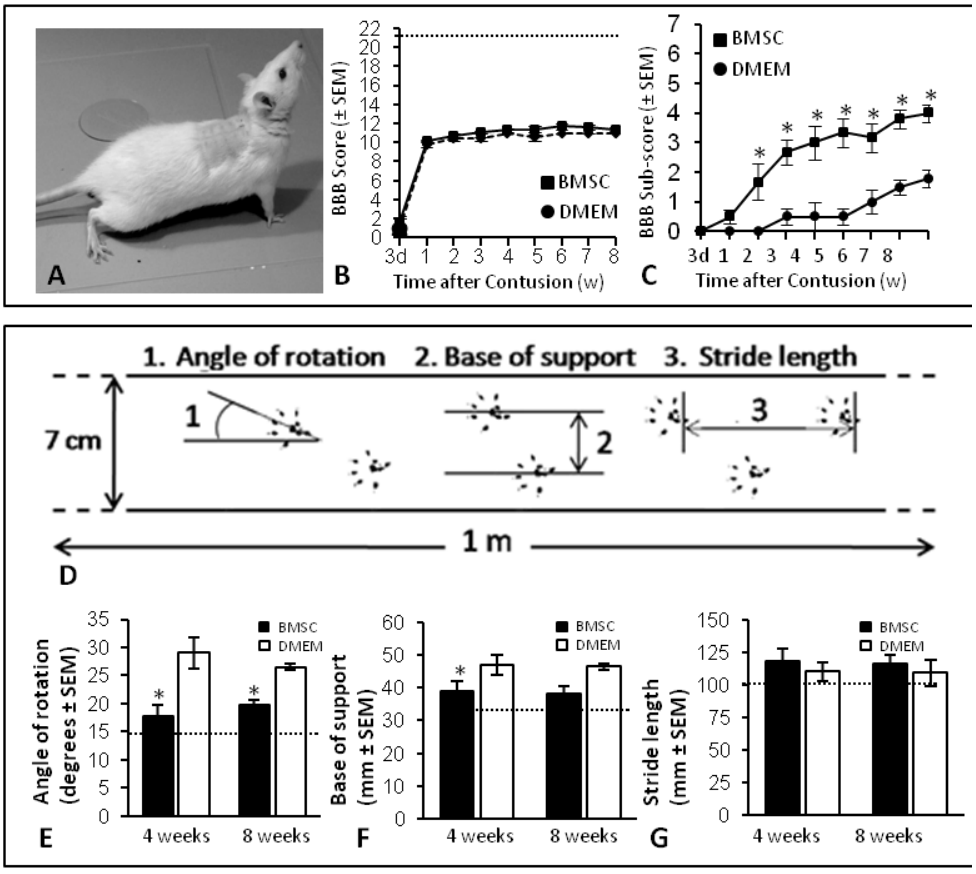

Fig. 2. BMSCs improved locomotion-related features/pattern but not overground walking. With the BBB test (A) we found that BMSC-transplanted and control rats exhibited similar overground walking abilities throughout survival (B). The baseline (21) is indicated by the horizontal broken line. The BBB sub-score revealed that higher motor functions in BMSC-transplanted rats were significantly ($p < 0.0001$) better than in controls (C). Footprint analysis was used to assess locomotion pattern by measuring the angle of hindpaw rotation, base of support, and stride length (D). Hindpaw rotation in BMSC-transplanted rats was significantly ($p < 0.01$) improved by 39 % at four and 26 % at eight weeks post-injection compared to controls (E). At eight weeks, external rotation of the hindpaws in BMSC-transplanted rats was increased compared to baseline (15 ± 2.1; horizontal broken line). Base of support was 17 % improved ($p < 0.01$) in rats with BMSCs compared to controls at four weeks but similar at eight weeks post-injection (F). At the eight week time point, the base of support in rats with BMSCs was 12 % larger than baseline (34.2 ± 2.2; horizontal broken line). Stride length was similar between groups at both measuring time points (G) and not different from baseline (105 ± 8.3; horizontal broken line in G).

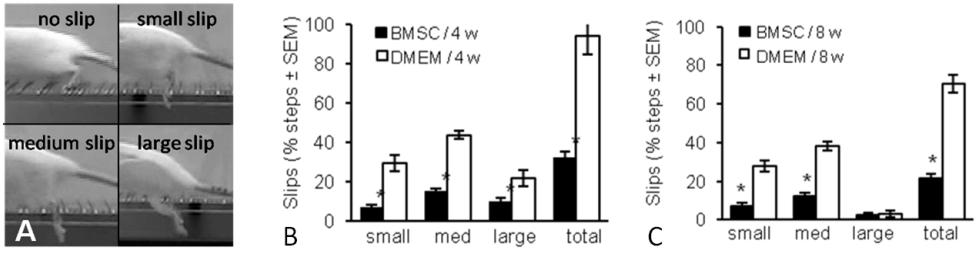

Fig. 3. BMSCs improved horizontal ladder walking performance. The number of small, medium, and large slips (A) at four and eight weeks post-injection were quantified and expressed as a percentage of the total number of steps used to cross the ladder. Rats with BMSCs performed significantly ($p < 0.0001$) better than controls at four (B) and eight (C) weeks post-injection. BMSC-transplanted rats made 66 % less total mistakes at four weeks and 70 % less at eight weeks post-transplantation.

Rats with BMSCs had more brainstem neurons projecting beyond the contusion

Retrograde FB tracing was used to identify brainstem and motor cortex neurons with an axon beyond the contusion site. Figure 6 provides examples of FB-labeled neurons present in the nucleus raphe obscurus (Fig. 6A, 6B) and the reticular formation (Fig. 6C, 6D). In transverse brainstem sections FB-labeled neurons were plotted and quantified in the red nucleus (Fig. 6E), reticular formation (Fig. 6F), vestibular nuclei (Fig. 6G), gigantocellular reticular nucleus (Fig. 6G), and raphe nuclei (Fig. 6H). No labeled neurons were found in the hindlimb area of the motor cortex. The total number of FB-labeled brainstem neurons in rats with BMSCs was 7650 ± 765; a significant twofold increase ($p < 0.05$) over controls (Fig. 6I). There were 2.2-fold more labeled neurons in the reticular formation ($p < 0.05$), threefold more in vestibular nuclei ($p < 0.05$), and twofold more in raphe nuclei (including the gigantocellular reticular formation; $p < 0.05$) in rats with BMSCs compared to controls (Fig. 6I). The number of FB-labeled neurons in the red nuclei was similar in both groups (Fig. 6I). With correlation analysis a strong significant association was identified between tissue sparing and total brainstem neurons ($r^2 = 0.57$, $p = 0.01$).

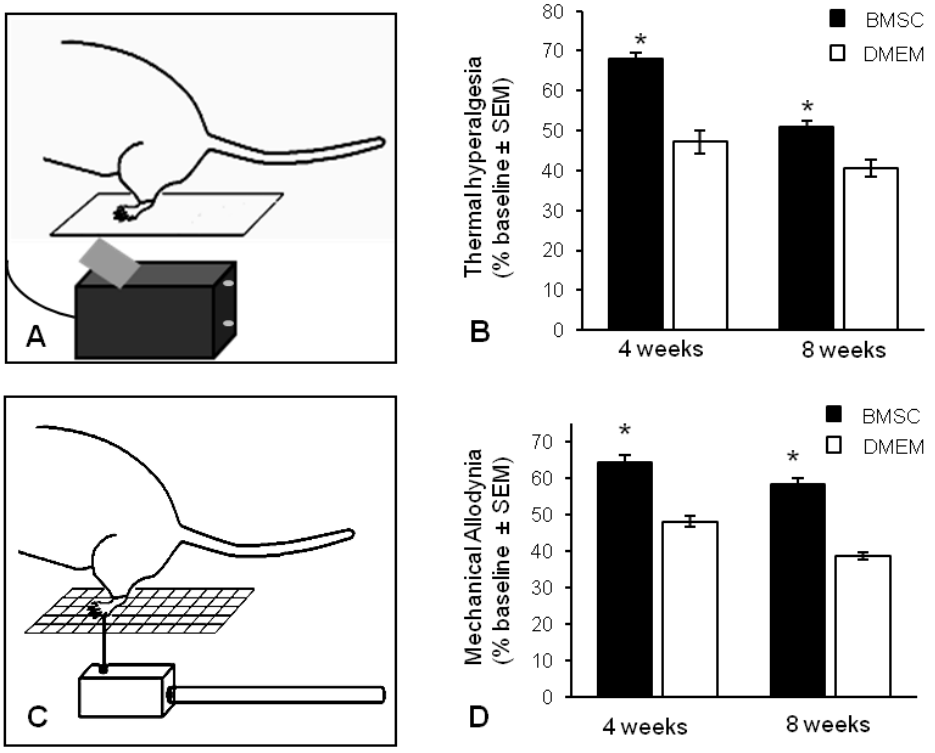

Fig. 4. BMSCs decreased thermal hyperalgesia and mechanical allodynia. Thermal hyperalgesia was assessed using a Hargreaves' heat source (A) which revealed that rats with a BMSC transplant had significantly ($p < 0.0001$) longer hindpaw withdrawal response times (i.e., improved tolerance) than controls (B). The improvement was 44 % at 4 weeks and 25 % at eight weeks after BMSC transplantation. At eight weeks post-injection, thermal hyperalgesia in BMSC-transplanted rats was 49 % above baseline (15 ± 1.8; horizontal broken line). With an electronic von Frey anesthesiometer (C) we found that mechanical allodynia in BMSC-transplanted rats was significantly ($p < 0.0001$) improved compared to controls (D). The improvement in tolerance to pressure to the hindpaws was 35 % at 4 weeks and 52 % at eight weeks after BMSC transplantation. At the latest time point, mechanical allodynia in rats with BMSC was 49 % above baseline (150 ± 13.5; horizontal broken line).

Table 1. Functional improvements at four and eight weeks after BMSC transplantation into the contused adult rat spinal cord.

	BBB	BBB subsc	Stride length	Base of support	Angle of rotation	Horizon. ladder	Thermal hyperalg	Mech. allodynia
4 wks	-	↑ 6.4x	-	↑ 17.0%	↑ 36.8%	↑ 66.0%	↑ 43.7%	↑ 34.7%
8 wks	-	↑ 2.2x	-	-	↑ 25.7%	↑ 69.6%	↑ 24.6%	↑ 51.7%

An upward arrow represents a statistically significant improvement in test performance of BMSC-transplanted rats compared to controls. A dash represents no difference between BMSC-treated rats and controls.

Relationships between tissue sparing, motor/sensory functions, blood vessel density, and brainstem neurons projecting beyond the contusion

Tissue sparing and motor/sensory functions (Table 2). A strong significant functional relationship was present between tissue sparing and BBB sub-score ($r^2 = 0.87$, $p < 0.001$), angle of rotation ($r^2 = 0.81$, $p = 0.001$), horizontal ladder walking ($r^2 = 0.74$, $p= 0.001$), and thermal hyperalgesia ($r^2 = 0.56$, $p = 0.013$). Relationships between tissue sparing and BBB, hindlimb stride length and base of support, and mechanical allodynia were not significant.

Motor/sensory function and brainstem neurons (Table 3). Strong significant functional relationships were found between raphe neurons and the BBB subscore ($r^2 = 0.78$, $p = 0.002$), angle of rotation ($r^2 = 0.68$, $p = 0.007$), and horizontal ladder ($r^2 = 0.54$, $p = 0.02$). Strong relationships were also found between the total brainstem neurons and the BBB subscore ($r^2 = 0.76$, $p = 0.002$), angle of rotation ($r^2 = 0.51$, $p = 0.03$), and horizontal ladder ($r^2 = 0.54$, $p = 0.02$). Reticular neurons had a strong significant relationship with BBB ($r^2 = 0.60$, $p = 0.008$) and BBB sub-score ($r^2 = 0.50$, $p = 0.03$).

Tissue sparing and blood vessel density. We found a strong significant functional relationship between spared tissue volumes and blood vessel density in the epicenter in general ($r^2 = 0.67$, $p = 0.004$) and in white matter only ($r^2 = 0.47$, $p = 0.04$).

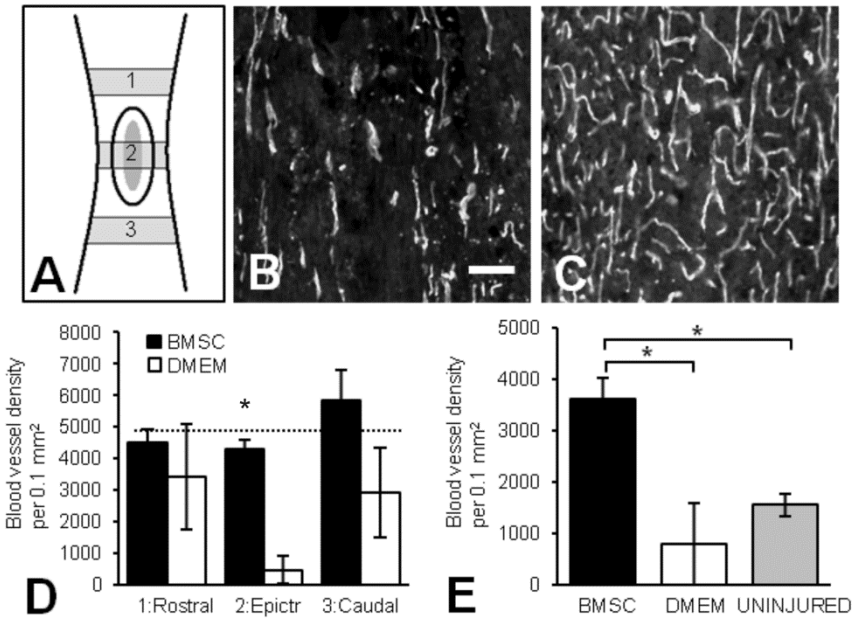

Fig. 5. BMSCs increased blood vessel density in the contusion epicenter. RECA-1 antibodies were used to identify blood vessels rostral, within, and caudal to the contusion epicenter (A). The blood vessels in the epicenter were generally longer, more straight, and with fewer branches (B) than outside the epicenter (C). In the epicenter blood vessel density was significantly ($p < 0.001$) higher in rats with BMSCs than in controls (D). Outside the epicenter the blood vessel density in both groups was similar and comparable to that in uninjured rats (average indicated by horizontal broken line). In white matter only in the epicenter, the blood vessel density was significantly ($p < 0.01$) higher in BMSC-transplanted rats than in controls or uninjured rats (E). Bar in B represents 35 µm in B and C.

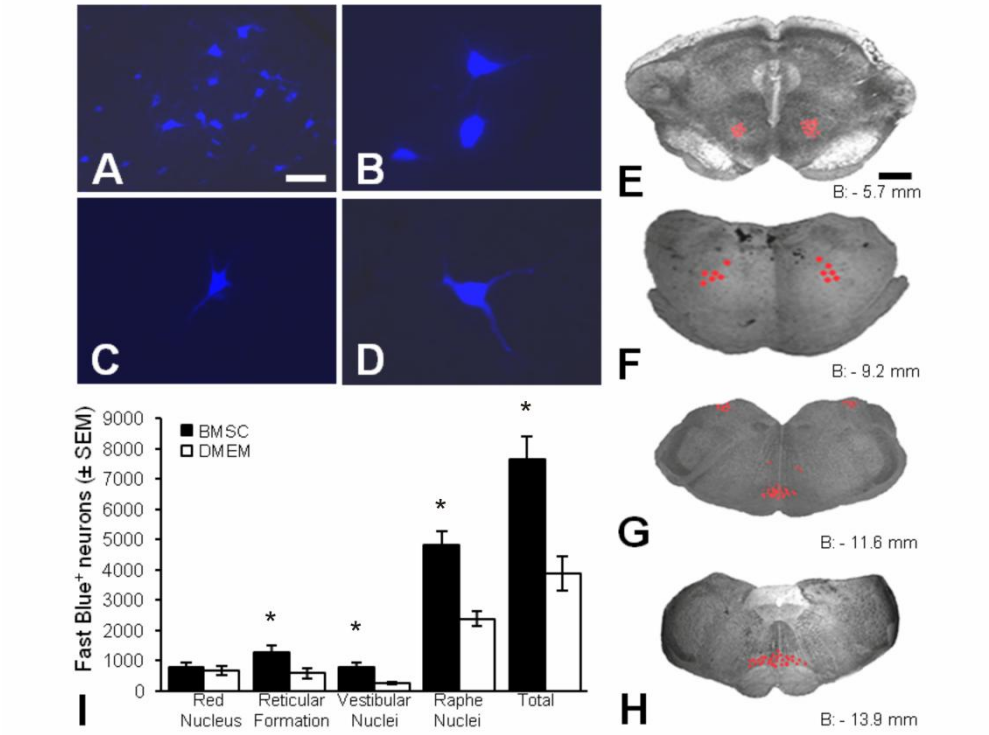

Fig. 6. BMSCs increased the number of brainstem axons caudal to the contusion. We used retrograde FB tracing to identify brainstem and motor cortex neurons projecting beyond the contusion. Labeled neurons were found in several brainstem nuclei including the nucleus raphe obscures, shown here at 10 X (A) and 20 X (B), and the reticular formation, shown here at 20 X (C, D). FB-labeled neurons (represented by red dots) were found in the red nucleus (E), reticular formation (F), vestibular and gigantocellular reticular nuclei (G), and the nucleus raphe obscurus and nucleus raphe pallidus (H). The level of brainstem sections shown in E-H is provided relative to Bregma (B). Rats with BMSCs had significantly ($p < 0.05$) more FB-labeled neurons in their brainstem than controls (I). Significantly higher numbers ($p < 0.05$) were found in the reticular formation, vestibular nuclei, and raphe nuclei, but not the red nucleus (I). On average the increase in FB-labeled neurons in BMSC-transplanted rats was 2.3-fold. Bar in A represents 50 µm in A and 25 µm in B-D. Bar in E represents 1 mm in E-H.

Table 2. Relationship between tissue sparing and motor/sensory functions at eight weeks after BMSC transplantation into the contused adult rat spinal cord.

	BBB	BBB subsc	Stride length	Base of support	Angle of rotation	Horizon. ladder	Thermal hyperal	Mech. allody
Tissue sparing	0.13	**0.87**	0.12	0.19	**0.81**	**0.74**	**0.56**	0.03
	0.31	**0.001**	0.40	0.24	**0.001**	**0.001**	**0.013**	0.61

Correlation analysis was employed to examine the functional relationships between the outcome measures at eight weeks post-injection. The table provides the coefficient of determination (r^2, top) and the significance level (P value, bottom). Strong statistically significant relationships between spared tissue volumes and neuron numbers ($r^2 > 0.25$, $P < 0.05$) are shaded in gray. Abbreviations: BBB = Basso-Beattie-Bresnahan.

Table 3. Relationship between brainstem neurons and motor/sensory functions.

	BBB	BBB sub	Stride length	Base of support	Angle rotation	Horizon. ladder	Therm hyper	Mech. allod
Red nucleus	0.00	0.11	0.25	0.11	0.08	0.08	0.07	0.08
	0.99	0.39	0.21	0.39	0.45	0.42	0.46	0.43
Reticular formation	**0.60**	**0.50**	0.49	0.00	0.09	0.13	0.01	0.16
	0.008	**0.03**	0.05	0.97	0.41	0.31	0.81	0.25
Vestibular nuclei	0.01	0.42	0.10	0.28	0.38	0.29	0.10	0.05
	0.44	0.06	0.44	0.14	0.08	0.10	0.39	0.54
Raphe nuclei	0.17	**0.78**	0.25	0.22	**0.68**	**0.54**	0.29	0.07
	0.24	**0.002**	0.21	0.19	**0.007**	**0.02**	0.11	0.45
Total brainstem	0.22	**0.76**	0.32	0.11	**0.51**	**0.44**	0.199	0.11
	0.18	**0.002**	0.14	0.39	**0.03**	**0.04**	0.20	0.34

We used correlation analysis to assess the functional relationships between the outcome measures at eight weeks post-transplantation. The table lists the coefficient of determination (r^2, top) and the significance level (P value, bottom). Statistically significant strong relationships between neuron numbers and function ($r^2 > 0.25$, $P < 0.05$) are shaded in gray. Abbreviations: BBB = Basso-Beattie-Bresnahan.

DISCUSSION

We found that a BMSC transplant in the contused spinal cord of adult rats elicits tissue sparing which correlated strongly with improvements in higher motor functions, hindpaw rotation, horizontal ladder walking, and thermal hyperalgesia. The data also revealed that rats with BMSC elicited an angiogenic response in the contusion epicenter which correlated with tissue sparing. Retrograde tracing revealed that rats with BMSCs had significantly more brainstem neurons with an axon projecting beyond the contusion which correlated with improved motor functions but not sensory functions. Together, the data indicate that BMSC transplants in the adult rat contused spinal cord result in tissue sparing which is differentially involved in functional restoration. Blood vessel preservation/formation and descending brainstem axon sparing/regeneration appear important mediators of the BMSC-mediated improved outcomes.

Transplantation of BMSCs into the contused adult rat spinal cord resulted in a significant 66 % increase in spared tissue volume in the injury epicenter. This finding is consistent with previous studies[25,30,34]. Tissue sparing was determined at eight weeks post-transplantation. At this time point almost all BMSCs had died; few were present in only three of the transplanted rats. Therefore, it is unlikely that the transplanted cells contributed significantly to the volume of spared tissue. We showed previously that BMSCs transplanted into the contusion at three days post-injury survive poorly with 50 % lost after one week and almost all lost after four weeks[34]. Interestingly, the relative short duration of BMSC presence in the contusion is sufficient to elicit a significant tissue sparing effect. The neuroprotective effect of BMSC transplants is not present with delayed (seven and twenty-one days) injection paradigms[34], which indicates the existence of a specific (sub)acute therapeutic window for BMSC-mediated neuroprotection.

The mechanisms underlying BMSC-mediated tissue sparing are largely unknown. In search for mediators of this effect we identified blood vessels to be a possible candidate. After injury the loss of blood vessels decreases the spinal cord blood flow which correlates with nervous tissue loss[56]. We show here that rats with BMSCs transplants have significant

higher blood vessel densities in the contusion epicenter and that this was strongly associated with spared tissue volumes. Transplanted BMSCs secrete the proangiogenic vascular endothelial growth factor (VEGF)[57] which is known to be involved in protection[58] and formation[e.g.,59] of blood vessels in injured nervous tissue. Through both mechanisms, BMSCs could have contributed to tissue sparing in the contusion epicenter.

A positive relationship between blood vessel density and spared tissue volumes in the injured spinal cord has been proposed before for adult rats[55] but not for adult guinea pigs[60]. The latter study revealed a correlation between blood vessel density and secondary loss of function indicating a role of hypervascularity in secondary pathology[60]. Our present results are in variance with this idea as they show greater improvements in neurological function in rats with higher blood vessel density in the contusion. Further studies are required to elucidate the role of hypervascularity in spinal cord repair.

BMSC transplantation resulted in improved motor, sensorimotor, and sensory functions which, with the exception of mechanical allodynia, strongly correlated with spared tissue volumes. In fact, based on our findings, over 70 % of the variability in higher motor and sensorimotor function and 56 % of that in thermal hyperalgesia can be attributed to a linear relationship with spared tissue volume. These results highlight a significant involvement of tissue sparing in functional restoration after a spinal cord contusion and emphasize the relevance of treatment that focus on tissue sparing as an early intervention in spinal cord repair strategies.

The behavioral improvements in our study seemed more pronounced during the first month after transplantation compared to the second month. Currently, it is unknown whether functional improvements due to BMSC transplantation remain present permanently. It is possible that the deteriorating general health due to spinal cord injury affects function negatively. Also, benefits from the BMSC transplant may be temporary due to the poor transplant survival. Future studies will be required to address these important clinically relevant issues.

The precise mechanisms underlying the relationship between spared tissue and motor and sensory functions are poorly understood. Tissue sparing likely includes a variety of cellular consequences which each exert their specific effect on behavioral recovery. We examined more closely to what extent descending axons from brainstem neurons were involved in the observed functional improvements. Different types of descending brainstem axons are involved in motor function[61-63]. We demonstrated in this study that the large majority of spared tissue in BMSC-transplanted rats is white matter. Thus it is possible that increased amounts of spared tissue reflect more spared axons descending to lower spinal cord segments involved in motor functions. We found here that rats transplanted with BMSC had a twofold increase in number of (retrogradely FB-labeled) brainstem neurons projecting their axon beyond the contusion compared to controls. The correlative analysis further indicated that these brainstem neurons were involved in over 40 % (coefficient of determination, $r2 > 0.4$) of the observed recovery in higher motor and sensorimotor function, but did not contribute to recovery in sensory functions ($r2 < 0.2$).

Among the brainstem neurons, raphe neurons were strongly correlated with higher motor functions and sensorimotor function and reticular formation neurons with higher motor functions and overground walking. Descending raphespinal axons have been implicated in initiating and modulating motor behaviors through the central pattern generator[64,65]. Reticulospinal axons have been associated with flexion during the swing phase and extension during the stance phase[66], which may explain their association with walking and higher motor functions.

BMSC-transplanted rats exhibited significantly improved higher motor functions as revealed with the BBB sub-scoring scale[48,64] and footprint analysis. Transplantation of BMSCs also significantly enhanced horizontal ladder walking with fewer small, medium, and total slips indicating enhanced integration of sensory and motor inputs[50]. Interestingly, the combined improvements did not result in improved overground walking as examined using the BBB test. A lack of improved walking in rats with a BMSC transplant was reported previously by some[30-33] but not by others[e.g., 23,24,27]. The absence of improved

walking could reflect the complexity of properly rating increasing degrees of forelimb-hindlimb coordination. This convolution has been recognized and resulted in the design of the BBB sub-scoring scale to increase the sensitivity of the BBB rating scale for rats that lack coordination[48,64]. An alternative explanation for the lack of improved walking in the present study is that mechanisms underlying recovery in overground walking are in part different from those underlying recovery of higher motor and sensorimotor functions. This possibility needs to be considered because elucidating the different mechanisms would be imperative for the design of more effective and comprehensive spinal cord repair approaches.

BMSC-transplanted rats exhibited less sensitivity to mechanical and thermal stimuli to the hindpaws, which is an improvement over the typically observed contusion-induced hypersensitivity. To our knowledge only one study has investigated the effects of a BMSC transplant on sensory function in rats with a contused spinal cord. Himes and colleagues[23] reported that transplantation of BMSCs did not affect thermal hyperalgesia. A possible explanation for this discordance with our study is the difference in time of BMSC transplantation; three days post-injury in our study and seven days post-injury in Himes et al[23]. We have previously shown that transplantation of BMSCs into a contused adult rat spinal cord at seven days post-injury leads to significantly less tissue sparing than transplantation at three days after injury[34]. We show in the present study that tissue sparing is correlated with thermal hyperalgesia reduction. Thus, less tissue sparing after a 7-day delayed BMSC transplantation may have caused the observed lack of improvements in thermal hyperalgesia in Himes et al.[23]

The injury-induced inflammatory response may contribute to the development of thermal hyperalgesia[e.g.,67]. BMSC transplantation has been associated with reduced inflammation[68,69]. Thus, it is possible that in our study the decrease in thermal hyperalgesia in transplanted rats reflects a BMSC-mediated decrease in inflammation, which may also contribute to tissue sparing[70]. On the other hand, we showed that BMSC transplantation into a three-day old spinal cord contusion does not lead to a decrease in

the number of macrophages[45]. Further studies are needed to elucidate the interactions between inflammation, thermal hypersensitivity, and tissue sparing following spinal cord injury and repair.

The present and previous studies suggest that optimal BMSC neuroprotective effects are obtained within a three-day (or acute) transplantation paradigm, suggesting clinical translation would favor allograft transplantation, because culturing an autograft would take more time. On the other hand, a human bone marrow biopsy, combined with cell sorting strategies, might provide an adequate amount of BSMCs to enable timely autograft transplantation. Additional research will be needed to investigate the applicability and efficacy of such autografts.

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Chapter 3

BDNF-overexpression augments bone marrow stromal cell-mediated neuroprotection in the contused rat spinal cord

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Submitted

ABSTRACT

The ability of bone marrow stromal cell (BMSC) transplants to elicit spinal cord repair is thought to result from paracrine effects by secreted trophic factors including brain-derived neurotrophic factor (BDNF). Here we used gene therapy to increase or silence BDNF production in BMSCs and investigated the involvement of BDNF in BMSC-mediated neuroprotection. In a spinal cord organotypic culture, conditioned medium of BMSCs with normal, increased, or decreased BDNF enhanced spinal motoneuron survival averagely 64 % compared with culture medium only. Only with conditioned medium of BMSCs with increased BDNF this neuroprotective effect was sustained. In a rat model of spinal cord contusion, at four weeks post-injury, tissue sparing was 68% improved with a sub-acute transplant of BMSCs with increased or normal BDNF compared with BMSCs with decreased BDNF and without transplant. Motoneuron survival in the contusion with BMSCs with increased BDNF was 33 % higher compared with any other group. Blood vessel density in the contusion with BMSCs with increased BDNF was 35 % higher compared with BMSCs with normal BDNF and 6-fold higher compared with no transplant. BMSCs with decreased BDNF resulted in a 76 % decrease in blood vessel density compared with BMSCs with normal BDNF. Our data show that increased BDNF augments BMSC-mediated neuroprotection in the contused adult rat spinal cord.

INTRODUCTION

Transplantation of bone marrow stromal cells (BMSCs)[1,2] in the injured spinal improves functional recovery[3-9]. BMSCs elicit neuroprotection[3-6] in the damaged spinal cord which is correlated with functional recovery[3]. How BMSCs elicit neuroprotection is poorly understood. It is thought that BMSCs secrete trophic factors exerting paracrine effects[9-16], but which and how trophic factors are involved in BMSC-mediated neuroprotection is unknown.

Protection of neurons in the damaged spinal cord nervous tissue may result from neurotrophic factors[17,18]. BMSCs secrete neurotrophic factors including brain-derived neurotrophic factor (BDNF)[14-16]. BDNF exerts trophic effects on neurons *in vitro* and *in vivo*[19-21]. Administration of BDNF to the injured spinal cord leads to neurotrophic effects including neuronal sparing[22-25]. Transplantation of BMSCs genetically modified to overexpress BDNF improves neuroprotection and repair[26-28]. BDNF is also known to be involved in vascular events[25,29-33], including protecting blood vessels from injury-induced loss, which could also support neuroprotection[34-36]. Together these data validate the candidacy of BDNF as a determinant in BMSC-mediated neuroprotection.

Here, we hypothesized that BDNF secreted by BMSCs is associated with neuronal and vascular sparing in the contused adult rat spinal cord. Gene therapy was used to modulate the secretion of BDNF by BMSCs. To assess the effect of the gene modulations we used a spinal cord organotypic culture to investigate motoneuron survival, and an adult rat model of spinal cord contusion to investigate motoneuron survival, blood vessel density, and nervous tissue sparing.

METHODS

BMSC culture and lentiviral transduction

BMSCs were harvested from femurs from adult female Sprague Dawley rats (200-250 g; Charles River Laboratories, Wilmington, MA) and cultured as previously described[3,37]. After one passage, BMSCs were transduced with lentiviral vectors (LV) encoding for BDNF and green fluorescent protein (GFP; LV-BDNF/GFP) to upregulate BDNF, LV-

BDNFshRNA/GFP to knock down BDNF (five different constructs; clones were purchased from OpenBiosystems; cat. #.: RMM4532-NM-012513), or LV-GFP for controls. LVs were produced by the Viral Vector Core Facility of the Miami Project to Cure Paralysis (University of Miami Miller School of Medicine, Miami, FL) and used at a multiplicity of infection of 100. Transduced BMSCs were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % Fetal Bovine Serum and 1 % Penicillin/Streptomycin and passaged every three days. Passage 4 (P 4) BMSCs were used for transplantation.

In vitro analysis of BMSCs

Lentiviral transduction of BMSCs in culture was confirmed by verifying stable GFP expression for seven passages. BMSCs in culture were characterized using immunostaining for CD90, CD105, CD45, TrkB and P75 (see below). Culture medium from confluent P 4 BMSC cultures (2 x 10^6 cells) was collected after 24 hours to quantify the amount of secreted BDNF using enzyme-linked immunosorbent assay (ELISA; Promega, Madison, Wi, USA) according to the manufacturer's guidelines. Unconditioned culture medium was used as a negative control. Three biological replicates were quantified per condition and each biological replicate was the average of two technical replicates.

Motoneuron survival in spinal cord organotypic cultures

The effect of culture medium conditioned by the differently transduced BMSCs on motoneuron survival was determined using spinal cord organotypic slices prepared from neonatal (postnatal days 1-4) B6.Cg-Tg(Thy1-YFP)16Jrs/J transgenic mice (Jackson Laboratories, Maine, MA) in which spinal motoneurons express (Thy1-driven) yellow fluorescence protein (YFP). The spinal cord was dissected and transferred into dissection medium containing Hank's balanced salt solution (Gibco, Carlsbad, CA) with 4.3 mM NaHCO3, 10 mM Hepes, 33 mM glucose, 0.03 % BSA, 0.15 % MgSO4-7H2O, 100 U/ml penicillin, and 100 U/ml streptomycin. After removal of dura and meninges, the spinal cord was transversely sliced at 350 µm on a McIllwain tissue chopper (Stoelting Co, Wood Dale, IL). Then, 5 slices were kept in inserts with collagen-coated transwell membranes (3.0 µm pore size; Corning, Lowell, MA). Inserts were placed in 6-well tissue culture plates

and incubated with BMSC conditioned culture medium or spinal cord slice culture medium containing 50 % MEM (Gibco), 25 % Hank's balanced salt solution (Gibco), 25 % Heat Inactivated Horse Serum (Hyclone), 25 mM Hepes (J.T. Baker), 200 mM L-glutamine (Gibco), 3.5 g/L d-glucose, 100 U/ml penicillin, and 100 U/ml streptomycin. Slices were kept for five days in vitro (DIV) in a humidified chamber with 5 % CO_2 at 37 °C. Medium was replaced every second day. After 5 DIV, cultures were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for two hours at room temperature. Cultures were then washed three times for 10 min with PBS and stored at 4 ˚C until imaging. Motoneuron survival in the slices was determined under the fluorescent microscope by quantifying YFP-positive motoneurons at 3 and 5 DIV. The numbers of slices per group were averaged.

Spinal cord contusion and BMSC transplantation

A total of 45 adult female Sprague Dawley rats (200-250 g; Charles River laboratories) were anaesthetized with an intraperitoneal injection of 60 mg/kg Ketamine (Butlerschein, Dublin, OH) and 0.5 mg/kg Dexdomitor (Pfizer, New York, NY)[3,38]. The backs were shaved and Lacrilube ophthalmic ointment was applied to the eyes to prevent drying during and after surgery. The skin and underlying muscles were incised and a laminectomy of the ninth thoracic (T9) vertebra was performed. The underlying T8 spinal cord was contused using the Infinite Horizon Impactor at an impact force of 200 kDyne. Subsequently, the muscles were sutured separately and the skin was closed with metal Michel wound clips (Butlerschein). Rats were given 1.5 mg/kg of Antisedan (Pfizer) subcutaneously to reverse Dexdomitor's sedative effect, 6 mg/ml of Gentamicin (VWR, Radnor, PA) intramuscularly and 10 ml of Ringer's solution subcutaneously (Butlerschein). Rats received daily injections of 5 ml Ringer's subcutaneously, 6 mg/kg Gentamicin intramuscularly and 5 mg/kg of Rimadyl subcutaneously (Pfizer) until BMSC transplantation[3,38]. Bladders were manually emptied twice daily until reflex voiding recovered (± 2 weeks).

Three days after the contusion, rats were anaesthetized as described above and the contused spinal cord was re-exposed. Next, 5 x 10^5 BMSCs in DMEM or DMEM alone (volume = 5 µl; n = 10/group) was injected into the contusion epicenter using a glass

pulled needle on a Hamilton syringe held within a micromanipulator[3,38]. After injection, the needle was kept in place for 3 min and then retracted slowly over a 2 min period to prevent back leakage of the injectate. The muscles and skin were closed and the rats treated postoperatively as described above. Daily injections of Ringer's and Rimadyl for three days and gentamicin for four days were given as described above[3,38]. There were 4 experimental groups: BMSC, BMSC-BDNF, BMSC-BDNFshRNA, and DMEM.

Histological and immunocytochemical procedures

One week (n = 3 per group) or 4 weeks (n = 7 per group) after injection, rats were deeply anaesthetized with intraperitoneal injection of 90 mg/kg Ketamine (Butlerschein) and 0.75 mg/kg of Dexdomitor and transcardially perfused with 250 ml of 0.1 M PBS followed by 400 ml of 4% paraformaldehyde[3]. Spinal cords were dissected out, postfixed overnight in the same fixative, and then cryoprotected for 24 h in phosphate-buffered 30 % sucrose for 24 h. Twelve mm long segments centered on the contusion epicenter were sectioned (20 µm) on a Leica CM 1950 cryostat. Every twelfth section was stained with cresyl violet to reveal the cytoarchitecture.

Sections were immuno-blocked and permeabilized in 5 % NGS and 0.03 % Triton for 30 min at room temperature. Subsequently, sections were incubated with primary antibodies for 2 h at room temperature and then overnight at 4 ˚C. Used primary antibodies were rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:200; DAKO) to stain reactive astrocytes, mouse monoclonal rat endothelial cell antibody (RECA-1; 1:100; Abd Serotec MCA 970 GA) to stain blood vessels. After washing twice in PBS, sections were incubated with secondary antibody Alexa Fluor 594 or 488 at a concentration of 1:200 for two h at room temperature. Sections were stained with DAPI (2 µl/ml) for 3 min to stain nuclei, covered with glass slips in fluorescent mounting medium (Dako North America, Inc. Carpinteria, CA, USA), and stored at 4 °C.

Quantification of motoneurons and blood vessels

The number of motoneurons (all cells > 25 µm) was determined in cresyl violet-stained sections. Motoneuron number was expressed as a percentage of the number of motoneurons in a comparable uninjured spinal cord segment.

Blood vessels were quantified in RECA-1-stained sections using MATLAB® Software (MathWorks)[39]. In the three middle sections, at 20 X magnification, a sample (320 µm x 420 µm) was taken in the gray matter about 600 µm laterocaudally to the injury epicenter and all RECA-1 positive structures were outlined[3]. The area fraction of the outlined structures was determined in each sample and averaged per rat. Averages for all rats per group were averaged.

Assessment of GFP-positive BMSC number and spared tissue volume

The number of GFP-positive BMSCs in the contusion was determined in DAPI-only stained sections using StereoInvestigator (MicroBrightField Inc., Colchester, Virginia, USA). In each section with GFP-positive cells, the transplanted area was outlined manually at 10 x magnification and covered with a 250 × 250 µm grid. At 60 X magnification with oil immersion, GFP-positive cells were marked using the optical fractionator with a 60 × 60 µm counting frame[38]. Results were averaged per group and expressed as a percentage of the number of transplanted cells.

The volume of spared nervous tissue was determined in cresyl violet-stained sections using the Cavalieri estimator function in StereoInvestigator® Software (MicroBrightField Inc). In each twelfth section the volume of spared tissue in a 3.4 mm long segment centered at the contusion epicenter was determined. Beyond the rostral and caudal borders of this segment cavities were absent. Within the analyzed segment, tissue was considered spared if it lacked cavities, areas with densely packed small cells resembling infiltrated neutrophils and lymphocytes, and neurons with darkly stained cytoplasmic Nissl bodies[38]. The Gundersen Coefficient of Error was < 0.05 for all measurements. The volume of spared tissue was expressed as a percentage of the average volume of a comparable uninjured spinal cord segment and displayed as mean ± SEM.

Statistical analysis

For all results, one-way ANOVA with Tukey's post-hoc analysis was used to compare differences among the experimental groups. Differences were accepted as statistical significant with $p < 0.05$.

Ethics and surgical approval

Rats used in this study were housed according to the National Institutes of Health and the United States Department of Agriculture guidelines. Rats were kept within a double-barrier facility, air in the cages was continuously refreshed and water and food were available *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

RESULTS

In vitro characterization of modified BMSCs

We characterized the transduced BMSCs in vitro and they were found to have stable GFP expression for at least seven passages. Transduction of BMSCs with LV-BDNF/GFP or LV-BDNFshRNA/GFP did not change the presence of typical BMSC surface markers CD90 (Fig. 1A, 1F, 1K, respectively) and CD105 (Fig. 1B, 1G, 1L, respectively) or the absence of the hematopoietic marker CD45 (Fig. 1E, 1J, 1O, respectively) compared to BMSCs transduced with LV-GFP[3,38]. BMSCs transduced with LV-BDNF exhibited an increase in staining for the BDNF receptors, TrkB (Fig. 1H) and P75 (Fig. 1I), while BMSCs transduced with LV-BDNFshRNA exhibited a decrease in staining for these receptors (Fig. 1M, 1N) compared to BMSCs transduced with LV-GFP. ELISA was used to determine the effectiveness of the used LVs in P4 BMSCs. BMSCs transduced with LV-BDNF/GFP secreted 60.4 ± 8.3 pg/ml BDNF in 24 h, which was over six-fold higher than that secreted by BSMCs transduced with LV-GFP (9.4 ± 0.3 pg/ml/24 h) (Fig. 1P; $p < 0.05$). BMSCs transduced with LV-BDNFshRNA secreted an amount of BDNF below the linear range of the ELISA (7.8 pg/ml; ig. 1P). DMEM does not contain BDNF.

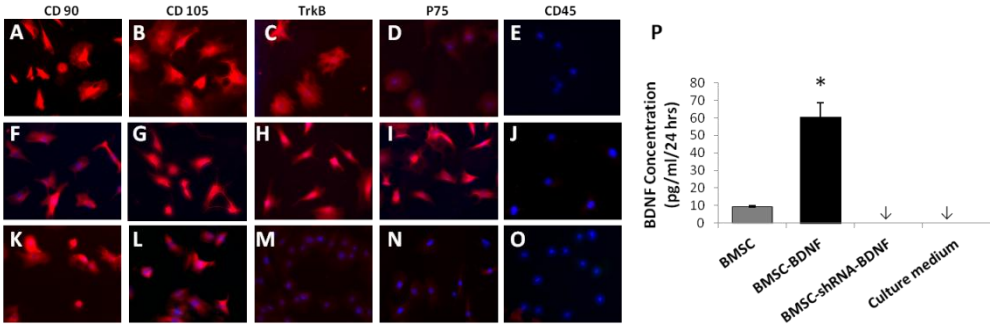

Fig. 1. Characterization of lentivirally modified BMSCs. Compared to control BMSCs (BMSC; A-E), lentiviral transduction of BMSCs with BDNF (BMSC-BDNF; F-J) or shRNA-BDNF (BMSC-shRNA-BDNF; K-O) does not change the expression of BMSC surface markers CD90 (A, F, K) and CD105 (B, G, L) or the absence of hematopoietic markers CD45 (E, J, O) but changes the expression of TrkB (C, H, M) and P75 (D, I, N). BMSC-BDNF exhibited increased staining for TrkB (H vs. C) and P75 (I vs. D) and BMSC-shRNA-BDNF exhibited decreased staining for TrkB (M vs. C) and P75 (N vs. D). Amounts of BDNF in conditioned medium of all types of modified BMSCs and DMEM (culture medium) was determined using ELISA (P). BDNF-hypersecreting BMSCs (BMSC-BDNF) secreted more than 6 times more BDNF than control BMSCs (BMSC). BDNF-hyposecreting BMSCs (shRNA-BDNF-BMSC) secreted amounts of BDNF undetectable for the ELISA. DMEM (Culture medium) also contained no detectable amounts of BDNF. Arrows indicates concentrations below the linear range of the ELISA (<7.8 pg/ml). Error bars display standard error of the mean (SEM). *$p < 0.05$.

Increased BDNF sustains BMSC-mediated neuroprotection

We used a spinal cord organotypic culture system to examine the neuroprotection potential of the modified BMSCs. Incubation with conditioned medium from cultured BMSCs transduced with LV-GFP, LV-BDNF, or LV-BDNFshRNA for 3 days resulted in a 58 % (M = 62.6 ± 6.4), 77 % (M = 70.1 ± 6.2), and 57 % (M = 62.1 ± 3.6) increase in YFP-positive motoneurons compared to incubation with spinal cord organotypic culture medium (Fig. 2A; $p < 0.05$). This result showed that the BMSCs, regardless of their respective genetic modification, secreted trophic factors leading to a similar degree of motoneuron sparing and that BDNF is not a necessary factor for motoneuron protection *in vitro*. Interestingly, at 5 days *in vitro* the protective effect on motoneurons was sustained with conditioned medium from BDNF-hypersecreting BMSCs (M = 66.8 ± 5.6; 87 % increased over incubation with spinal cord organotypic culture medium) but not with conditioned medium from BDNF-hyposecreting BMSCs (M = 35.6 ± 4.4) or from control BMSCs (M =

49.8 ± 3.6) (Fig. 2B). Photographic examples are provided of spinal cord slices and YFP-positive ventral motoneurons in conditioned medium from normal BMSCs (C and G, respectively), BDNF-hypersecreting BMSCs (D and H), BDNF-hyposecreting BMSCs (E and I), and with incubation in the defined spinal cord organotypic culture medium (Culture medium) (F and J). The data demonstrated that increased BDNF levels sustain spinal motoneuron sparing *in vitro*.

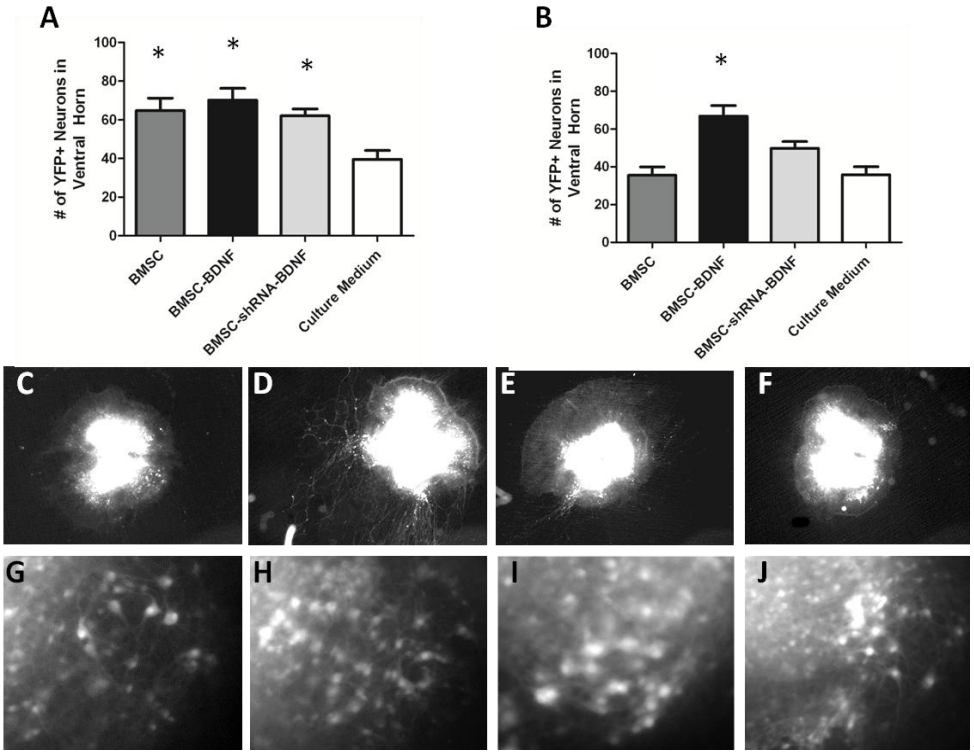

Fig. 2. Sustained in vitro protection of ventral horn motor neurons by BMSC-BDNF. (A) At day three in vitro all types of BMSC conditioned medium resulted in more live YFP-positive neurons in the ventral horn of organotypic spinal slices than unconditioned medium. At five days in vitro, conditioned medium from BDNF-hypersecreting BMSCs (BMSC-BDNF), but not that from the other BMSC types, sustained motoneuron protection. (C-F) shows an example of a spinal cord slice and (G-J) shows YFP-positive ventral motoneurons in conditioned medium from normal BMSCs (C and G, respectively), BDNF-hypersecreting BMSCs (D and H), BDNF-hyposecreting BMSCs (E and I), and with incubation in the defined spinal cord organotypic culture medium (Culture medium) (F and J). Error bars display standard error of the mean (SEM). *$p < 0.05$.

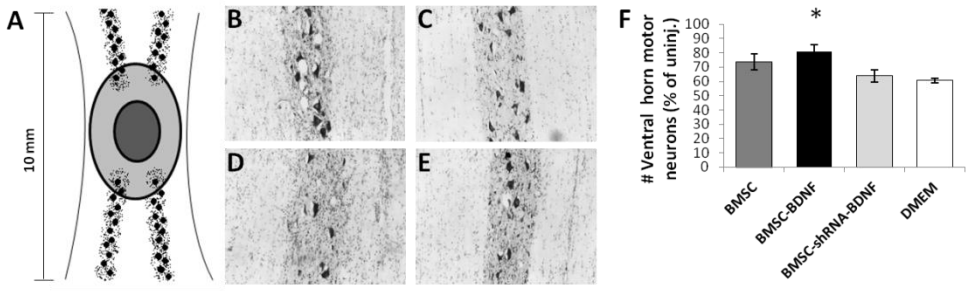

Fig. 3. BDNF-hypersecretion enhances motoneuron survival in a contused spinal cord. (A) Schematic representation of the quantification of ventral motoneurons in cresyl violet-stained sections of a 10-mm long spinal cord segment centered on the contusion epicenter. Motoneurons were found in the ventral spinal cord of rats injected with normal BMSCs (B), BDNF-hypersecreting BMSCs (C), BDNF-hyposecreting BMSCs (D), and DMEM (E). (F) Bar graph showing that 33% more motoneurons were spared in the group of rats that received BDNF-hypersecreting BMSCs compared to the knockdown and DMEM groups. Error bars display standard error of the mean (SEM). *$p < 0.05$.

BDNF-hypersecretion enhanced motoneuron survival in a contused spinal cord

Our in vitro data and previous studies showed that BDNF has protective effects on spinal motoneurons. We examined whether manipulation of BDNF secretion in a BMSC transplant would affect motoneuron survival in a 10-mm long spinal cord segment centered on the contusion epicenter (Fig. 3A). In rats with transplants of BMSCs transduced with LV-BDNF (Fig. 3B), 80.6 % ± 5.3 motoneurons relative to the number in a comparable uninjured spinal cord segment were present compared to 60.7 % ± 1.6 in rats with DMEM injections (Fig. 3E), which represents a 33 % increase in motoneuron number ($p<0.05$; Fig. 3F). Transplantation of BMSCs transduced with LV-GFP (Fig. 3C) or LV-BDNFshRNA (Fig. 3D) did not result in a statistically significant increase in the number of motoneurons (M = 73.9 ± 5.6, M = 63.9 ± 4.3, respectively; Fig. 3F).

BDNF-hypersecreting BMSCs augment BMSC-mediated blood vessel protection

Previously, we have demonstrated that BMSC transplants results in increased blood vessel numbers in a contused spinal cord[3]. Here, we examined the role of BDNF in this protective effect by determining the density of blood vessels near the contusion (Fig. 4A). Blood vessels were present in rats with BMSCs transduced with LV-GFP (Fig. 4B), LV-BDNF (Fig. 4C), LV-BDNFshRNA (Fig. 4D), and in rats with DMEM injections (Fig. 4E). Quantification

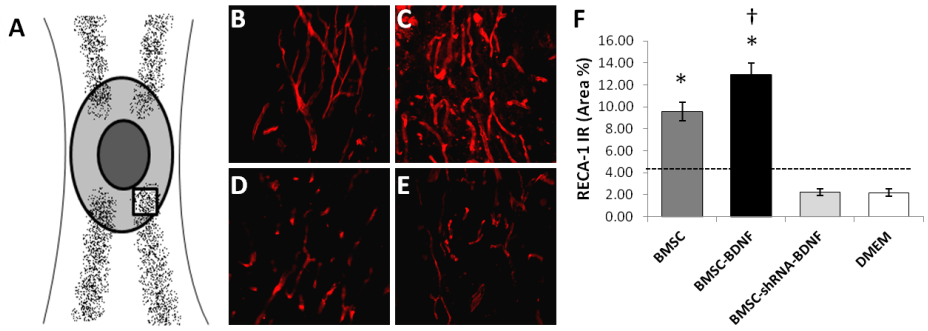

Fig. 4. BDNF-hypersecretion augment BMSC-mediated blood vessel sparing in a contused spinal cord. (A) Schematic of the contusion injury with gray matter and location where blood vessel density was determined. RECA-1-positive blood vessels were found in gray matter near the contusion in rats with normal BMSCs (B), BDNF-hypersecreting BMSCs (C), BDNF-hyposecreting BMSCs (D), and DMEM (E). (F) Bar graph showing that rats with BDNF-hypersecreting BMSCs had a 6-fold increase and rats with an injection of normal BMSCs had a 4-fold increase in blood vessel density compared to DMEM. Rats that received BDNF-hyposecreting BMSCs had a lower density of blood vessels compared to rats with normal BMSCs. Dotted line in F indicates mean blood vessel density in uninjured controls. Error bars display standard error of the mean (SEM). *$p < 0.05$ compared to DMEM and $^{\dagger}p < 0.05$ compared to BMSC.

revealed a significant six-fold increase ($p < 0.05$) in blood vessel density in rats with BDNF-hypersecreting BMSCs compared to rats with DMEM injections (Fig. 4F). Rats with BMSCs transduced with LV-GFP had a significant four-fold increase in blood vessel density compared with rats with DMEM injections (Fig. 4F). Overexpressing BDNF in a BMSC transplant resulted in a 35 % increase and silencing BDNF expression in a 76 % decrease in blood vessel density compared to normal BMSCs (Fig. 4F).

Tissue sparing 4 weeks after BMSC transplantation

Previous research showed that intraspinal BMSC transplants result in larger volumes of spared nervous tissue[3,38]. Here, we examined the role of BDNF in BMSC-mediated neuroprotection in the contused spinal cord. At four weeks post-injection, the volume of spared tissue in rats that received BMSCs transduced with LV-GFP or LV-BDNF was 68 % larger ($p < 0.05$) in both groups compared to that in rats with DMEM injections (Fig. 5). Spared tissue volume in rats with BDNF-hyposecreting BMSCs was similar as in rats with DMEM injection (Fig. 5). These results show that BDNF is necessary for BMSC-mediated tissue sparing but overexpressing BDNF does not lead to larger volumes of spared nervous tissue.

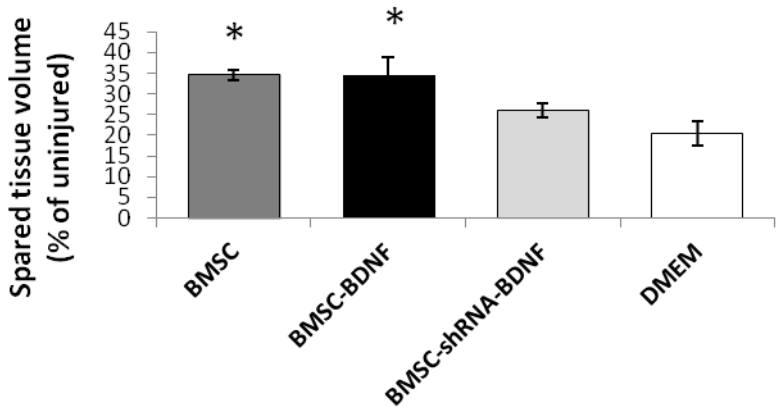

Fig. 5. Tissue sparing 4 weeks after BMSC transplantation. Four weeks after transplantation, a protective effect of BMSCs on nervous tissue is present. The bar graph shows that transplantation of normal BMSCs (BMSC) and BDNF-hypersecreting BMSCs (BMSC-BDNF) resulted in increased volumes of spared tissue compared with rats with DMEM or BDNF-hyposecreting BMSCs. The latter group was not significantly different from the DMEM group. Error bars display standard error of the mean (SEM). *$p < 0.05$.

BDNF-hyposecretion affects BMSC survival in a spinal cord contusion

Survival of a BMSC transplant in damaged nervous tissue is limited[38]. We examined whether increased or decreased BDNF secretion would affect BMSC survival in a contusion environment. One week after transplantation into a three-day old spinal cord contusion, transplanted BMSCs transduced with LV-GFP (Fig. 6A) or LV-BDNF (Fig. 6B) appeared to be mostly spindle-shaped, which was in agreement with our previous study[38]. Stereology-based quantification showed that 13.7 ± 2.2 % of the transplanted BMSCs transduced with LV-GFP and 17.2 ± 4.9 % of BMSCs transduced with LV-GFP were present in the contusion (Fig. 6D). Transplanted BMSCs transduced with LV-BDNFshRNA were not found in the contusion (Fig. 6C). The data show that BDNF is necessary for BMSC survival in a spinal cord contusion but overexpressing BDNF does not lead to improved survival.

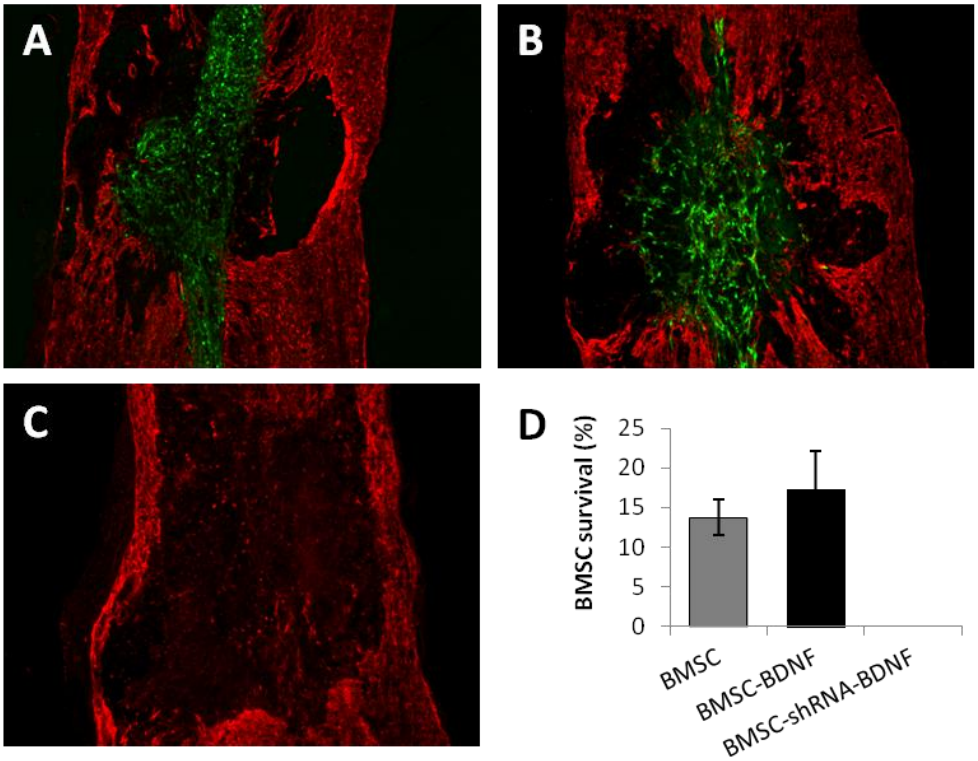

Fig. 6. BDNF-hyposecretion affects BMSC survival in a contusion. One week after transplantation into the contused spinal cord, BMSCs appeared mostly as spindle-shaped cells in transplants of normal BMSCs (BMSC) and of BDNF-hypersecreting BMSCs (BMSC-BDNF) (A,B). BDNF-hyposecreting BMSCs were not detected in the contusion (C). Quantification revealed similar numbers in transplants of normal and BDNF-hypersecreting BMSCs (D). Error bars display standard error of the mean (SEM).

DISCUSSION

The repair-supporting effects of BMSC transplants in the injured spinal cord[3-9] are thought to result from paracrine actions by secreted trophic factors[9-16]. We used gene therapy to manipulate BDNF production by BMSCs and assess its role in BMSC-mediated neuroprotection. Our experiments revealed that *in vitro* increased BDNF amounts sustained motoneuron protection. In the contused spinal cord, BDNF overexpression by grafted BMSCs protected motoneurons and blood vessels; spared tissue volume was unaffected. The data suggest that enhancing BDNF levels in BMSC transplants elicit neuronal and vascular effects that may be beneficial for recovery after spinal cord injury.

With an organotypic culture system we showed that motoneuron survival in spinal cord slices was similar in conditioned medium with normal, increased, or decreased amounts of BDNF. This finding indicated that BDNF is not necessary for *in vitro* spinal motoneuron survival and that other trophic factors secreted by BMSCs are sufficient for this neuroprotective effect. After the first days in culture, motoneuron survival was only sustained with increased BDNF amounts. Thus increased BDNF is vital to maintain the motoneuron survival-promoting effects exerted by the trophic molecules secreted by BMSCs. Possibly; BDNF sustains the survival of motoneurons by preventing or delaying apoptotic pathways[21,40].

Transplantation of BDNF-hypersecreting BMSCs enhanced motoneuron survival in the ventral gray matter in the contused adult rat spinal cord. This finding is in agreement with our *in vitro* observation that conditioned medium from BDNF-hypersecreting BMSCs promoted and sustained motoneuron survival in spinal cord organotypic cultures. BDNF is known to exert neurotrophic actions on neurons[9-16] and treatment of injured spinal cord with BDNF[22-25] or with BDNF-hypersecreting cells promotes neuron survival[26-28,41-44]. In our spinal cord organotypic culture, BDNF was found not to be a necessary factor for motoneuron sparing; other BMSC-derived factors were sufficient. We were unable to verify whether this finding would translate to the *in vivo* spinal cord injury model because silencing BDNF production in BMSCs resulted in rapid loss of transplanted BMSCs.

The finding that survival of BMSCs with decreased BDNF was inferior to that of BMSCs with normal or increased BDNF in a contusion environment is intriguing. To our knowledge, this is the first demonstration of transplantation of BMSCs with silenced BDNF production into the injured spinal cord. In culture, we observed no difference in survival of any of the differently modified BMSCs used in this study. Also, BDNF hypersecretion did not enhance BMSC survival *in vitro* or *in vivo*. How can we explain the decrease in BMSC transplant survival in the absence of BDNF? It is possible that grafted BMSCs are protected by BDNF from apoptotic death[21,40] due to injury-related events including oxidative stress, hypoxia, and/or transplant preparation-related events such as detachment which induces

anoikis. Possibly, the observed lower expression of the BDNF receptors, TrkB and P75 in BDNF-hyposecreting BMSCs contributed to the lower survival in the contusion. In culture, injury-related events are absent and cells are attached; thus, BDNF would not be required for their protection. Further research is needed to elucidate the role of BDNF in BMSC transplant survival in a spinal cord contusion.

In the contused spinal cord with a transplant of BDNF-hypersecreting BMSCs the volume of spared nervous tissue was similar as with normal BMSCs. Our finding that a transplant of normal BMSCs resulted in improved tissue sparing corroborated with data from our previous studies[3,38] as well as that from other groups[4-6]. The lack of additional nervous tissue sparing with increased BDNF was also shown with a complete spinal cord transection[25] but not in a partial, dorsal column, transection[44]. Possibly, the severity of the injury is a determinant in BDNF-mediated tissue sparing. As discussed above, early death of BMSCs with decreased BDNF production prevented determining whether BDNF is necessary for BMSC-mediated tissue sparing.

A transplant of BMSCs with increased BDNF production significantly augmented BMSC-mediated increases in blood vessel density near the injury epicenter. Previously, we showed that BMSC transplants resulted in increased vascularization in the contused spinal cord[3]. Here, we extend this observation and show that BMSC-secreted BDNF possibly plays a role to elicit this effect. Other studies have also implicated BDNF in vascular events in the injured nervous tissue[25,29-33], possibly through upregulation of vascular endothelial growth factor[30]. It is possible that the effects of increased BDNF on motoneuron sparing and blood vessel sparing as we demonstrate in our study are related. Enhancing blood vessel number through angiogenic/angioprotective effects may have resulted in neuroprotection[34-36]. Future research will need to elucidate the interplay between BDNF-mediated vascular and neuronal sparing.

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Chapter 4

Reducing Macrophages to Improve Bone Marrow Stromal Cell Survival in the Contused Spinal Cord

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ABSTRACT

We tested whether reducing macrophage infiltration would improve the survival of allogeneic bone marrow stromal cells (BMSC) transplanted in the contused adult rat thoracic spinal cord. Treatment with cyclosporine, minocycline, or methylprednisolone all resulted in a significant decrease in macrophage infiltration at 3 days postinjury. However, when BMSC were injected at that time point, survival 7 days later was similar between treatment groups and saline-injected controls. In fact, we found that the presence of BMSC resulted in a significant increase in macrophage infiltration into the contusion.

INTRODUCTION

A contusive spinal cord injury causes immediate death of neural cells and disruption of axon circuits[1]. The number of macrophages in the injury site rapidly increases over the ensuing days and contribute to further destruction of local nervous tissue[2]. There is no therapy available that effectively improves function after spinal cord injury. Transplantation of bone marrow stromal cells (BMSC) has been explored for spinal cord repair[3]. In different model systems, BMSC transplantation resulted in tissue sparing and, in some cases, improved motor function[4-7]. These results warrant further investigation of BMSC for nervous system repair. Survival of allogeneic BMSC transplanted into the damaged spinal cord is low[4,7,8]. Several studies have pointed at a role of macrophages in the loss of BMSC after injection into the injured nervous system[8,9]. Thus, we reasoned that a reduction in macrophage number in an injury site would increase the survival of transplanted BMSC. Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment decreases macrophage infiltration into a spinal cord lesion[8,10-13]. This study focuses on the efficacy of these three drugs to reduce macrophage infiltration and whether this would result in increased survival of subsequently transplanted BMSC.

METHODS

Spinal cord contusion and postsurgery care

Adult female Sprague–Dawley rats (n=76, 200–230 g; Harlan, Indianapolis, Indiana, USA) were anaesthetized with an intraperitoneal injection of 60 mg/kg of ketamine HCl (Phoenix Pharmaceuticals, St Joseph, Maryland, USA) and 0.4 mg/kg medetomidine (Domitor, an a-2- adrenergic agonist; Orion Corp., Espoo, Finland). The 10th thoracic spinal cord segment was exposed and contused using the Infinite Horizon Impactor (Precision System & Instrumentation, Lexington, Kentucky, USA) at a force of 200 kDyn (Fig. 1a). Consistency between animals was guaranteed by registering the impact force and spinal cord displacement. The wound was closed and the rats were given antisedan (atipamezole hydrochloride; 1.25 mg/kg, intramuscular), an a-2-adrenergic antagonist that reverses

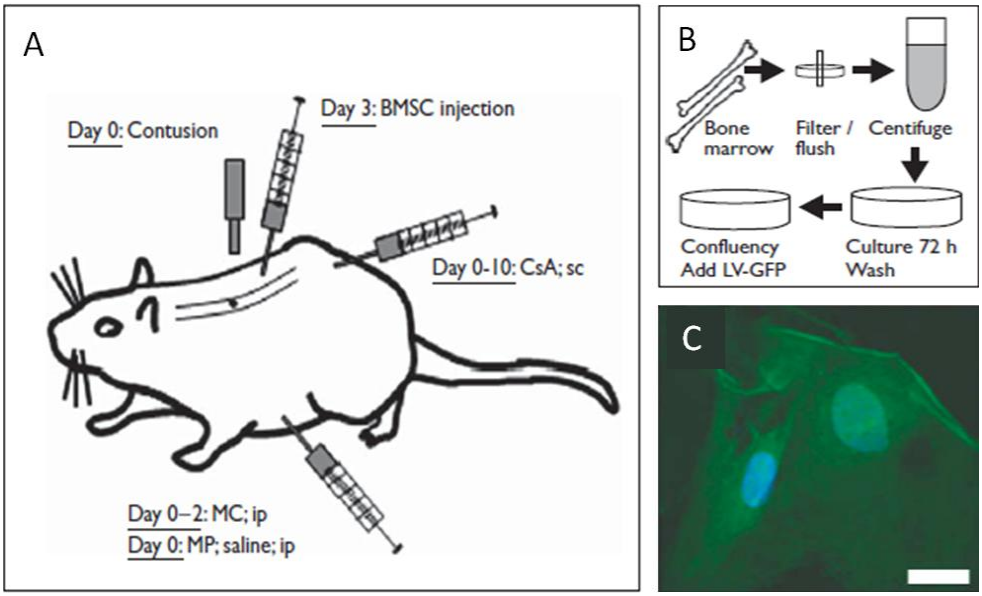

Fig. 1. Schematic representation of the experiments and BMSC harvest and transduction. A. Rats were contused at the 10th thoracic spinal cord segment and then divided into 4 groups that received CsA, MC, MP or saline. B. BMSC were harvested, transduced to express GFP using lentiviral vectors, and grown in DMEM. C. GFP-expressing BMSC in a passage 3 culture which were used for transplantation into the epicenter of the 3-day old contusion. Abbreviations: BMSC, bone marrow stromal cells; CsA, cyclosporine A; ip, intraperitoneal; MC, minocylcine; MP, methylprednisolone; sc, subcutaneous. Bar in C represents 10 μm.

sedative and analgesic effects of medetomidine. All surgical procedures were performed by the same investigator. Postsurgery maintenance was as described earlier[7].

Drug administration

Contused rats were divided into four groups that received CsA, MC, MP, or saline (n=18 each; Fig. 1a) starting 5 min after the contusion. All injections were performed by the same investigator. CsA (Bedford Labs, Bedford, Ohio, USA) was administered subcutaneously once per day at a dose of 30 mg/kg for the first three days and 15 mg/kg for the next 7 days[8]. MC (Sigma-Aldrich, St Louis, Missouri, USA) was administered intraperitoneally at a dose of 50 mg/kg twice a day for the first two days[12]. MP (Sigma-Aldrich) was administered intraperitoneally once at a dose of 30 mg/kg[10]. Saline was given to controls following the same regime as for MP.

Bone marrow stromal cell culture and lentiviral transduction

BMSC were obtained from femurs of adult female Sprague-Dawley rats (n=4) as described earlier[7,14] (Fig. 1b). BMSC at passage 0 were transduced overnight using lentiviral vectors encoding for green fluorescent protein (LV-GFP) at an MOI of 150[7,15]. Transduced BMSC were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 371C/5% CO2. BMSC from the third passage (Fig. 1c) were used for the transplantation experiments. The transduction rate of the BMSC was determined using a FACScan/FACSorter [Becton Dickinson Immunocytometry Systems (BDIS) Biosciences, San Jose, California, USA]. The number of viable GFP-positive cells relative to the total number of cells was 63%.

Transplantation of bone marrow stromal cells

At 3 days after injury, 24 rats (six rats from each group) were anaesthetized with intraperitoneal injections of 60mg/kg of ketamine HCl (Phoenix Pharmaceuticals) and 0.4mg/kg of medetomidine (Domitor; Orion Corporation). The 10th thoracic spinal cord segment was exposed and 5 ml Dulbecco's modified Eagle's medium with 1 x 10^6 BMSC (Fig. 1a) was injected into the contusion epicenter[14]. Four additionally-contused rats were similarly injected and perfused with fixative (see below) 15min later. These rats were used to determine the number of BMSC in the contusion at 15min postinjection. All BMSC injections were given by the same investigator. After the injections, the rats were maintained as described previously[7].

General histology

Three days (n=24) and 10 days (n=48) after injury, rats were anaesthetized with an intraperitoneal injection of 90 mg/kg of ketamine HCl (Phoenix Pharmaceuticals) and 0.6 mg/kg of meditomidine (Orion Corporation). After deep sedation was confirmed, 0.1 ml heparine (500 IU; Henry Schein, Melville, New York, USA) was injected into the left ventricle of the heart. Then, 500 ml saline followed by 500 ml ice-cold 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) was pumped through the vascular system. Spinal cords were removed without damaging the anatomical integrity,

postfixed for 24 h in the same fixative, and transferred to 30% sucrose in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) for 48 h. A 12-mm-long spinal cord segment centered at the contusion was cut into 20-mm-thick horizontal cryostat sections, which were mounted on glass slides.

Immunocytochemical procedures

For characterization of the BMSC, eight-well chamber glass slides (BD Falcon; BD Biosciences, Bedford, Massachusetts, USA) were coated with 100 ml/ml poly- D-lysine for 1 h at room temperature. After washing 2 x 5 min with double-distilled water, 3000 BMSC in 250 ml D-10 medium were plated per well. After 2 days at 37 °C/ 5% CO2 the cultures were washed 3 x 5 min with PBS and fixed with 4% paraformaldehyde in PB (10 min, room temperature. Next, the cultures were washed 5 x 5 min with PBS, incubated with 5% normal goat serum (NGS) in PBS for 30 min, and then incubated overnight at 4 °C with antibodies against CD90 (1 : 100; Immunotech, Brussels, Belgium), CD105 (1 : 100; N1G1, Becton Dickenson), CD34 (1 : 100; 8G12 clone IgG1, Becton Dickenson), CD45 (1 : 100; H130 clone IgG1, Becton Dickenson), and HLA-DR (1 : 100; Dako, Clostrup, Denmark) diluted in PB with 5% NGS. Some cultures were incubated with PB with 5% NGS only and served as negative (no primary antibody) controls to exclude a a false-positive outcome. Next, cultures were washed 3 x 5 min with PB and then incubated with goat-anti-mouse IgG-Alexa 594 (1:500 in PB; Molecular Probes, Carlsbad, CA) for 2 h at room temperature. Afterwards, cultures were washed 3 x 5 min with PB and covered with a glass slip with Vectashield and DAPI (Vector Laboratories, Inc., Burlingame, CA). The slides were examined and images were taken with an Olympus Fluoview FV1000 confocal microscope.

For immunostaining of activated macrophages, every 10th cryostat section was pre-incubated at room temperature for 30 min in 5 % NGS and 0.3 % Triton X-100 in 0.01 M PBS (pH 7.4) and then incubated with antibodies against ED1 (1:200; Serotec, Raleigh, NC) in 5 % NGS for 2 h at room temperature followed by overnight incubation at 4 °C. After washing 3 x 5 min with PBS, sections were incubated with goat anti-mouse Alexa 594 antibodies (1:200; Molecular Probes) in PBS (0.01 M; pH 7.4) at room temperature for 2 h.

The sections were then washed and covered with a glass slip in Vectashield with DAPI (Vector Laboratories, Inc.). The coverslips were sealed with nail polish. All sections were stored at -20 °C until analysis.

Quantitative assessments

For analysis of GFP-positive BMSC, every 10th cryostat section was covered with a glass slip with Vectashield mounting medium with DAPI (Vector Laboratories, Inc). Stereoinvestigator (MicroBrightField Inc., Colchester, VA, USA) was used to determine the numbers of surviving BMSC in the contusion[16]. The sections were 200 mm apart spanning the width of the spinal cord. In every section containing GFP positive cells, the transplanted area was outlined manually at 4 X magnification and covered by a 250 x 250 µm grid. At 60 X magnification with oil immersion, GFP-positive cells with a discernable DAPI-positive nucleus were marked using the optical fractionator with a 60 x 60 µm counting frame. Numbers were corrected for the 63 % transduction rate of the BMSC with LV-GFP. For each of the groups, BMSC survival was calculated as the number of BMSC relative to the number of BMSC at 15 min after transplantation (which was 168159 ± 31129; SEM, n=4). The effect of treatment on BMSC survival was assessed by expressing BMSC survival for each group as a percentage of that in controls. For analysis of macrophages we employed a method described by Hayashi and colleagues[17]. This method uses three sections per rat for examination: one section through the center of the contusion/transplant (with the densest cellular staining), and sections 200 µm dorsal and ventral to the center. The area fraction of staining in these sections was determined using SlideBook 4.1.0.12 (Intelligent Imaging Innovations, Inc, Santa Monica, CA, USA) and expressed as a percentage of that in control animals.

Statistical analysis

Sigmastat® (Systat Software, Inc., San Jose, CA, USA) was used for statistical analyses using one-way ANOVA and the Bonferroni post-hoc test. Differences were accepted at $p<0.05$.

Ethics and surgical approval

All rats used in this study were housed according to the guidelines of the National Institute of Health and United States Department of Agriculture. The described animal procedures were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University.

RESULTS

Characterization of BMSC in vitro

Cultured GFP-positive cells expressed CD90 (Fig. 2a) and CD105 (Fig. 2b). These two extracellular molecules are both well-known BMSC markers[18,19]. None of the cells expressed the blood cell markers, CD34 (Fig. 2c) and CD45 or the immune cell marker, HLA-DR. No staining was visible if the primary antibody was omitted. The data characterize the cells used for transplantation as BMSC.

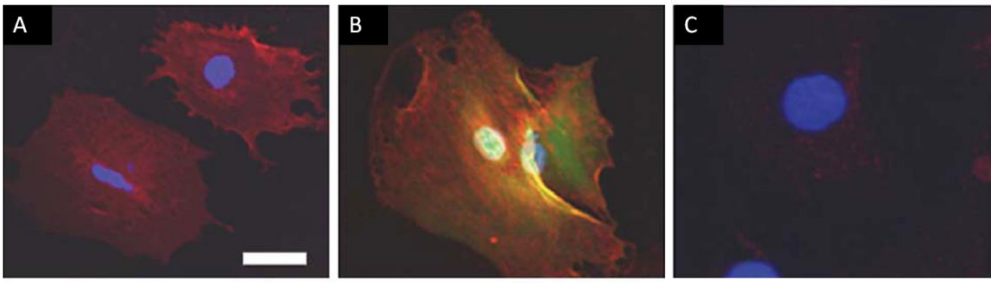

Fig. 3. Characterization of bone marrow stromal cells (BMSC) in vitro. Cultured cells expressed the BMSC markers, CD90 (A) and CD105(B), but not the blood cell marker, CD34 (C). Scale bar =10 mm.

CsA, MC, and MP reduce macrophage infiltration into the spinal cord contusion

Microscopic analysis of macrophage presence in the contusion revealed high numbers in controls (Fig. 3a) compared to treated rats (Fig. 3b) at 3 days after injury. The numbers appeared increased in treated rats at 10 days (Fig. 3c) compared to 3 days (Fig. 3b) after contusion. Quantitative analysis demonstrated that at three days post-injury, relative to controls, macrophage infiltration was 46 ± 10 % with CsA-, 47 ± 3 % with MC-, and 63 ± 3 % with MP-treatment (Fig. 3d). ANOVA revealed that the number for each of the

treatment groups was significantly smaller ($p<0.001$) than that for controls. Thus treatment-induced reduction was 54 % with CsA, 53 % with MC, and 37 % with MP compared to controls. Treatment-induced reduction was not significantly different from controls at ten days post-injury (Fig. 3d). These results showed that all three drugs when administered following the treatment regime described above reduced macrophage infiltration into the adult rat spinal cord contusion at three days post-injury.

BMSC survival in contusion is not affected by CsA, MC, or MP treatment

BMSC were present in the contusion at seven days post-injection (Fig. 4a). Quantitative analysis revealed that BMSC survival in the contusion at seven days post-injection was 27 ± 4 % with CsA, 24 ± 4 % with MC, and 33 ± 2 % with MP treated rats. In saline-injected control rats, BMSC survival was 21 ± 7 %. To assess the effects of treatment, we expressed BMSC survival in each treatment group relative to that in the control group (Fig. 4b). We found that BMSC survival was 126 ± 17 % with CsA, 111 ± 19 % with MC, and 155 ± 12 % with MP treated rats relative to control rats (Fig. 4b). ANOVA revealed no difference in BMSC survival between treatment and control groups ($p=0.16$).

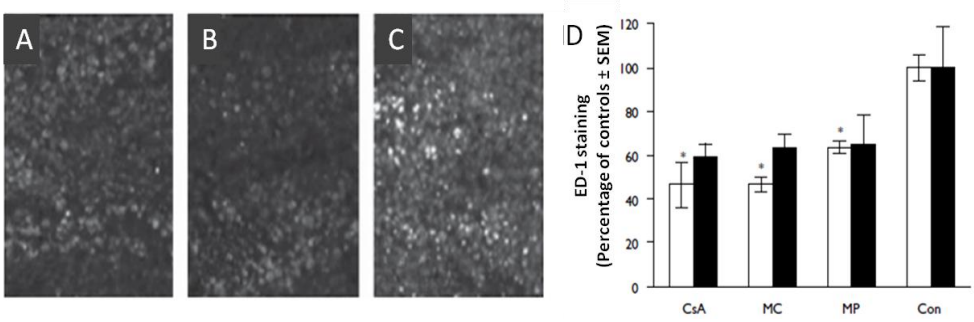

Fig. 4. Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment reduced macrophage infiltration into the contusion. Photomicrographs of ED-1-positive cells in control rats (A) and CsA-treated rats (b) at 3 days postcontusion and in CsA-treated rats at 10 days postcontusion (B). In panel (C) the more intense staining was found associated with cellular debris. (D) Bar graph shows that at 3 days postinjury (open bars) relative to controls (Con), macrophages infiltration in the contusion was decreased significantly with CsA, MC, and MP treatment. At 10 days postinjury (solid bars), the decrease in macrophage presence in the contusion of treated rats was not statistically different from that in Con. *Significant difference between treated and Con groups at 3 days postinjury with $P<0.001$.

BMSC presence initiates macrophage infiltration into the spinal cord contusion.

We assessed the effect of a BMSC transplant on macrophage presence in the contusion at ten days post-injury relative to that at three days post-injury. With a BMSC transplant, macrophage infiltration relative to controls was 2.4 fold in CsA- and MP-treated rats, and 6.9 fold in MC-treated rats. These differences were statistically significant ($p<0.001$). In the absence of a BMSC transplant, macrophage presence in treated rats relative to controls was unchanged. Our data demonstrated that the presence of a BMSC transplant significantly increased macrophage infiltration into the contused adult rat spinal cord.

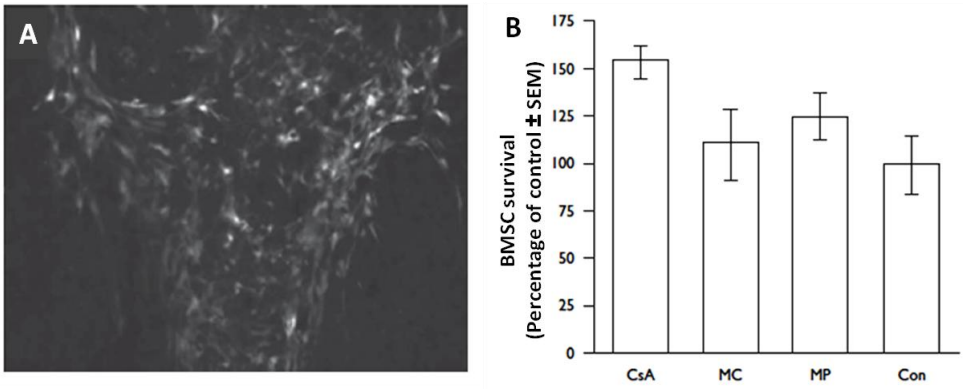

Fig. 5. Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment did not improve bone marrow stromal cell (BMSC) survival. (a) Photomicrograph of green fluorescent protein-positive cells within the contusion at 7 days postinjection. (b) Bar graph showing BMSC survival relative to controls (Con). The differences were not statistically different, although there was a trend towards higher numbers in CsA-treated rats.

DISCUSSION

Quantitative investigations have demonstrated that survival of BMSC transplanted into the contused adult rat spinal cord is low[4,7]. Previously, it was proposed that macrophages which are naturally present within an injury site are involved in the loss of BMSC transplanted into the central nervous system[9]. Our present results showed that a decreased presence of activated macrophages at the time of BMSC injection (three days post-injury) does not increase survival of grafted BMSC. It is possible that our treatment regimens failed to lower macrophage infiltration to a level where BMSC survival would have been improved. The maximum reduction which was achieved here was 54%. Because

macrophage invasion into a spinal cord injury site is typically large[1,2,11], this reduction may still leave many macrophages that could potentially contribute to BMSC loss. An additional observation is that the treatment effect which was present at three days post-injury was not significant at ten days post-injury. Although we used treatment protocols known to effectively reduce the presence of macrophages[8,10-13] they might not have been effective enough for sufficient and prolonged reduction of macrophages.

An alternative explanation for the observed lack of improved BMSC survival would be that any treatment-induced reduction in macrophage presence was masked by a subsequent increase in macrophage infiltration due to the introduction of BMSC into the environment. This notion is supported by our data because in animals with a BMSC transplant we found that macrophage infiltration was drastically increased. It is likely that these extra macrophages have exacerbated the loss of BMSC[20]. It is important to keep in mind that other factors than invaded macrophages are most likely also involved in transplanted BMSC loss such as the lack of oxygen and/or nutrients within the damaged tissue[21].

Previously, it was reported that BMSC are hypo-immunogenic; they suppress the proliferation and function of T-cells, B-cells, natural killer cells, and dendritic cells[22]. However, these publications did not investigate possible effects of BMSC on macrophage invasion. It is possible that the immunosuppressive properties of BMSC affect only the adaptive immunity due to the low expression level of human leukocyte antigen (HLA) major histocompatibility (MHC) class I and the absence of co-stimulatory molecules[22]. This would explain why the recruitment of macrophages (acquired immunity) would not be affected by BMSC.

Lowering the number of macrophages in the injured spinal cord needs to be addressed with caution. It is well known that macrophages can support spinal cord repair by promoting axon regeneration and myelination which may be accompanied by improved function[23,24]. These constructive effects occur while macrophages also exert destructive effects such as neural cell death[25]. Because of this dual role, decreasing the number of

macrophages within a spinal cord injury could lead simultaneously to beneficial and detrimental effects[24]. Thus it is important to aim for a reduction in macrophages that would not jeopardize their positive contributions to spinal cord repair.

CONCLUSION

After spinal cord contusive injury, concurrent macrophage reducing treatments did not improve survival of transplanted BMSC. The presence of BMSC in the contusion site resulted in a four-fold increase in macrophage numbers.

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Chapter 5

The effect of a polyurethane-based reverse thermal gel on bone marrow stromal cell transplant survival and spinal cord repair

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ABSTRACT

Cell therapy for nervous tissue repair is limited by low transplant survival. We investigated the effects of a polyurethane-based reverse thermal gel, poly(ethylene glycol)-poly(serinol hexamethylene urethane) (ESHU) on bone marrow stromal cell (BMSC) transplant survival and repair using a rat model of spinal cord contusion. Transplantation of BMSCs in ESHU at three days post-contusion resulted in a 3.5-fold increase in BMSC survival at one week post-injury and a 66% increase in spared nervous tissue volume at four weeks post-injury. These improvements were accompanied by enhanced hindlimb motor and sensorimotor recovery. In vitro, we found that ESHU protected BMSCs from hydrogen peroxide-mediated death, resulting in a four-fold increase in BMSC survival with two-fold fewer BMSCs expressing the apoptosis marker, caspase 3 and the DNA oxidation marker, 8-Oxo-deoxyguanosine. We argue that ESHU protected BMSCs transplanted is a spinal cord contusion from death thereby augmenting their effects on neuroprotection leading to improved behavioral restoration. The data show that the repair effects of intraneural BMSC transplants depend on the degree of their survival and may have a widespread impact on cell-based regenerative medicine.

INTRODUCTION

Cell therapy is promising for repair of the damaged nervous system[1-3]. Bone marrow stromal cells (BMSCs) are candidate cells for such therapies because of their repair proficiency and relative accessibility[4-7]. BMSCs support repair of a myriad of other ailments including cardiomyopathy, muscle dystrophy, and wound healing[8-10]. Intraneural BMSC transplants are thought to elicit repair through paracrine effects[11-14]. However, these effects are likely limited due to low transplant survival in damaged nervous tissue[15-18].

Cell transplants may be lost due to various events including inflammation[19] and oxidative stress[20-24], which are initiated rapidly after injury. Thus, measures to protect cell transplants against these death-mediating mechanisms may increase transplant survival and potentially improve their reparative effects. One strategy to improve transplanted cell survival is by using the synthetic poly(ethylene glycol)-poly(serinol hexamethylene urethane) or ESHU, which is a reverse thermal gel with good biocompatibility and degradability[25,26]. ESHU is a copolymer with two hydrophilic poly(ethylene glycol) blocks flanking a hydrophobic poly(serinol hexamethylene urethane) block[25]. The presence of polyurethane[28,29] may provide ESHU with antioxidant capacity. ESHU dissolves in water and undergoes phase transition with increasing temperatures to form a physical gel at 37°C[25], which makes it especially practical for treatment of closed injuries[30].

ESHU was shown to have good biocompatibility with nervous tissue in the ocular system[26,27]. A beneficial feature of ESHU is that the repeating units of the polymer contain protected amine groups providing an easy path to functionalization using biomolecules or other signaling molecules that can offer enhanced bioactivity of the gel in vivo. One example is to functionalize ESHU through these amine groups with the pentapeptide, IKVAV, which in pilot experiments was shown to produce a neural interface similar to laminin.

We hypothesized that ESHU protects intraneural BMSC transplants from death leading to improved repair. This premise was tested in vivo using an adult rat model of spinal cord contusion[7,15,16] assessing BMSC transplant survival, inflammation, anatomical restoration, and functional recovery and in vitro using BMSC cultures determining the effects of ESHU on survival of BMSCs under oxidative stress.

MATERIALS AND METHODS

Ethics and surgical approval

Before and after surgery, rats were housed following guidelines of the National Institutes of Health and the United States Department of Agriculture. The rats were kept within a double-barrier facility in standard rat cages with continuous supply of fresh air, water, and food. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Transplant preparation

We harvested BMSCs from femurs of female adult Sprague Dawley rats according to a previously described protocol (Fig. 1a)[7,15,31]. Isolated cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Allentown, PA, USA) with 10% fetal bovine serum (Mediatech, Manassas, VA, USA) and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY, USA). To enable detection after transplantation, first passage cells were transduced to express green fluorescent protein (GFP) using lentiviral vectors (Fig. 1a')[7,15,31]. Fourth passage cells positive for the BMSC markers, CD90 and CD105 and negative for blood cell markers, CD34, CD45 and HLA-DR[16,32,33] were used for the experiments.

ESHU

The preparation of ESHU (Fig. 1b) was previously described[25]. In brief, polyurethane blocks were synthesized by melting N-BOC-serinol (Sigma-Aldrich) under nitrogen and slowly adding hexamethylene diisocyanate (HDI; TCI America, Wellesley Hills, MA, USA) to initiate polymerization via urethane bonds. Both ends of polyurethane were capped with an isocyanate group using additional HDI and then dissolved in anhydrous dimethylformamide. Diethyl ether (Fisher Scientific, Pittsburgh, PA, USA) was used to precipitate out the polymer and remove unreacted hexamethylene diisocyanate. Polyethylene glycol (Alfa Aesar, Ward Hill, MA, USA) was coupled onto the polyurethane blocks under nitrogen, dissolved in dimethylformamide (EMD, Gibbstown, NJ, USA), and precipitated in and washed with diethyl ether. For purification, ESHU was dissolved in

water and dialyzed (3500 MWCO) for 48h and finally freeze-dried. In our experiments, a 16% w/v ESHU solution in phosphate-buffered saline (PBS; pH 7.4) was prepared and sterile-filtered before use.

Surgical procedures

A model of adult rat spinal cord contusion[34,35] was used to test our hypothesis. Female adult Sprague Dawley rats (200 g, n=80; Charles Rivers Laboratory, Wilmington, MA, USA) were anaesthetized using intraperitoneal injection Ketamine (60 mg/ml; Butlerschein, Dublin, OH, USA) and Dexdomitor (0.5 mg/kg; Pfizer, New York, NY, USA)[7,15]. The tenth thoracic spinal cord segment was contused using a force of 200 kDyne (Infinite Horizon IH-0400 impactor; Precision Systems and Instrumentation, LLC, Versailles, KY, USA)[7,15,36]. The wound site was rinsed with sterile PBS with 0.1% gentamicin (VWR, Radnor, PA), the muscles were sutured in layers, and the skin was closed with Michel wound clips (Fine Science Tools, Foster City, CA, USA). All rats included in the studies had an impact within 5% of the intended force resulting in a 0.9-1.8 mm spinal cord compression and a Basso-Beattie-Bresnahan (BBB)[37,38] score ≤ 1 at day 1 and ≤ 5 at day 3 post-impact. Three days post-injury, rats were sedated and injected into the contusion[7,15,16] with 5 µl ESHU or PBS with 5x105 BMSCs, or ESHU or PBS only.

Post-surgery procedures

Antisedan (1.5 mg/kg; Pfizer) was injected subcutaneously to reverse the effects of Dexdomitor[7,15]. An intramuscular injection of gentamicin (6mg/kg; VWR), a subcutaneous injection of Rimadyl (5 mg/kg; Pfizer), and a subcutaneous injection of Ringer's solution (10 ml on surgery day, 5 ml thereafter; Butlerschein) were administered daily for the first three days post-injury[7,15]. After the intraspinal injection at three days post-injury, the rats received gentamicin for four days and Ringer's and Rimadyl for three days[7,15]. Bladders were manually emptied twice daily until reflex voiding occurred. Rats were monitored daily throughout the experiments. Rats were fixed at 15 min, one, four, or six weeks after injection. All rats survived without requiring pain or distress treatment.

Motor function assessment

Overground walking ability was assessed using the BBB test[37,38] weekly for six weeks post-injection (n = 10/group). Rats were tested for 4 min by two testers unaware of the treatments. Rats were familiarized with the open field and baseline values were determined before surgery. Scores were averaged per experimental group. Higher motor functions were assessed at six weeks post-injury using the BBB sub-score[39,40] as previously described (n = 10/group)[7]. Scores were averaged per experimental group. Sensorimotor function of the hind limbs was assessed before (baseline) and at six weeks post-injection using horizontal ladder walking (n = 10/group)[7,41,42]. Slips of the foot and part of lower leg and slips of the full leg were counted and expressed as a percentage of the total number of steps. Scores were averaged per experimental group.

Histological procedures

Rats were anaesthetized and transcardially perfused with 300 ml PBS followed by 400 ml 4 % paraformaldehyde (Sigma-Aldrich) in PBS. Spinal cords were dissected, post-fixed overnight in the same fixative, and transferred to 30% sucrose (Fisher Scientific) in PBS for 48 h. A 12 mm-long spinal cord segment centered at the injury epicenter was cut in 20 µm-thick horizontal cryostat sections (CM 1950; Leica Biosystems, Buffalo Grove, IL, USA). Every twelfth section was stained with cresyl violet (0.5 %; Sigma-Aldrich) for cytoarchitecture analysis and spared tissue volume assessment. Other section series were used for immunocytochemistry. Sections were analyzed using an Axio Observer Z1 fluorescent microscope (Zeiss, Thornwood, NY, USA) with StereoInvestigator® (MicroBrightField, Inc., Williston, VT, USA).

Immunocytochemistry

Tissue sections were incubated in 5% normal goat serum (Vector Labs, Burlingame, CA, USA) and 0.03% Triton X-100 (Sigma-Aldrich) in PBS for 1 h followed by the primary antibody for 2 h at room temperature and then overnight at 4 °C. Rabbit polyclonal antibodies against glial-fibrillary acidic protein (GFAP) were used to detect astrocytes (1:200; Dako North America, Inc., Carpinteria, CA). Mouse monoclonal antibodies against

ED1 were used to detect macrophages (1:100; Millipore, Temecula, CA). BMSCs in vitro on 8-chamber culture slides (BD Falcon, Franklin Lakes, NJ; see also below) were fixed with 4 % paraformaldehyde in PBS for 10 min and stained with monoclonal antibodies against caspase 3 (rabbit, clone D3E9) to detect apoptotic cells (1:100; Millipore) and 8-Oxo-2'-deoxyguanosine (8-oxo-dG; mouse, clone 483.15) to detect cells with DNA damage (1:200; Millipore). After washing twice in PBS for 20 min, sections or cells were incubated with goat-anti-rabbit and goat anti-mouse Alexa Fluor 594 (1:200; Life Technologies, Grand Island, NY, USA) for 2 h at room temperature. DAPI (0.2 µl/ml; Sigma-Aldrich) was used to stain nuclei. Sections were covered with glass slips in fluorescent mounting medium (Dako North America, Inc.) and stored at 4 °C. Sections were analyzed using an Axio Observer Z1 fluorescent microscope (Zeiss, Thornwood, NY, USA) with StereoInvestigator® (MicroBrightField, Inc., Williston, VT, USA).

Cell quantification

StereoInvestigator® (MicroBrightField, Inc.) was used to determine the numbers of GFP-positive BMSCs in the injury site[15,43] at seven days post-transplantation (n = 6/group) in every twelfth section and the numbers of caspase 3- and 8-oxo-dG-positive BMSCs in cultures (see below). All assessments were done by personnel blinded to the treatment groups. For GFP-positive BMSCs in the contusion, sections were 240 µm apart spanning the width of the spinal cord. In every section the area containing GFP-positive cells was outlined manually at 2.5 X magnification and covered with a 250 x 250 µm grid. At 60 X magnification with oil immersion, GFP-positive cells with a discernible DAPI-stained nucleus were marked using the optical fractionator with a 60 x 60 µm counting frame[7,15,43]. The numbers of immunostained cells in cultures were similarly determined. The numbers of GFP-positive cells were expressed as a percentage of the number of transplanted cells (± SEM). The numbers of caspase 3- and 8-oxo-dG-positive cells were expressed as a percentage of the number of seeded cells (± SEM). The numbers were averaged per experimental group. Image J Software was used to determine the number of ED1-immunoreactive macrophages in the injury site at one and four weeks post-

transplantation in every twelfth section by persons blinded to the treatment groups[16]. Numbers were averaged per experimental group.

Measurement of nervous tissue sparing

Cresyl violet-stained sections of rats that survived for four weeks post-injection were used to determine the volume of spared tissue in the damaged spinal cord segment using the Cavalieri estimator function of StereoInvestigator® (MicroBrightField, Inc.)[7,43]. Analysis was performed by personnel blinded to the experimental groups (n = 6/group). The Gundersen Coefficient of Error was < 0.05 for all measurements. Spared tissue volume was expressed as a percentage of the volume (± SEM) of an equally-sized comparable uninjured spinal cord segment and averaged per experimental group[7].

In vitro assessment of the protective effect of ESHU

To assess ESHU's cell protective ability we kept BMSCs in vitro under oxidative stress, which is known to contribute to intraneural cell transplant loss[23,24]. A total of 4x105 cells were incubated in 100 µl ESHU or PBS with 200 µM hydrogen peroxide (H2O2; Sigma-Aldrich) for 24 h at 37°C. Then, 100 µl Trypan Blue (Sigma-Aldrich) was added and viable (Trypan Blue-negative) cells were quantified in a hematocytometer and expressed as a percentage of all counted cells. Results from nine samples from three independent experiments were averaged. In nine samples from three independent experiments, the average number of BMSCs expressing caspase 3, a marker for apoptosis, and 8-Oxo-2'-deoxyguanosine (8-oxo-dG), a marker for DNA damage, were determined (see above). Details on caspase 3 and 8-oxo-dG staining are described in 2.8. Immunocytochemistry.

Quantification of ESHU's antioxidant ability

ESHU's ability to scavenge H2O2 relative to PBS was measured using a H2O2 quantification kit (National Diagnostics, Atlanta, GA, USA) which colorimetrically measures Xylenol Orange-Ferric iron complex resulting from H2O2-mediated oxidation of ferrous iron. The linear standard curve of this assay is 15-100 ng/ml. We added 30 ng/ml H2O2 (Sigma-Aldrich) to ESHU or PBS which was kept in reagent buffer for 30 min following the

manufacture's guidelines. Absorbance was measured (Victor 2V 1420; Perkin-Elmer, Waltham, MA, USA) and the values from three independent experiments were averaged.

Statistical analysis

Two-tailed Student's T-test was used to determine differences in cell numbers in vivo and in vitro and in H2O2 concentrations in vitro. One-way ANOVA with Tukey's post-hoc test was used to assess differences in macrophages and nervous tissue sparing. Repeated measures ANOVA with Tukey's post hoc test determined differences in functional performances. Differences between groups were considered significant when $p < 0.05$.

RESULTS

BMSC transplant survival

We investigated whether ESHU protects transplanted BMSCs from death in damaged nervous tissue using a spinal cord contusion model. At 15 min post-injection, rounded BMSCs were present in the injury when mixed in either ESHU (Fig. 2a) or PBS (Fig. 2b). One week post-injection, in both groups many spindle-shaped cells were also found (Figs. 2c, 2d). At 4 and 6 weeks, hardly any cells could be found in or near the contusion site after injection of BMSCs in ESHU (Fig. 2e) or in PBS (Fig. 2f). The temporal morphological profile of the grafted cells is in accordance with earlier observations[15]. Also, GFAP staining (Figs. 2a-f) was similar as previously described[7,15]. We found that 73 ± 17 % (SEM; n =6) of transplanted cells had survived in ESHU while 21 ± 8 % (SEM; n = 6) survived in PBS (Fig. 2g), which represents a significant ($p < 0.05$) 3.5-fold increase in survival in ESHU compared with PBS. At four weeks post-injection, in both groups < 1 % of the cells has survived in the injury site. The data show that ESHU does not affect BMSC transplant morphology and protects against early death resulting in increased transplant presence at one week post-injection.

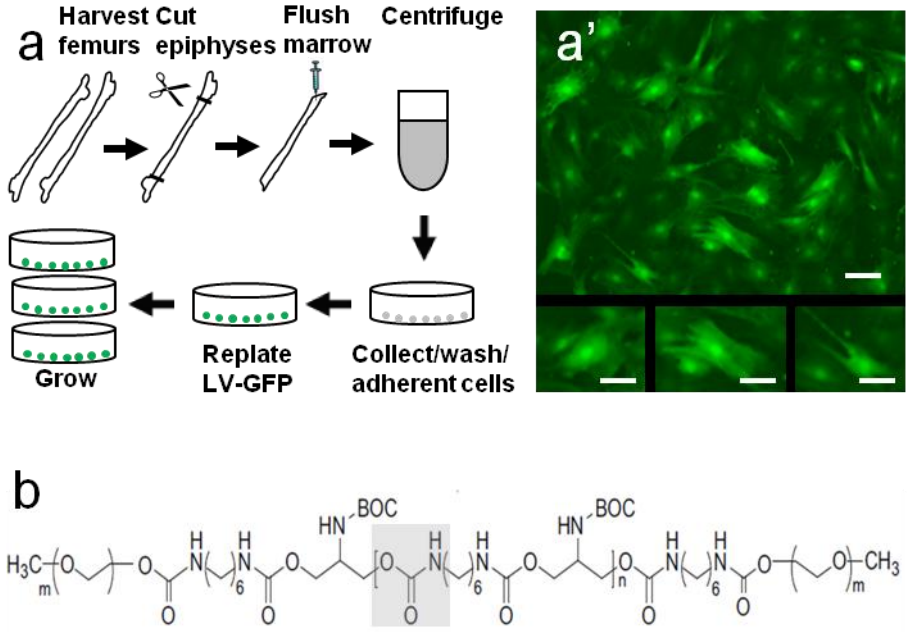

Fig. 1. Schematic representations of transplant preparation and ESHU. (a) Isolation and transduction of bone marrow stromal cells. Plastic-adherent cells from femurs of adult Sprague-Dawley rats were collected, lentivirally transduced with green fluorescent protein, and passaged four times before used for transplantation. (a') Green fluorescent protein-expressing bone marrow stromal cells in culture. Smaller panels show examples of cultured BMSC morphologies. (b) Structural formula of ESHU with the urethane bond in gray box. Bar = 50 µm in a' and 25 µm in panels below.

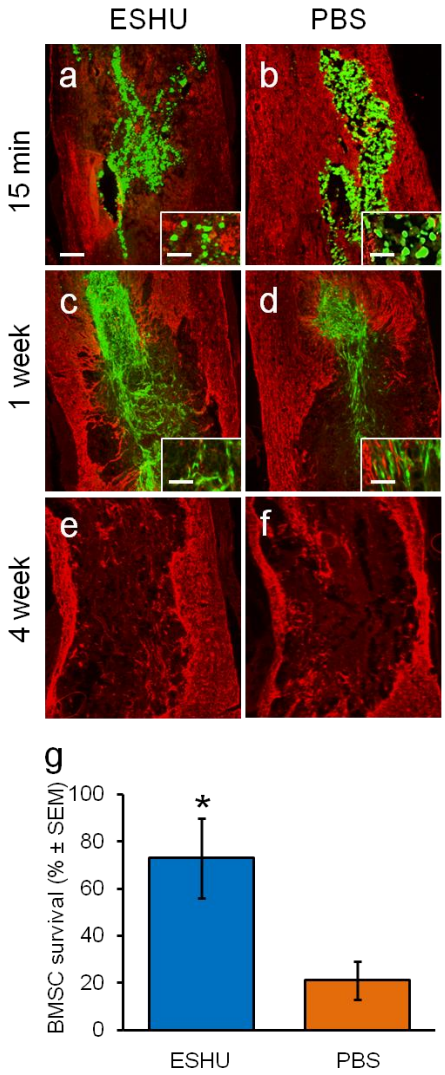

Fig. 2. ESHU improves the survival of bone marrow stromal cell transplants in a spinal cord contusion. Fifteen minutes after injection, transplanted cells (green) occupies most of the contusion regardless whether they were suspended in ESHU (a) or phosphate-buffered saline (PBS) (b). Staining for glial-fibrillary acidic protein (GFAP, red) was used to outline the contusion. Transplanted cells were mostly rounded in ESHU (insert panel a) and PBS (insert panel b). One week after injection, the transplant occupies only part of the contusion site but more so when suspended in ESHU (c) than PBS (d). The transplanted cells at this time point were mostly elongated with bipolar morphologies in ESHU (insert panel c) and PBS (insert panel d). Four weeks after injection, hardly any cells were detected in the contusion when injected with ESHU (e) or PBS (f). Similar results were observed after six weeks (not shown). (g) More transplanted cells survive in the contusion site the first week after injection when suspended in ESHU than PBS. Survival rate was measured against total number of injected cells. Error bars in bar graph display standard error of the mean (SEM). Asterisk = $p < 0.05$. Bar in a = 350 µm in a-d and 30 µm in inserts.

Effect of BMSC transplant survival on neuroprotection

Because BMSC transplant survival is associated with neuroprotection[15] we assessed whether ESHU-promoted transplant survival rendered enhanced tissue sparing (Fig. 3a-d). The results demonstrated that the volume of spared tissue in rats with the transplant in ESHU is 66 % larger ($p < 0.05$; n = 6/group) than in rats with the transplant in PBS at four weeks post-transplantation (Fig. 3e). ESHU only had no effect on spared tissue volume in the damaged area (Fig. 3e). The data suggest that increased survival of intraneural BMSC transplants early after injection enhances neuroprotection of nervous tissue.

Effect of BMSC transplant survival on motor recovery

After spinal cord contusion, motor performance depends in part on the amount of nervous tissue at the injury site[7]. We examined whether augmented neuroprotection by BMSC transplants with ESHU-enhanced survival affected motor function recovery. We found that rats with the transplant in ESHU performed significantly ($p<0.05$; n = 10/group) better in overground walking than rats with BMSC in PBS at 4-6 weeks post-injury (Fig. 4a). Rats with BMSC in ESHU performed better than rats with ESHU or PBS alone at 1-6 weeks post-injury and rats with BMSCs in PBS walked better overground than rats with ESHU or PBS alone only at 1-3 weeks post-injury (Fig. 4a). At 6 weeks, rats with BMSCs in ESHU showed consistent (>95%) weight-supported plantar steps with frontlimb-hindlimb coordination. The control transplanted rats were less consistent (50-95%) making such steps, whereas rats with ESHU or PBS only were less consistent and lacked frontlimb-hindlimb coordination. During the 4th-6th week after injection, overground walking was increased by 1.7 ± 0.4 points on the BBB scale in rats with the transplant in ESHU which was significantly ($p < 0.05$; n = 10/group) higher than the increase in the other groups (Fig. 4b). Higher motor functions of the hindlimbs were significantly improved ($p < 0.05$; n = 10/group) by 74% in rats with BMSC in ESHU compared with BMSCs in PBS at 6 weeks post-injury (Fig. 4c). Sensorimotor function was significantly increased ($p < 0.05$; n = 10/group) in rats with the transplant in ESHU compared with the other three groups at 6 weeks post-injury (Fig. 4d). Rats receiving the transplant in PBS had significantly improved sensorimotor function compared with rats with ESHU or PBS alone (Fig. 4d).

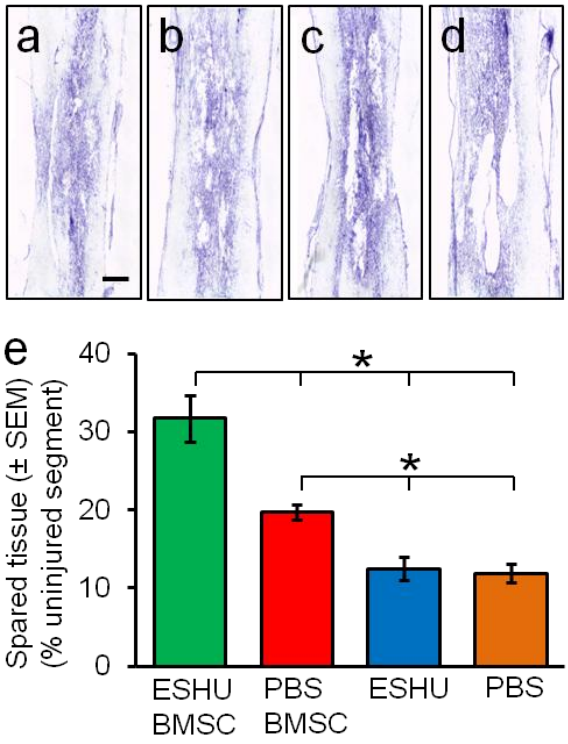

Fig. 3. ESHU augments neuroprotection by bone marrow stromal cell transplants in the contused spinal cord. Damage and loss of nervous tissue was observed at four weeks after a bone marrow stromal cell (BMSC) transplant in ESHU (a) or PBS (b) or ESHU (c) or PBS (d) alone into the contused spinal cord. (e) Spared tissue volume was larger with the transplant in ESHU compared with all other groups. Error bars in bar graph display standard error of the mean (SEM). Asterisks = $p < 0.05$. Bar in a = 600 µm in a-d.

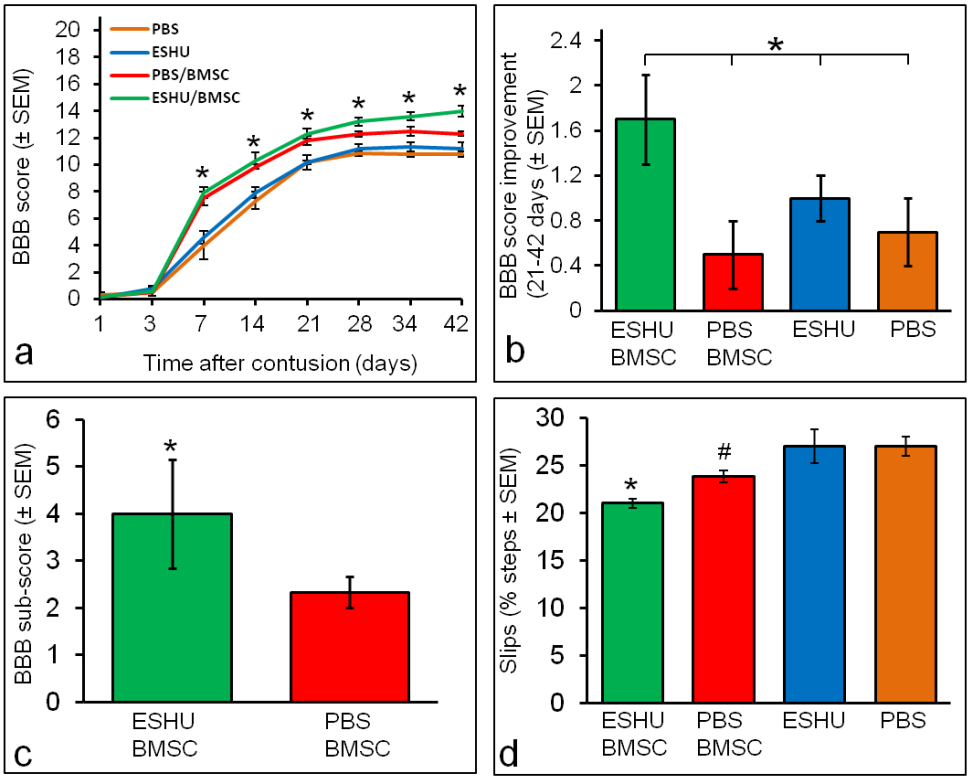

Fig. 4. ESHU leads to enhancement of motor function recovery by a bone marrow stromal cell transplant in the contused spinal cord. (a) Overground walking ability was significantly improved in rats with a bone marrow stromal cell (BMSC) transplant in ESHU compared with BMSC in PBS at 4-6 weeks post-injury. Rats with BMSC in ESHU performed better than rats with ESHU or PBS alone at 1-6 weeks post-injury. Rats with BMSCs in PBS walked better overground than rats with ESHU or PBS alone only at 1-3 weeks post-injury. (b) Improvement in overground walking ability during the 4th-6th week post-injury was significantly improved in rats with a transplant in ESHU compared with all other groups. (c) Improved higher motor functions in rats with BMSCs in ESHU compared with PBS at 6 weeks post-injury. (d) Improved sensorimotor recovery in rats with the transplant in ESHU over all other groups and in rats with BMSCs in PBS over the control groups without BMSCs at 6 weeks post-injury. Error bars in bar graph display standard error of the mean (SEM). Asterisk = $p < 0.05$. Number sign = $p < 0.05$.

Inflammatory response

Macrophages invade damaged nervous tissue and contribute to cell death[19]. ESHU breakdown products could carry negative charges and so affect macrophage presence[44]. We tested the possible influence of macrophages on ESHU's protective capacity by assessing their presence in the injury after injection of BMSCs in ESHU (Fig. 5a-c) or PBS (Fig. 5d-f), or ESHU or PBS only. The results demonstrated similar macrophage presence

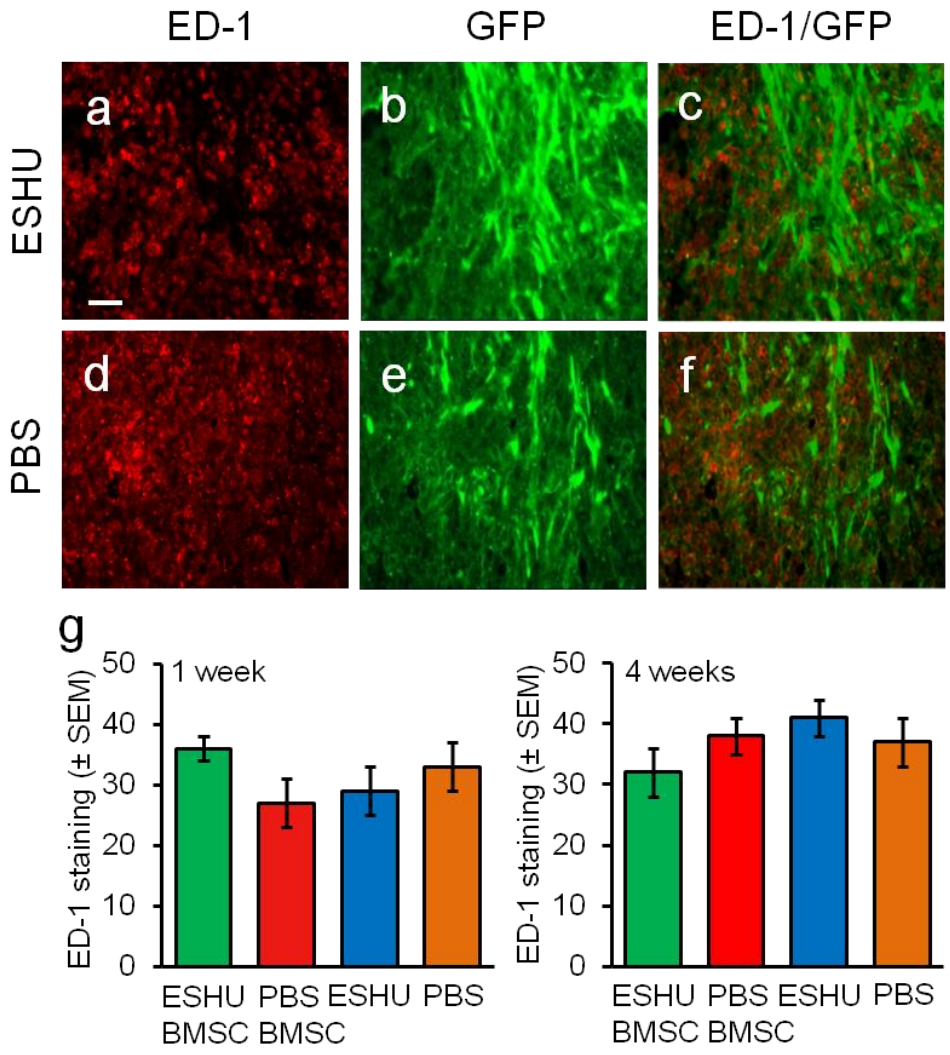

Fig. 5. ESHU does not affect the injury-induced macrophage response. Macrophages (ED-1+, red) were found in the contusion with transplanted bone marrow stromal cell (green) in ESHU (a-c) or PBS (d-f). (g) ESHU as a transplant matrix or alone did not affect the presence of macrophages in the contusion at one and four weeks after transplantation. Error bars in bar graphs display standard error of the mean (SEM). Bar in a = 15 µm in a-f.

between all groups at one (Fig. 5g) and four (Fig. 5h) weeks post-injection, suggesting that ESHU is non-immunogenic. The data indicate that macrophages are not implicated in ESHU-mediated transplant survival.

Oxidative stress-mediated cell death in vitro

We tested whether ESHU protects BMSCs from H2O2-mediated death in vitro (Fig. 6a) and found that survival was increased four-fold in ESHU (62 ± 6%, SEM; n = 9) compared with PBS (15 ± 2%, $p < 0.05$, n = 9; Fig. 6b). ESHU resulted in an almost two-fold decrease in BMSCs positive for caspase 3 or 8-oxo-dG ($p < 0.05$, n = 9; Fig. 6c). To explore ESHU's cell protective effect we assessed its proficiency in scavenging H2O2. We found that ESHU decreased the amount of H2O2 by 10 % (3 ng/ml) in 30 min compared with PBS ($p < 0.05$, n = 9; Fig. 6d), suggesting ESHU-mediated oxidation of H2O2. The data show that ESHU scavenges H2O2 and protects against oxidative stress-mediated cell death.

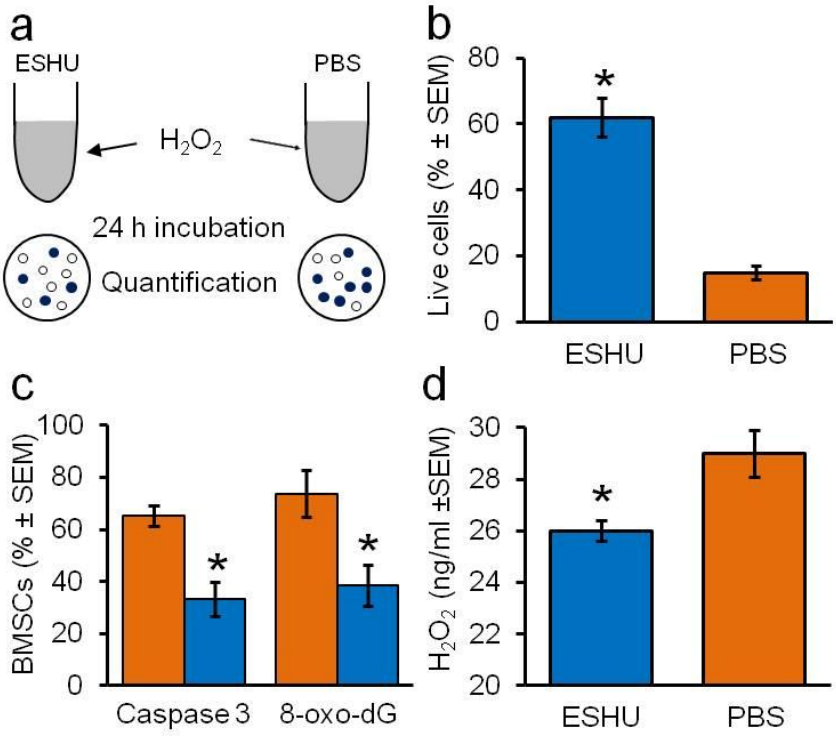

Fig. 6. ESHU protects bone marrow stromal cells in suspension and scavenges hydrogen peroxide in vitro. (a) Schematic representation of in vitro assay of ESHU's ability to protect bone marrow stromal cells from hydrogen peroxide (H2O2)-induced death. (b) Cell survival from H2O2-induced oxidative stress is better in ESHU than phosphate-buffered saline (PBS). (c) Fewer BMSCs positive for caspase 3 and 8-oxo-dG with ESHU (blue) than with PBS (orange). (d) ESHU scavenges H2O2 in PBS. Error bars in bar graphs display standard error of the mean (SEM). Asterisks = $p < 0.05$.

DISCUSSION

We show that ESHU, a synthetic injectable reverse thermal gel, protects transplanted BMSCs from death thereby prolonging their presence in damaged nervous tissue and leading to enhanced tissue sparing accompanied by improved motor function recovery. Our study demonstrates that improved intraneural BMSC transplant survival enhances their effects on repair, which may have widespread impact on BMSC-based therapies for tissue repair.

The inclusion of ESHU enhanced BMSC presence in a contusion in the adult rat spinal cord. This effect was transient possibly due to degradation of ESHU[25]. When mixed in culture medium, BMSC presence was significantly lower in the contusion site[15-18,47]. Possibly ESHU retains BMSCs better in the contusion (i.e., the site of injection) compared with culture medium, resulting in the higher numbers. Previously we showed that about 2.4% of GFP-expressing BMSCs in culture medium leaked or migrated away from a contusion[15]. Therefore, the present results suggest that ESHU protects transplants in the contused spinal cord tissue during the first week post-injection, which is a critical time period for BMSC-mediated neuroprotection[15].

The ESHU-mediated increase in BMSC survival in the contused spinal cord resulted in anatomical (tissue sparing) and functional (motor/sensorimotor) improvements. Spared tissue volume was not affected by ESHU alone, indicating that the neuroprotection was elicited by the increased survival of the transplant. Previously, we showed that the neuroprotective effects of BMSC transplants are greatest during the first week post-injury[15]. The current finding demonstrates that the efficacy of an intraspinal BMSC transplant to elicit neuroprotection depends on its degree of survival and that increased survival leads to increased spared tissue volumes. Neuroprotection by intraneural BMSC transplants is thought to result from paracrine effects[11-14]. Our finding that increased transplant survival results in increased tissue sparing may imply that the magnitude of neuroprotection elicited by the transplants depends on the concentration and/or availability of secreted growth factors mediating paracrine actions.

Overground walking and higher motor and sensorimotor functions of the paralyzed hindlimbs were further improved in rats that received BMSCs in ESHU. The improvements in overground walking were particularly evident during the second half of the 6-week period. It was demonstrated that BMSC transplant-mediated improvements in motor function recovery after spinal cord[4-7,18] and brain[50-52] injury are correlated with the amount of spared nervous tissue[7,53]. Thus, in our study, neuroprotection elicited by BMSC transplants with ESHU-increased survival likely contributes to the observed improved motor function recovery. At present the anatomical correlates underlying improved motor recovery are not completely known but may involve increased numbers of descending axons conducting the actual motor activity[7] and/or increased myelination providing better signal conduction[53,54], which both could result from neuroprotection.

In search of potential mechanisms underlying ESHU-mediated BMSC protection, we assessed macrophage presence in the contusion. Macrophages are naturally present in damaged nervous tissue and contribute to the death of neural and transplanted cells[16,17,19]. Oxidation of ESHU could lead to carboxylates whose negative charges might inhibit adhesion of macrophages[44] thereby limiting their contribution to cell death. We found that the number of macrophages in the contusion was similar with or without the presence of ESHU. BMSCs are hypoimmunogenic, lacking MHC class II and co-stimulatory molecules for effector T cell induction[48], and suppress T cell proliferation[49]; thus the adaptive immune response is unlikely to be largely involved in allogeneic BMSC death. Our data suggest that the protective effects of ESHU result from direct effects on the transplant rather than indirect effects involving macrophages.

Another possible mechanism underlying ESHU-promoted BMSC survival is antioxidation. Reactive oxygen species (ROS) accumulate rapidly in damaged nervous tissue and induce oxidative stress leading to cell death[20-24]. We used H2O2 to determine whether ESHU has the ability to scavenge ROS and thus mediate antioxidant effects. H2O2 is amply present in damaged nervous tissue[45]. We found that a 16 % ESHU solution removed 3 ng H2O2 in a 30 min time period. Assuming continuous activity at 3 ng/30 min, ESHU removed ~ 20% of

added H2O2 in our in vitro assay of ESHU's ability to protect BMSCs, which elicited a 47 % increase in their survival relative to PBS. The ability to scavenge ROS may be exerted through its urethane groups[28,29]. Possibly ESHU's antioxidant effects may be increased with higher concentrations[46]. Our observations point at antioxidation as a potential mechanism of ESHU-promoted BMSC transplant survival. ROS are known to contribute to transplanted cell death[23,24]. Future studies need to define molecular factors in ESHU's protective actions and whether the protection by ESHU in vivo is concentration-dependent.

CONCLUSIONS

We demonstrate that the reparative effects of a BMSC transplant are enhanced by promoting their survival. This finding critically impacts current and future BMSC-based therapies for the central nervous system. ESHU's ability to gel at body temperature allows for injection (i.e., minimally invasive) into closed injuries. Besides reducing oxidative stress and serving as a matrix for cells, ESHU can also be used to deliver drugs and/or functionalized with bioactive molecules to affect targeted biological events. These benefits render ESHU an important candidate in future therapies for the traumatized or degenerated nervous system. Furthermore, because oxidative stress is part of many diseases where BMSCs can be effective, such as cardiac myopathy and peripheral arterial disease, ESHU may have wide therapeutic relevance.

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Chapter 6

Discussion and Concluding Remarks

Spinal cord injuries result in permanent anatomical and functional damage which are so far untreatable. The present thesis aimed to enhance our understanding of BMSC therapy for spinal cord injury and to investigate approaches to increase the therapeutic efficacy of BMSC transplants for spinal cord repair. Using an adult rat model of spinal cord contusion, which is clinically the most relevant type of spinal cord injury, the efficacy of BMSCs to repair the contused spinal cord was studied. The results confirmed and further expanded previous data demonstrating that a BMSC transplant results in neuroprotection (i.e., tissue sparing) and improved motor, sensorimotor, ands sensory function recovery. Most functional improvements were strongly correlated with the neuroprotective effects, which included sparing of descending raphespinal axons from the brainstem. Moreover, increased blood vessel density at the injury epicenter was identified as a potential mediator of BMSC-mediated tissue sparing. Although BDNF was not found to be a necessary factor in BMSC-mediated tissue sparing, genetically modifying BMSCs to hypersecrete BDNF were found to further increase the neuroprotective effects. Importantly, increasing BMSC transplant survival using ESHU, a reverse thermal gel with anti-oxidant abilities, was found to augment the effects of a BMSC transplant on anatomical and functional repair of the contused spinal cord.

PARACRINE FUNCTIONS OF BMSCs

The efficacy of BMSCs is thought to be due to the paracrine actions of secreted factors. BSMCs secrete a variety of growth factors and cytokines that can be grouped into three repair-promoting categories. The first group is comprised of factors that affects blood vessels. Among these factors are vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and angiopoietin-1 (ANG-1).[1,2] We found increased blood vessel density at a contusion site after BMSC transplantation (Chapter 2) suggesting that BMSC-mediated angiogenesis is involved in its neuroprotective actions. Further studies need to elucidate which of the abovementioned BMSC-derived factors play a role in the formation, repair, or sparing of blood vessels near an injury site. One possible mechanism by which BMSC transplants may elicit vascular repair is by secretion of Ang-1 which is known to stabilize blood vessels thereby decreasing their permeability.

The second group consists of factors that affect cell survival. This group includes BDNF, glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and ß-fibroblast growth factor (ß-FGF).[3,4] We found that BDNF is not a necessary factor for BMSC-mediated neuroprotection but the therapeutic efficacy of BMSC transplants can be enhanced using BDNF-hypersecreting BMSCs (Chapter 3). A specific repair-related event may be affected by many different trophic factors as was confirmed in Chapter 3. Conversely, a particular trophic factor may affect multiple events. It is important to acquire thorough understanding of the role(s) of a particular repair-supporting factor, including the benefits and detriments, before approaches can be developed to enhance BMSC-based spinal cord repair.

The third group of repair-supporting factors secreted by BMSC affect the immune response. This group includes interleukin-10 (IL-10) and transforming growth factor ß-1 (TGF-ß1). The inflammatory response plays a dual role in spinal cord injury. After the initial impact, a massive influx and proliferation of macrophages is evident. These macrophages are needed to clear cellular debris and reorganize tissue at the injury site. In doing so, they secrete molecules that increase oxidative stress and exacerbate secondary tissue degeneration. Currently, the differential role of M1 macrophages, with mainly deteriorating effects, and M2 macrophages, with predominantly beneficial effects, are being investigated. Within the setting of this complex inflammatory response, the role of immune modulatory factors secreted by BMSCs remains to be elucidated.

FACTORS DETERMING OUTCOME

Interestingly, investigations of BMSC transplants in spinal cord injury have led to different and at times conflicting conclusions. Many groups, but not all, have reported anatomical or functional improvements after BMSC transplantation in the injured spinal cord. Some groups report BMSC-mediated effects on axonal regeneration in the injured spinal cord. Many different aspects can influence the effects of BMSCs on repair in models of spinal cord injury. Firstly, the age of BMSCs affects their genetic expression profile, including expression of genes involved in neural repair. Previously, we characterized the gene

expression profiles of BMSCs that were passaged three (P3) or fourteen (P14) times and revealed a decrease in plasticity and repair aptitude of long-term cultured BMSCs .[5] In addition, the age of the rat from which the BMSCs are harvested affect BMSC plasticity and their proliferative life span. BMSCs from younger rats have higher telomerase activity and higher expression of Sox-2 and Nanog, increasing their proliferative life span and cell plasticity, respectively.[6.7] Also, human bone marrow stromal cells exhibit donor variations in secretion patterns of growth factors and cytokines, affecting axons growth and functional recovery in rat spinal cord injury.[8] Clearly, determination and standardization of the optimal BMSC age and donor lot is necessary to validly compare studies and move forward with BMSC therapy research.

Another major factor determining outcome after BMSC transplantation is the model system used. The strain and gender of the rats used affect the immune response to the transplanted BMSCs. In the present thesis, female rats were used because their short urethra makes manual bladder expression more practical, and their more gentile temperament makes handling easier. Sprague Dawley rats were used, which is an outbred strain, resulting in greater surgery survival rates and less complications. Allogeneic transplantations in Sprague Dawley rats could possibly result in different immune responses than using more inbred strains, thus allowing for more syngeneic transplantations. Different injury devices, injury types and injury levels used result in different baseline functional deficits. Future research needs to determine which types of injury model best predicts functional recovery in humans, and the site, dose and timing of BMSC injection influence cell survival and cell dynamics. Differences in any of these factors can impact the observed outcome. A golden standard model system for testing cellular transplants for spinal cord injury does not exist and further research is needed to determine the true therapeutic efficacy of BMSCs. On the other hand, there is a high degree of variation between humans and having differences between models of spinal cord injury may in fact support our understanding of the potential of BMSC-based spinal cord repair.

Although the rat contusion model of spinal cord injury shows considerable anatomical similarities to human spinal cord injury, and is generally considered to be a suitable model system, there are limitations that affect the interpretation of the repair effects of BMSCs, especially in light of their potential for human spinal cord repair. Firstly, rats show some degree of functional recovery even in the complete absence of supraspinal input, likely due to the presence of a locomotor central pattern generator (CPG) in the lumbar spinal cord segments. Within days after injury, rats will start to show hindlimb joint movements, followed by stepping movements, and, depending on the severity of the injury, weight supported stepping. Reorganization of the CPG is believed to underlie this functional recovery. Humans do not seem to reorganize their lumbar spinal neurons in a way that leads to functional recovery, even though a lumbar CPG is present. Rats are quadrupeds and following awakening from anesthesia after a spinal cord injury, they begin to move around using their forelimbs while dragging their hind limbs. This constant sensory input to the hind limbs is believed to positively affect functional recovery. Indeed, a recent study shows that hind limb immobilization and hind limb stretching therapy in rats hinders the functional recovery of spinal cord injured rats.[9] Treatments tested in rats that improve functional repair, might do so by positively affecting spinal cord reorganization below the level of injury. Humans might be more dependent on supraspinal input for effective functional recovery. The widely used BBB locomotor recovery scale used in rats, also used in this thesis, may not adequately reflect these differences. In the 21-point BBB scale small changes in tissue can be correlated to changes on the scale. In humans, no scale exists in which the extent of tissue damage/sparing can be correlated with a functional rating scale. Although it seems plausible that neuroprotective interventions that are so closely correlated to functional recovery in rats would also be beneficial for humans, no such evidence exists to date.

Understanding the factors underlying the observed differences in recovery between rats and humans as well as gaining insight in the mechanisms of action of proposed treatments will help us predict which (combination of) therapies may restore function in humans. Conversily, data from the few spinal cord injured patients injected with cellular transplants

so far, both from the discontinued Geron trial, as well as from the ongoing StemCell trial may provide us with insights regarding the questions we need to focus on in the laboratory. However, caution is warranted when efforts to translate therapies into the clinic are taken too prematurely, since lack of efficacy in unfully understood treatments, might unduely discourage patients, the scientific community as well as funding agencies, and decrease the progress of basic research.

COMBINATION STRATEGIES

Clearly, BMSCs or any stem cell by itself will not provide the 'silver bullet' for restoring functional repair after spinal cord injury. The neuroprotective properties of these cells will have to be combined with regenerative and rehabilitative strategies to regain function after paralysis. One recent study showing the additive strength of combination strategies was by Van Den Brand et al[10]. Monoamine agonist, epidural electrical stimulation and neurorehabilitation within a robotic harness were successfully combined to considerably improve walking after spinal cord injury in a rat model.

CONCLUSION

BMSC transplantation is a promising cell-based strategy to promote repair of the injured spinal cord. The knowledge we gain from studying BMSC transplants within spinal cord injury models, provide valuable insights into cell-based treatments for central nervous system disorders that can one day be translated into the clinic providing treatments to improve the quality of life of spinal cord injured patients.

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Chapter 7

Summary

INTRODUCTION

A spinal cord injury leads to loss of neural cells and interruption of axonal tracts, resulting in partial or complete motor, sensory, and autonomous dysfunction below the level of injury. The most prevalent causes of traumatic spinal cord injuries include traffic accidents, sport injuries, and violence. Non-traumatic causes include tumor compression and infections. The initial impact results in immediate death of neurons and glial cells, and damage to axons and blood vessels. The impact further sets off a series of pathophysiological events causing progressive secondary damage which continues for days to months. Contributing events to secondary damage are inflammatory cells and release of cytotoxic molecules. The presence of growth-inhibitory molecules in scar tissue at the injury site and the gradual formation of a fluid-filled cyst contribute to a hostile environment for repair-supporting events including axonal regeneration. Currently, there is no therapy available that effectively restores the damaged axonal tracts, so that most people with spinal cord injury suffer permanent paralysis.

STEM CELL THERAPY

Chapter 1 provides a general introduction on stem cells for repair of the central nervous system. Different types of stem cells can be used for repair of the central nervous system; each type with its specific advantages and disadvantages. Embryonic and fetal stem cells have the advantage of the potential to differentiate into almost any cell type and can potentially replace damaged and lost cells in the nervous system. Disadvantages of these cells include ethical considerations regarding cell harvest and the risk of tumor formation as a result of uninhibited proliferation. Adult stem cells are less tumorigenic but also have less differentiation capacity. Stem cells can exert their beneficial effect by secreting growth factors with protective and/or proliferative effects on neural tissue. One easy to harvest adult stem cell is the bone marrow-derived mesenchymal stem cell, also called bone marrow stromal cell (BMSC). Part two of the introduction specifically focuses on these BMSCs as a therapy for spinal cord repair. BMSCs are relatively easy to harvest from adult bone marrow and can be cultured quickly in defined growth medium. They secrete

various growth factors which include neurotrophic factors and have a protective effect on neural tissue after transplantation into the injured adult rat spinal cord. This thesis has two main goals: (1) to expand the our knowledge of BMSC therapy for spinal cord repair, and (2) to investigate approaches to enhance the therapeutic efficacy of intraspinal BMSC transplants. The experiments in this thesis are conducted using an adult rat model of spinal cord injury involving a T9 laminectomy and subsequent contusion of the exposed spinal cord using an automated impactor which results in hindlimb and tail paralysis. Anatomically, the impact results in loss of neurons and glia cells, damage to axons and blood vessel, and an influx of inflammatory cells which contribute to progressive secondary tissue loss. This model mimics the consequences of spinal cord injury in humans.

BMSC-MEDIATED NEUROPROTECTION AND REPAIR

Chapter 2 examined whether BMSC-mediated neuroprotection enhances motor and sensory function recovery after transplantation into an adult rat model of spinal cord contusion. The relationship between BMSC-mediated tissue sparing and different aspects of functional recovery were investigated. The results showed that BMSC transplantation positively affects different parameters for (sensori)motor function (BBB subscore, foot print analysis, horizontal ladder walking) and sensation (thermal hyperalgesia and mechanical allodynia) and that these effects are correlated with the volume of spared nervous tissue in the contused segment. In addition, we found that the BMSC-transplanted segment contained more blood vessels than control (not-transplanted) rats, which may contribute to the increased volumes of spared tissue. We also found that rats with BMSCs contained more axons originating from the raphe nuclei in the brain stem, which may contribute to the observed improvements in motor recovery.

DE ROLE OF BDNF IN BMSC-MEDIATED NEUROPROTECTION

In **chapter 3** we studied the role of BDNF, one of the growth factors secreted by BMSCs, in BMSC-mediated neuroprotection by increasing and decreasing the expression of BDNF in

BMSCs through lentiviral transduction. *In vitro,* BDNF was shown not to be a necessary factor for the observed protective effect on spinal motoneurons. BMSCs with silenced BDNF production had a similar beneficial effect on neural survival as control BMSCs, possibly due to compensatory effects of other secreted trophic factors. BMSCs overexpressing BDNF resulted in an increase in motoneuron survival that was not seen with control BMSCs or with BMSCs with silenced BDNF production. *In vivo*, this beneficial effect of BDNF-overexpressing BMSCs was confirmed; more motoneurons survived in the spinal cord after transplantation of BDNF-hypersecreting BMSCs, compared to unmodified BMSCs or BMSCs with silenced BDNF production. Rats that received BDNF-hypersecreting BMSCs were found to have a higher density of grey matter blood vessels, which could have been a mediating factor in the improved motoneuron survival. In addition, we found BDNF to be a necessary factor for BMSC survival *in vivo.* BMSCs with silenced BDNF production did not survive the first week of transplantation, which may explain the lack of tissue sparing in these rats.

BMSC SURVIVAL

Survival of BMSCs in the damaged spinal cord is poor and limits their repair efficacy. One week after transplantation, about twenty percent of the transplanted cells survive. Different factors contribute to this poor BMSC survival, including phagocytosis by macrophages, lack of oxygen and nutrients by ruptured blood vessels, and the presence of reactive oxygen species and other cytotoxic molecules at the injury site. In **chapter 4** we investigated whether BMSC survival could be improved by suppressing the inflammatory response. Three clinically used anti-inflammatory drugs, Minocycline, Methylprednisolone, and Cyclosporine were tested for their ability to suppress the number of activated macrophages in the injured spinal cord and thereby increase BMSC survival. All three drugs were effective in decreasing the macrophage response, but this did not improve transplanted BMSC survival.

In **Chapter 5** we investigated whether BMSC survival could be improved by transplanting the cells in the reverse thermal gel, poly(ethylene glycol) -poly(serinol hexamethylene

urethane), or ESHU, which has anti-oxidative properties. We showed that BMSCs survived the first week of transplantation better when transplanted in ESHU and that this improved survival was associated with increased tissue sparing and improved motor and sensorimotor function recovery. A likely contributor to the improved BMSC survival is the anti-oxidative ability of the poly-urethane group of ESHU. This antioxidant effect was confirmed *in vitro*.

CONCLUSION

BSMC transplantation has beneficial anatomical effects associated with improved motor, sensorimotor, and sensory function recovery in a rat model of spinal cord contusion. Some of these effects can be further enhanced by overexpressing BDNF in the transplanted BMSCs. Short term BMSC survival can be improved by transplanting the cells in ESHU and this leads to increased tissue sparing and functional improvement, indicating that survival is a determinant in the therapeutic efficacy of BMSC transplants. Future research will need to focus on combinations of neuroprotective BMSC transplants with axonal regenerating promoting therapies to further optimize BMSC-based therapy for spinal cord injury.

Chapter 8

Samenvatting

INTRODUCTIE

Een dwarslaesie leidt tot verlies van zenuwcellen en een onderbreking van zenuwbanen in het ruggenmerg, leidend tot gedeeltelijke of volledige verlamming, gevoelsverlies en autonome functiestoornissen onder het niveau van het letsel. Tot de meest voorkomende oorzaken van traumatische dwarslaesies behoren verkeersongelukken, sportongevallen en geweld. Niet-traumatische oorzaken zijn onder andere tumorcompressie en infecties. De initiele impact leidt tot direct verlies van neuronen en gliacellen en schade aan axonen enz bloedvaten. De impact zet vervolgens een pathofysiologische cascade op gang die nog dagen tot maanden tot secundaire weefselschade kan leiden, onder andere door influx van ontstekingscellen en vrijkomen van cytotoxische moleculen. De formatie van littekenweefsel en een met vocht gevulde cyste dragen bij aan een vijandig milieu waarin geen regeneratie van axonen plaatsvindt. Er is vooralsnog geen therapie beschikbaar die deze verbindingen kan herstellen, waardoor de meeste mensen met een dwarslaesie moeten leven met permanente verlamming.

STAMCELTHERAPIE

In **hoofdstuk 1** wordt in deel 1 een algemene introductie gegeven over stamcellen voor herstel van het centrale zenuwstelsel (CZS). Verschillende typen stamcellen kunnen worden gebruikt als therapie voor CZS schade, elk met speciefieke voor-en nadelen. Embryonale en foetale stamcellen hebben het voordeel van mogelijkheid tot differentiatie tot bijna elke cel in het lichaam en daarmee de potentie om beschadigde/verloren zenuwcellen te vervangen. Nadelen van deze cellen zijn ethische problemen met celoogst en een risico op tumorformatie door ongeremde proliferatie. Volwassen stamcellen en precursorcellen hebben doorgaans minder differentiatiepotentie, maar tonen tegelijkertijd ook minder risico op tumorvorming. De werkzaamheid van deze volwassen stamcellen is gelegen in de afgifte van groeifactoren die een beschermend en/ of proliferatief effect of neuraal weefsel hebben. Een makkelijk te oogsten type volwassen stamcel is de uit het beenmerg afkomstige mesenchymale stamcel, ook wel BeenMerg Stromale Cel (BMSC) genoemd. In deel 2 van de introductie wordt specifiek ingegaan op

deze BMSC's als therapie voor het beschadige ruggenmerg. BMSC's zijn relatief makkelijk te verkrijgen uit volwassen beenmerg en eenvoudig te kweken in groeimedium. Zij geven diverse groeihormonen af, waaronder neurotrofe factoren, en hebben na transplantatie in het beschadigde ruggenmerg een beschermend effect op neuraal weefsel in ratmodellen van dwarslaesie. Dit proefschrift heeft twee hoofddoelstellingen: (1) om onze kennis over BMSC therapie voor herstel van het ruggenmerg te vergroten en (2) om middelen te vinden om de werkzaamheid van intraspinale BMSC transplantaten te vergroten. De experimenten in dit proefschrift maakten gebruik van een in vivo rattenmodel, waarbij na laminectomie van thoracaal segment T9, met behulp van een geautomatiseerde impactor een contusielaesie wordt aangebracht. Dit resulteert in paralyse van achterpoten en staart. Histologisch leidt de impact tot schade aan neuronen, axonen, gliacellen en bloedvaten en tot influx van ontstekingscellen, wat bijdraagt aan progressieve secundaire weefselschade. Dit model bootst de consequenties van ruggenmergletsel in mensen na.

BMSC-GEMEDIEERDE NEUROPROTECTIE EN HERSTEL

In **hoofdstuk 2** is gekeken of de door BMSC-gemedieerde neuroprotectie een positief effect heeft op herstel van motoriek en sensibiliteit na transplantatie in een ratmodel van dwarslaesie. Het verband tussen BMSC-gemedieerde weefselsparing en verschillende facetten van functie werd onderzocht. De resultaten toonden dat BMSC transplantatie een positief effect heeft op verschillende parameters voor (sensori)motoriek (BBB-subscore, voetprintanalyse, vaardigheid op een horizontale ladder) en sensibiliteit (thermische hyperalgesie en mechanische allodynie) en dat deze effecten gecorreleerd zijn aan het gespaarde weefselvolume. Daarnaast vonden we dat BMSC-getransplanteerde segmenten meer bloedvaten bevatten dan controles, wat mogelijk een bijdrage heeft geleverd aan de grotere volumes gespaard weefsel. Ook bleek dat ratten met BMSC meer axonen hadden afkomstig van de raphe kernen in de herstenstam, wat mogelijk een bijdrage heeft geleverd aan de verbeterde locomotie.

DE ROL VAN BDNF IN BMSC-GEMEDIEERDE NEUROPROTECTIE

In **hoofdstuk 3** werd de rol onderzocht die Brain-derived neurotrophic factor (BDNF), een van de neurotrofe factoren die BMSC uitscheidt, speelt in BMSC-gemedieerde neuroprotectie. Hiertoe werd de expressie van BDNF in BMSC door middel van lentivirale factoren verlaagd en verhoogd. *In vitro* bleek BDNF geen noodzakelijke factor voor het geobserveerde beschermende effect op motoneuronen in de ventrale horn; ook BMSC zonder BDNF (BMSC-shRNA-BDNF) hadden een gunstig effect op neurale overleving, mogelijk door het compensatoire effect van andere uitgescheide groeifactoren. Echter, overexpressie van BDNF (BMSC-BDNF) resulteerde wel in een geprolongeerd effect op motoneuron overleving dat met BMSC en BMSC-shRNA-BDNF niet werd gezien. Ook *in vivo* bleek overexpressie van BDNF voordelig; meer motoneuronen overleefden in de ventrale hoorn na BMSC-BDNF therapie, een effect dat met BMSC en BMSC-shRNA-BDNF niet werd gezien. Ook werden meer bloedvaten gevonden in de grijze stof van deze BMSC-BDNF behandelde ratten, wat mogelijk een mediator is geweest voor de verbeterde motoneuron overleving. Verder bleek BDNF expressie noodzakelijk voor BMSC overleving *in vivo*. BMSC-shRNA-BDNF overleefden de eerste week na transplantatie niet, wat mogelijk het gebrek aan een weefselsparend effect in deze ratten verklaart.

OVERLEVING BMSC

De overleving van BMSC's in het beschadigde ruggemerg is matig en beperkt de therapeutische werking. Een week na transplantie overleeft ongeveer 20% van de getransplanteerde cellen. Verschillende factoren dragen mogelijk bij aan deze matige overleving van BMSC's, waaronder fagocytose door macrofagen, gebrek aan voedingsstoffen en zuurstof door gescheurde bloedvaten en de aanwezigheid van reactieve zuurstofverbindingen en andere toxische moleculen ter plaatse van het letsel. In **hoofdstuk 4** werd gekeken of de overleving van BMSC's kon worden verbeterd door onderdrukking van de ontstekingsreactie. Drie klinisch beschikbare ontstekingsremmers, Minocycline, Methylprednisolon en Cyclosporine, werden onderzocht op hun vermogen om het aantal macrofagen in het beschadigde ruggenmerg te onderdrukken en zodoende

de overleving van BMSC's te verbeteren. Alle drie medicijnen waren effectief in het remmen van de macrofaagrespons, maar we detecteerden geen verbeterde overleving van BMSC's.

In **hoofdstuk 5** werd onderzocht of de overleving van BMSC's kon worden verbeterd door de cellen te transplanteren in de thermaal reversibele biogel poly(ethylene glycol) - poly(serinol hexamethylene urethane), ofwel ESHU, een gel met anti-oxidatieve eigenschappen. BMSC's overleefden de eerste week na transplantatie beter wanneer de cellen getransplanteerd werden in ESHU en deze verbeterde overleving ging gepaard met meer weefselsparing en verbeterd motorisch herstel. Een mogelijke verklaring voor de verbeterde BMSC overleving is het anti-oxidatieve effect van het poly-urethaan element van ESHU. Het anti-oxidatieve effect werd bevestigd *in vitro*.

CONCLUSIE

BMSC transplantatie heeft positieve histologische effecten, geassocieerd met verbeterd herstel van motorische en sensibele functies in een rattenmodel van dwarslaesie. Sommige van deze effecten kunnen verder verbeterd worden door upregelatie van BDNF in BMSC's. Korte termijn overleving van BMSC kan verbeterd worden door de cellen te transplanteren in ESHU, en deze verbeterde overleving gaat gepaard met meer weefselsparing en functioneel herstel, wat impliceert dat celoverleving een determinant is voor de werkzaamheid van BMSC transplantaten. Toekomstig onderzoek zal zich moeten richten op het combineren van de neuroprotectieve BMSC therapie met axon regenererende therapieen om BMSC-gebaseerde therapieen voor dwarslaesies verder te verbeteren.

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CURRICULUM VITAE

Gaby Ritfeld was born on February 8th, 1985 in Paramaribo, Suriname. She attended pre-university secondary education at Oranje Nassau College in Zoetermeer, and was also an exchange student at E.L. Crossley Secondary School in Fonthill, Canada for one semester. She graduated *Atheneum* in 2002 and received an award from the Dutch Classical Association for her outstanding studies of the classical languages. In 2002 en 2003, she volunteered for Teaching and Projects Abroad in Mexico, Guadalajara, teaching English at a secondary school and providing child care in an orphanage. In 2003, she started to study medicine at Leiden University Medical Center (LUMC) and completed her 'propedeuse' *cum laude* in 2004. In 2005, she was selected for the LUMC Excellent Student Program, and started working in the laboratory of dr. Ron de Kloet on a project that involved predicting cortisol responsive genes in the brain. During her studies she was an active badminton player, assistant trainer and member of the board and several committees of her badminton club. In 2007 she was invited to work in Dr. Martin Oudega's Laboratory at The Johns Hopkins University in Baltimore, where she started her research on Bone Marrow Stromal Cell treatment for spinal cord repair. Her research was nominated for best science internship by LUMC. She completed her 'doctoraal' medicine *cum laude* and graduated medical school in October 2009. Gaby was selected to continue her research on spinal cord injury within the LUMC MD/PhD Program for Excellent Students, and moved back to the United States in 2009 to further her research with Dr. Oudega at the University of Pittsburgh, under LUMC supervision of Prof. Dr. Raymund Roos. In 2012 she returned to the Netherlands to start her neurology residency at LUMC.