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Collagen Scaffolds Populated with Human Marrow Stromal Cells Reduce Lesion Volume and Improve Functional Outcome after Traumatic Brain Injury

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Abstract

Objective—Traumatic brain injury (TBI) causes extensive loss of cerebral parenchyma; however, no strategy for reconstruction has been clinically effective. We and others have employed human marrow stromal cells (hMSCs) to treat rats subjected to TBI and found no significant changes in the lesion volume, although functional outcome was improved significantly. To identify new ways of delivering hMSCs into the injured brain and to maximize the therapeutic benefits of hMSC treatment, we investigated the use of collagen scaffolds implanted with hMSCs as a cell delivery system for treatment of TBI.

Methods—Collagen scaffolds populated with 3×10^6 hMSCs were transplanted into the lesion cavity of the injured cortex 4 days after TBI, and the rats were sacrificed 35 days after TBI. Sensorimotor function and spatial learning were measured using an array of function tests, and the brain tissue was processed for histopathology analysis.

Conclusions—The data show that scaffolds populated by hMSCs improve spatial learning and sensorimotor function, reduce the lesion volume, and foster the migration of hMSCs into the lesion boundary zone after TBI in rats. hMSC-populated scaffolds may be a new way to reconstruct injured brain and improve neurological function after TBI.

Keywords

Scaffold; Human marrow stromal cells; Lesion volume; Traumatic brain injury

Introduction

Neuro- and cell transplantation have become widely recognized as powerful experimental tools for studying structure-function relationships, development, neuroplasticity, and regeneration in the adult central nervous system (CNS),²¹ and have recently shown promise in the repair of brain injury and in the restoration of function after traumatic brain injury (TBI).^{11,14} Tissue and cell therapy may have advantages over single pharmacotherapy in that: 1) the grafts may anatomically reconstruct the injured brain; 2) the grafts may establish connections with host cells; 3) exogenous cells entering brain may produce neurotrophins and growth factors^{3,8}; 4)

exogenous cells provoke endogenous cell to express neurotrophins and growth factors⁸; 5) the interaction between exogenous cells and endogenous cells is dynamic and sensitive to the microenvironment; 6) exogenous cells target the injured area⁵ and 7) transplantation may also activate latent or silent pathways that exist in brain or promote newly formed pathways after injury, and thereafter benefit functional improvement.²³ Our group has employed human bone marrow stromal cells (hMSCs) to treat TBI and promising results have been achieved.^{14–16}

We have employed three different routes (i.e., intraarterial, intravenous and intracranial) to administer hMSCs into injured brain.^{10,15,16} However, problems persist (e.g. relatively few hMSCs (less 1×10^6) can be injected intracranially,¹⁰ intraarterial injection of hMSCs can cause brain ischemia,¹⁶ and intravenous injection results in body-wide distribution of hMSCs^{14,15}). Although hMSC transplantation after TBI reduces neurological functional deficits, the lesion volume of cortex in the controlled cortical impact model employed is not significantly reduced.^{2,10,17} Therefore, in an effort to optimize hMSC therapy of TBI, we tested the use of hMSC-impregnated collagen scaffolds as hMSC delivery vehicles. The engineered scaffolds have been used for delivery of neural stem cells after ischemia²⁰ and for treating spinal cord injury²; however to date, this approach has not been utilized for the delivery of hMSCs within TBI sites. We have previously demonstrated the efficacy of silk fibroin scaffolds in the delivery of hMSCs for repair of various skeletal tissues including bone, cartilage, and ligament tissue both *in vitro* and *in vivo*.^{1,6,7,17–19,22} We hypothesize that the scaffolds provide an effective platform for therapeutic delivery of hMSCs to injured tissue, likely due to enhanced anchoring, which leads to improved cellular survival and overall function for the intended repair.

Materials and Methods

All experimental procedures have been approved by the IACUC of Henry Ford Hospital.

Animal Models

A controlled cortical impact model of TBI in the rat was utilized for the present study.^{4,14} Male Wistar rats (300–400 g) were anesthetized intraperitoneally with 350 mg/kg/body weight chloral hydrate. Rectal temperature was kept at 37°C with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce the injury. Rats were placed in a stereotactic frame. Two 10-mm diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The second craniotomy allowed for movement of cortical tissue laterally. The dura was kept intact over the cortex. Injury was induced by impacting the left cortex (ipsilateral cortex) with a pneumatic piston containing a 6-mm diameter tip at a rate of 4 m/s and 2.5 mm of compression. Velocity was measured with a linear velocity displacement transducer.

Transplantation

Scaffolds were seeded with 3×10^6 hMSCs and transplanted into the lesion cavity of rats 4 days after TBI. Under aseptic conditions and general anesthesia with ketamine (40 mg/kg) and xylazine (8 mg/kg), a 1-cm incision was made along the middle line of the scalp. The lesion cavity induced by TBI was exposed in the left hemisphere. A scaffold seeded with hMSCs was placed directly into the cavity without removal of additional brain tissue, and subsequently covered by surgical foam (polyurethane foam), and the incision was closed with 4-0 absorbable gut surgical suture. The animals were sacrificed 35 days after TBI.

Experimental Groups

Twenty-four male Wistar rats were randomly divided into four groups with 6 rats per group (i.e. TBI + saline, TBI + scaffold, TBI + hMSCs and TBI + scaffold/hMSCs). All treatments

were performed four days after TBI. Rats in the TBI + saline group were subjected to TBI and injected intracranially with saline. Rats in the TBI + scaffold group were subjected to TBI, and four days later, a scaffold was directly transplanted into the lesion core. Rats in the TBI + hMSCs group received 3×10^6 hMSCs injected directly into the lesion core 4 days after TBI. Rats in the TBI + scaffold/hMSCs group received transplantation of a scaffold seeded with 3×10^6 hMSCs. All rats were tested on the Morris Water Maze (MWM) test during the last five days before sacrifice and the neurological severity scores (NSS) were measured before and after TBI at different time points.

Cells

hMSCs were isolated and expanded, as previously described.¹ Briefly, human bone marrow aspirates obtained from a 25-year-old, consenting, non-smoking donor (Clonetics-Poietics, Walkersville, MD) were resuspended in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 100 U/ml penicillin and streptomycin, and 1 ng/ml basic fibroblast growth factor (bFGF), plated at $10 \mu\text{l}$ aspirate/ cm^2 in tissue culture flasks, and maintained at 37°C in an atmosphere of 95% air and 5% carbon dioxide. All tissue culture components were obtained from Life Technologies, Rockville, MD. hMSCs were selected based on their ability to adhere to the tissue culture plastic after approximately 10 days in culture; non-adherent hematopoietic cells were removed during medium replacement. Medium was changed twice per week thereafter. hMSCs were subsequently detached using 0.25% trypsin/1 mM EDTA, replated at 5×10^3 cells/ cm^2 , and cultured to 90% confluency to passage-one cells. hMSCs were subsequently frozen in 10% fetal calf serum (FCS) and 8% dimethyl sulfoxide (DMSO) in DMEM. hMSCs were then thawed and ex vivo expanded to passage-three cells as described above and subsequently utilized for scaffold seeding.

Scaffold Preparation and Cell Seeding

Ultrafoam scaffolds, collagen type I, were obtained from commercial sources (Daval, RI, USA). Scaffolds were pre-wetted overnight at 4°C (approximately 12 hours) in culture medium consisting of DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1 mM nonessential amino acids, and 1 ng/ml of basic fibroblast growth factor (bFGF) (Life Technologies, Rockville, MD), and then aseptically transferred using tweezers (1 scaffold per tube) to a 50-ml sterile centrifuge tube (Falcon) allowing the scaffolds to sit at the bottom of the tube. Following trypsinization of hMSCs from ex vivo expansion conditions, hMSCs were resuspended thoroughly and transferred gently (3×10^6 hMSCs per scaffold) into 200 μl of culture medium. 100 μl of culture medium was then applied two times successively to opposite sides of the body of the cylindrical scaffold. The scaffold and cell solution were incubated for 30 minutes in a humidified incubator to facilitate primary cell seeding. The scaffolds were agitated gently within the solution manually twice every 15 minutes during this time. Following primary seeding, the centrifuge tubes were filled with an additional 3 ml of culture medium and placed within a humidified incubator and incubated overnight until scaffold implantation.

Morris Water Maze Tests

All functional tests were performed by investigators blinded to the treatment. The testing procedure is a modification of that described previously.¹³ Data collection was automated by the HVS Image 2020 Plus Tracking System (US HVS Image, San Diego, CA.). For descriptive data collection, the pool was subdivided into four equal quadrants formed by imaging lines. All animals were tested during the last five days before sacrifice. At the start of a trial, the rat was placed randomly at one of four fixed starting points, randomly facing either toward the wall or the center (designated North, South, East and West) and allowed to swim for 90 seconds

or until they could find the platform. The platform was located in a randomly changing position within the NE quadrant throughout the test period (e.g., sometimes, equidistant from the center and edge of the pool, against the wall, near the center of the pool, and at the edges of the NE quadrant). If the animal was unable to find the platform within 90 sec, the training was terminated and a maximum score of 90 seconds was assigned. The percentage of time traveled within the NE (correct) quadrant was calculated relative to the total amount of time spent swimming before reaching the platform and employed for statistical analysis.

Neurological Functional Evaluation

Neurological functional measurement was performed using a modified Neurological Severity Scores (mNSS).¹² The test was carried out on all rats preinjury and on days 1, 7, 14 and 35 after TBI. The mNSS is a composite of the motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive) and reflex tests. This test has been employed in previous studies. Motor tests of the mNSS include seven items with a maximum of 6 points, which mainly reflect the function of the motor representation area in the contralateral cortex. Damage to this area causes contralateral limb paralysis, leading to high scores on the mNSS motor tests. Sensory tests include two items with a maximum score of 2, reflecting a combination of visual, tactile and deep sensation. Unilateral lesion of the sensory and motor representations of the forelimb in the somatosensory cortex can produce contralateral asymmetry. Indeed, the placing test included in sensory tests of the mNSS also reflects an aspect of the motor function, because the corticospinal pathway mediates the execution of the placing reaction²³ and their lesions produce an enduring forelimb placing deficit. Beam Balance Tests, as part of the asymmetry test, have seven items with a maximum score of 6, mainly reflecting hindlimb placing performance, which is controlled by the contralateral cortical representation of motor function. Damage to this area causes dragging of the contralateral hindlimb (the hindlimb is not placed on the beam), or the hindlimb is placed on the vertical surface of the beam to help support the animal's weight and to aid in maintaining balance, which results in a high score on Beam Balance Tests. The last part of the mNSS includes reflexes such as pinna, corneal and startle reflexes, and abnormal movements. In this model, injury in the left hemisphere cortex of rats causes sensory and motor functional deficiency with elevated scores on motor, sensory, and Beam Balance Tests in the early phase after injury (day 1 after injury). Absent reflexes and abnormal movements can be measured on rats with severe injury. Slow recovery in asymmetry deficiency as reflected by Beam Balance Test results has been reported in unilateral brain injuries including brain trauma, brain hemorrhage and ischemia. This test is suitable for evaluating long-term neurological functional after unilateral brain injury.

Tissue preparation and measurement of lesion volume

Rats were anesthetized intraperitoneally with ketamine and xylazine, and perfused transcardially first with saline solution containing heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Their brains were removed, post-fixed in 10% formalin for 1–2 d at room temperature, and then processed for paraffin sectioning. A series of 6- μ m thick sections were cut with a microtome through each seven standard sections. One section from each block was processed for hematoxylin and eosin staining, and then used for calculation of lesion volume using a MCID system.⁹

Immunohistochemistry staining

Single staining was performed for identification of hMSCs using a primary mouse anti-human nuclei monoclonal antibody (MAB1281), specific for all human cell types, and a secondary Cy5-conjugated F(ab')₂ Fragment rabbit anti-mouse IgG in the coronal sections of brains. Briefly, 6- μ m thick sections from the TBI + hMSCs and TBI + scaffolds/hMSC groups were deparaffinized and the sections were put in boiling citrate buffer (pH = 6) in a microwave oven

for 10 minutes for identification of neurons. After cooling at room temperature, the sections were incubated with a primary mouse anti-human nuclei monoclonal antibody (a human cellular nuclei antigen, MAB1281, dilution, 1: 200) and a Cy5-conjugated F(ab')₂ fragment mouse anti-human IgG (dilution, 1: 20). The slides were analyzed using a fluorescent microscope (Olympus, BH-2). Negative control sections from each animal received identical staining preparation, except that the primary or secondary antibodies were omitted.

Estimates of Cell Number

For measurement of MAB 1281 reactive cells, an average number of five equally spaced slides (approximately 100- μ m interval) were obtained from brain block E, and MAB 1281 reactive cells were counted within the boundary zone. To reduce biases introduced by sampling parameters, all sections for MAB 1281 identification were stained simultaneously. The criteria for MAB 1281 positive cells were defined before the cells were counted by observers blinded to the individual treatment. To test the effect of the implant on the hippocampus, the density (cells/mm) of granular cells in the dentate gyrus and pyramidal cells in the CA3 region was calculated on the H&E stained slides.

Results

hMSC-populated scaffolds improve spatial learning

To test whether scaffold/hMSC treatment promotes spatial learning in the relatively late phase (approximately 1 month) after TBI, rats were tested on the MWM during the last five days (from day 31 to day 35 after TBI) before sacrifice. The mean percentages of time spent in the correct quadrant were significantly higher in the hMSC-treated group (TBI + hMSC) than in the saline-treated (TBI + saline) and scaffold-treated (TBI + scaffold) group at days 34 and 35 after TBI ($p < 0.05$, Figure 1). However, treatment with scaffold/hMSCs significantly improved spatial learning as early as 33 days after TBI and the improvement continued at days 34 and 35. In addition, the percentage of time spent in the correct quadrant was significantly higher than that of the TBI + hMSCs group ($p < 0.05$) (Figure 1). These data demonstrate that both hMSCs and scaffold/hMSCs promote restoration of spatial learning in the relatively late phase after TBI. However, scaffold/hMSCs appear to be more effective in improving spatial learning therapy than hMSCs only.

hMSC-populated scaffolds improve sensorimotor functional outcome after TBI

Figure 2 shows that there is no significant difference in the mNSS scores between TBI + scaffold and TBI + saline groups ($p < 0.05$). However, significantly improved scores were measured at days 7, 14 and 35 after TBI in both TBI + hMSCs and TBI + scaffold/hMSC groups, compared with TBI + saline and TBI + scaffold groups ($p < 0.05$). At days 14 and 35, significantly lower (i.e., improved) scores were measured in the TBI + scaffold/hMSC group versus TBI + hMSC group ($p < 0.05$). These data demonstrate that both hMSCs and hMSC-populated scaffolds promote recovery of sensorimotor functional deficits, and scaffold/hMSC treatment is superior to hMSCs only.

hMSC-populated scaffolds reduce the lesion volume and neuronal loss after TBI

No scaffolds were found in the cavity of injured cortex, demonstrating the degradation of the scaffold in the brain. There were no significant differences in lesion volume among TBI + MSC ($16.2 \pm 1.4\%$), TBI + scaffold ($17.7 \pm 2.1\%$) and TBI + saline ($18.1 \pm 2.3\%$) groups ($p < 0.05$) (Figures 3 and 4). Treatment with scaffold/hMSCs significantly reduced lesion volume to $11 \pm 1.6\%$ when compared to the other three groups ($p < 0.05$). Figure 4 also shows that scaffold/hMSC treatment reduces the tissue loss in the injured cortex. These data demonstrate that seeding scaffolds with hMSCs reduces lesion volume of the injured cortex after TBI. The

table shows that hMSC transplantation and combination of scaffold and hMSCs increase cell densities in the dentate gyrus and hippocampal CA3 region, with the combination treatment showing more efficacy in protecting neurons in the hippocampus after TBI than hMSC treatment alone.

hMSCs migrate into the lesion boundary zone from scaffold

Since no MAB 1281 positive cells were detected in the lesion boundary zone in both TBI + scaffold and TBI + saline groups, we only counted MAB 1281 positive cells in the lesion boundary zone in the TBI + hMSC and the TBI + scaffold/hMSC groups. Some scattered MAB 1281 positive cells were detected in other areas of the ipsilateral cortex, but we focused on the lesion boundary zone. Figures 5 and 6 show MAB 1281 positive cells in the lesion boundary zone in the TBI + MSC and the TBI + scaffold/hMSC groups. A significantly increased number of MAB 1281 positive cells were present in the TBI + scaffold/hMSC group ($14.3 \pm 2.1/\text{mm}^2$) compared to the TBI + hMSC group ($4 \pm 1.3/\text{mm}^2$) ($p < 0.05$). These data demonstrate that scaffolds enhance the presence of hMSCs in the lesion boundary zone.

Discussion

Our data demonstrate that scaffolds suffused with hMSCs improve spatial learning and sensorimotor function, and reduce the lesion volume as well as increase the numbers of hMSCs into the lesion boundary zone after TBI compared to saline-, scaffold- and hMSC-treated rats.

Our previous studies have shown that intracranial transplantation of 1×10^6 hMSCs was an effective treatment for TBI.¹⁶ However, there was no significant change of the lesion volume with intracranial, or intravenous administration of hMSCs compared with controls.^{15,16} Since hMSCs were injected in the lesion boundary zone, a limited volume of solution (10 μl) was used to dilute the hMSCs, which restricts the use of this approach. Large numbers of hMSCs (from 2×10^6 to 8×10^6) have been intravenously injected; however, hMSCs have been detected in almost all organs in the recipient rats.¹⁵ In the present study the scaffold was seeded with 3×10^6 hMSCs, three times more than the single injection of intracranial hMSCs, thereby increasing the magnitude of hMSC delivery to the TBI defect site.

This is the first study to find that transplantation of scaffolds populated with hMSCs reduces deficits of spatial learning and sensorimotor function after TBI. However, transplantation of scaffolds alone has no significant effect on functional outcome when compared with saline injection. Although hMSC treatment significantly improves the functional outcome compared to controls, the effect is reduced compared to that of the scaffolds populated with hMSCs, demonstrating that transplantation of scaffolds populated with hMSCs may be an effective cell delivery vehicle for restoration and repair of neural injury after TBI. Mechanically engineered hydrogel scaffolds without cells have been used for treatment of spinal cord injury in the rat and these scaffolds promote axonal growth and angiogenesis.² However, there are no current data relating to whether this treatment promotes functional recovery. A synthetic biodegradable polymer (polyglycolic acid) has been employed to recruit neural stem cells and to reconstitute lost tissue caused by hypoxia-ischemia in mice.²⁰ These neural stem cell-scaffold complexes appeared to augment the reparative response by facilitating a series of reciprocal interactions between donor neural stem cells and host brain tissue; however, the behavioral responses of the recipients were not provided.

Reduction of the lesion volume by complexes of scaffolds and hMSCs may be mediated by the following mechanisms: 1) hMSCs within the scaffolds may provide neurotrophic support for the surrounding brain tissues, and 2) hMSCs which migrate into the lesion boundary zone may promote remodeling of the injured brain tissue. The scaffolds, by temporally fixing the hMSCs in space and maintaining them in proximity to the injured tissue, likely foster such

constitutive reciprocal interactions between hMSCs and the injured brain tissue. The fate of hMSCs is regulated by multiple interactions with the microenvironment (i.e., neighboring cells, soluble trophic factors and mechanical stimuli).¹¹ hMSCs in brain evoke increased expression of an array of neurotrophic and growth factors, such as NGF, VEGF and BDNF.¹¹ This may be one of the mechanisms by which hMSCs inhibit the progressive chronic processes of cell death, or alternatively, repair the injured cerebral tissue. In addition, these factors are both secreted by hMSCs as well as induced within parenchymal cells by activation of hMSCs' intrinsic neurorestorative mechanisms, which include angiogenesis, neurogenesis and synaptogenesis.¹¹ In turn, the surrounding injured brain tissue generates cytokines that not only stimulate and activate hMSCs to secrete growth factors, but also attract hMSCs to migrate into the lesion boundary zone. This may be one of the reasons that many more hMSCs have been detected in the lesion boundary zone in the TBI + scaffold/hMSC group than in the TBI + hMSC group. Additional studies are required to determine other possible mechanisms underlying the therapeutic benefit of collagen scaffold suffused with hMSCs in the treatment of TBI.

Conclusion

The present study shows that scaffolds impregnated by hMSCs improve spatial learning and sensorimotor functions, reduce lesion volume, and benefit migration of hMSCs into the lesion boundary zone after TBI in rats.

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Spatial learning -Scaffold/ hMSCs-TBI-Rat

Figure 1.

The plot shows deficits of spatial learning of rats from day 31 to 35 after TBI, and after TBI with different treatments. Rats in the saline-treated and scaffold groups after TBI spent less time in the correct quadrant with a maximum of ~30% at the last day of training. Rats in the hMSC- and scaffold-treated groups as well as hMSC-only groups showed significant increases in time spent in the correct quadrant from days 33 to 35 after TBI when compared to the saline- and scaffold-treated groups ($* < 0.05$). However, hMSC- and scaffold-treated rats showed significant increases of the time spent in the correct quadrant at days 34 and 35 when compared to the hMSC-only group ($\#p < 0.05$). These data demonstrate that: 1) TBI caused spatial learning deficits which persist to 31–35 days after TBI; and (2) treatment with both hMSCs only and hMSCs combined with scaffold restores spatial learning caused by TBI, with scaffold/hMSCs showing a greater effect than hMSCs only.

mNSS Scores -Scaffold/hMSCs-TBI

Figure 2.

The plot shows the functional improvement detected on the modified neurological severity scores (mNSS). Treatment of TBI rats with hMSCs after TBI significantly promotes improvement of mNSS scores at 7, 14 and 35 days after TBI when compared with the saline- and the scaffold-treated groups (* $p < 0.05$), whereas, treatment with hMSC-impregnated scaffolds shows significantly lower scores than the hMSC-treated group at 14 and 35 days after TBI (# $p < 0.05$). Therefore, hMSC-impregnated scaffolds are superior to hMSCs in the improvement of neurological function after TBI.

Figure 3.

The bar graph shows changes of lesion volume among different groups. Treatment with hMSCs or scaffold alone does not significantly reduce lesion volume compared with treatment with saline in rats subjected to TBI. However, treatment with scaffold/hMSCs significantly reduces lesion volume when compared with hMSC, scaffold and saline treatments, respectively ($p < 0.05$).

Figure 4. Hematoxylin and eosin staining shows the representative sections from TBI + hMSC (a) and TBI + scaffold/hMSC (b) groups. Treatment with scaffold/hMSCs reduces lesion volume and prevents tissue loss in the cortex and hippocampus (b).

MAB 1281 -Scaffold/hMSCs -TBI

Figure 5.

The bar graph shows significantly increased MAB 1281 positive cells detected in the lesion boundary zone in the scaffold/hMSC-treated group versus hMSC-alone treated group (*p, 0.05).

Figure 6. Immunohistochemical staining shows the MAB 1281 positive cells in the lesion boundary zone from hMSC- (a) and scaffold/hMSC- (b) treated groups. Arrows show the positive cells (yellow).