TREATMENT OF TRAUMATIC BRAIN INJURY IN ADULT RATS WITH INTRAVENOUS ADMINISTRATION OF HUMAN BONE MARROW STROMAL CELLS

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Received, September 30, 2002.
Accepted, April 23, 2003.

OBJECTIVE: We investigated the effect of human bone marrow stromal cells (hMSCs) administered intravenously on functional outcome after traumatic brain injury in adult rats.

METHODS: hMSCs were harvested from three human donors. A controlled cortical impact was delivered to 27 adult male rats to induce traumatic brain injury, and 24 hours after injury, hMSCs were injected into the tail veins of the rats (n = 18). These rats were divided into two groups: Group 1 was administered 1×10^6 hMSCs, and Group 2 was administered 2×10^6 hMSCs. Group 3 (control) rats received saline intravenously. Neurological function was evaluated according to the rotarod test and modified neurological severity score. All rats were killed 1 month after injury, and immunohistochemical staining was performed on the brain sections to identify donor hMSCs. To study the phenotypic differentiation of hMSCs, coronal brain sections were stained for neuronal (Tuj1) and astrocytic (glial fibrillary acidic protein) markers.

RESULTS: Treatment with 2×10^6 hMSCs significantly improved the rats' functional outcomes ($P < 0.05$). The transplanted cells successfully migrated into injured brain and were preferentially localized around the injury site. Some of the donor cells also expressed the neuronal and astrocytic markers.

CONCLUSION: These data suggest that hMSCs may be a potential therapy for patients who have sustained traumatic brain injuries.

KEY WORDS: Human marrow stromal cells, Neural transplantation, Traumatic brain injury

Neurosurgery 53:697-703, 2003 DOI: 10.1227/01.NEU.0000079333.61863.AA www.neurosurgery-online.com

This study was designed to study the effects of the intravenous administration of human marrow stromal cells (hMSCs) to improve functional outcome in adult rats after traumatic brain injury (TBI). In previous studies, we demonstrated that intracerebral as well as intravenous administration of rat MSCs improves neurological outcome in rats subjected to TBI (14, 16–18). The present study was performed to test whether hMSCs have a similar beneficial effect in the treatment of TBI. We recently demonstrated that hMSCs provide significant therapeutic benefit to rats subjected to middle cerebral artery occlusion, that these cells apparently do not evoke graft versus host disease, and that they are not rejected (12). Bearing in mind the notion that any improvement in functional outcome produced by the administration of hMSCs may be dose-dependent, we administered two different doses of hMSCs. In addition to following the functional outcomes of the rats, the survival and differentiation of donor hMSCs were evaluated on the basis of immunohistochemical studies.

In recent years, there has been increasing interest in MSCs because they may have multiple potential therapeutic applications. In addition to their ability to support hematopoiesis, MSCs can differentiate into osteocytes, chondrocytes, tenocytes, adipocytes, and smooth muscle cells (4, 5). They also have the ability to secrete an array of growth factors (2, 12), and their potential for central nervous system repair has been recognized (10, 12). This study is the first to investigate the effects of hMSCs on functional outcome in animals after TBI and represents an extension of our research evaluating the potential use of MSCs as a therapy for TBI.

MATERIALS AND METHODS

Preparation of hMSCs

Our Institutional Review Board and Animal Protocol Committee approved the use of hMSCs for the treatment of TBI in rats. hMSCs were harvested from posterior iliac crest of healthy human donors under local anesthesia in the hematology oncology department solely for research purposes. Preparation of hMSCs was performed as described by Digirolamo et al. (6) and Li et al. (12). Briefly, mononuclear cells were plated at a concentration of 1×10^6 cells/75 cm^2 in tissue culture flasks with 20 ml low-glucose Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) and were supplemented with 20% fetal bovine serum (Gibco BRL), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mmol/L L-glutamine. After 72 hours of incubation, nonadherent cells were removed from the cultures, and fresh medium was added to the flasks. The plastic-adherent hMSCs were split on Day 14 and every 7 days thereafter to assess cell growth and cell yield. After harvesting the cells from the flasks and performing centrifugation at 1000 rpm for 10 minutes at 4°C, the supernatant was removed, the cells were washed twice with 0.1 mol/L phosphate-buffered saline (PBS) and diluted in PBS. Thirty microliters of the cell suspension was mixed with 30 μl 0.4% trypan blue stain, and the number of viable cells was counted with a hemacytometer and a counter under a phase contrast microscope. hMSCs suspended in saline were injected into the rats' tail veins.

Animal Models and Injection of hMSCs

Male Wistar rats (n = 27) were anesthetized by intraperitoneal administration of 350 mg/kg body weight chloral hydrate. Their rectal temperature was controlled at 37°C with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce TBI (7). The rats were placed in a stereotactic frame. Two 10-mm diameter craniotomies were performed adjacent to the sagittal suture, midway between lambda and bregma. The second craniotomy allowed for the lateral movement of cortical tissue (19). The dura was kept intact over the cortex. TBI was induced by delivering an impact at a rate of 4 m/s and with 2.5 mm of compression to the left cortex (i.e., the ipsilateral cortex) with a pneumatic piston containing a 6-mm diameter tip. Velocity was measured with a linear velocity displacement transducer (7, 19). The rats were anesthetized with chloral hydrate administered intraperitoneally 24 hours after TBI. The hMSCs suspended in 1 ml saline were injected slowly with a 1-ml syringe into the tail veins of the rats. The control animals received saline only. The rats were divided into three groups:

Group 1 (n = 9): TBI + 1×10^6 hMSCs
Group 2 (n = 9): TBI + 2×10^6 hMSCs
Group 3 (n = 9): TBI + saline

All rats were killed 28 days after TBI was induced.

Immunohistochemical Staining

Single staining on coronal brain sections was performed to identify hMSCs with the use of a primary mouse antihuman nuclei monoclonal antibody (MAB1281), which is specific for all human cell types, and a secondary Cy5-conjugated $F(ab')_2$ fragment rabbit antimouse immunoglobulin G. To confirm the results of this fluorescent staining, 3,3'-diaminobenzidine staining also was performed on the same sections using the avidin-biotin-peroxidase technique. Brain sections were first incubated in a solution containing 2% volume-to-volume hydrogen peroxide and 60% volume-to-volume methanol in PBS to block endogenous peroxidase activity. Then the tissue was incubated with mouse MAB1281 (1:200 dilution) of the primary antibody and subsequently incubated with a biotinylated antimouse immunoglobulin G (1:200; Sigma Chemical Co., St. Louis, MO). Biotin was detected with the avidin-biotin-peroxidase system (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), using 3,3'-diaminobenzidine as the chromogen. The slides were mounted with mount medium and visualized under light microscopy. Double staining also was performed to identify the expression of neuronal and astrocytic markers as described previously (15). For this step, the brain sections were initially stained for the neuronal marker Tuj1 or an astrocytic marker, glial fibrillary acidic protein (GFAP), with the corresponding primary antibodies and the secondary fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment. These sections subsequently were double stained with primary MAB1281 antibody and secondary antibodies of Cy5-conjugated-$F(ab')_2$ fragment for the identification of hMSCs. Briefly, 6-μm-thick sections from TBI-positive saline and TBI-positive hMSC groups were deparaffinized, and the sections were placed into boiling citrate buffer solution (pH 6) in a microwave oven for 10 minutes for the identification of neurons. After the sections were cooled at room temperature, they were incubated in 0.1% saponin PBS at 4°C overnight for monoclonal antibody Tuj1 (dilution 1:400; Sigma Chemical Co.). Antimouse FITC-conjugated $F(ab')_2$ fragment (dilution 1:20; Calbiochem, San Diego, CA) was then added and incubated for 1 week. To identify astrocytes, the sections were treated with 0.1% pepsin at 37°C for 15 minutes and then polyclonal antibody GFAP (dilution 1:400, Dakopatts AB, Stockholm, Sweden) was added on separated sections. The sections were incubated with anti-rabbit FITC-conjugated $F(ab')_2$ fragment (dilution 1:20, Calbiochem, CA) for 1 week. The above sections stained with FITC-conjugated $F(ab')_2$ fragment were subsequently processed for the identification of a human cellular nuclei antigen with a primary mouse antihuman nuclei monoclonal antibody, MAB1281 (dilution, 1:200) and a Cy5-conjugated $F(ab')_2$ fragment rabbit antimouse immunoglobulin G (dilution 1:20). The slides were analyzed under a fluorescent microscope (Olympus BH-2; Olympus America, Long Beach, CA). Because the primary antibodies used against MAB1281 and Tuj1 were monoclonal antibodies, a nonspecific positive reaction could occur in double-labeled staining. Therefore, a series of nega-

tive controls were used to assess and evaluate the immunohistochemical staining results. Negative control sections from each animal underwent identical staining preparation, except that the primary or secondary antibodies were omitted.

Estimates of Cell Number

To measure MAB1281-reactive cells, an average number of five equally spaced slides (at approximately 100-μm intervals) were obtained from each brain block, and MAB1281-reactive cells were counted within the seven 2-mm-thick blocks (labeled A–G) encompassing the forebrain. The percentage of 100 MAB1281-reactive cells colabeled with Tuj1 and GFAP was calculated with the use of fluorescent microscopy within block D. To reduce biases introduced by sampling parameters, all sections obtained from the rats for MAB1281 identification were stained simultaneously. The criteria for defining MAB1281-positive cells were established before the observers blinded to individual treatment counted the cells.

Neurological Function Evaluation

An accelerating rotarod test was performed to measure neurological motor function (9). To obtain baseline values, the rats were tested before TBI was induced. After TBI, the tests were performed on Day 1 (before administration of hMSCs or saline) and then on Days 4, 7, 14, and 28. The motor test data were expressed as percentages of the pre-TBI baseline values. In addition to the rotarod motor test, the rats were evaluated on the basis of modified neurological severity score, which is a composite of motor, sensory, and reflex tests (21). One point is tallied for failure to perform a task, and the maximum score is 14 points. Modified neurological severity scores were obtained before and on Days 1, 4, 7, 14, and 28 after TBI. Observers who were blinded to individual treatment performed all measurements. These tests also were performed in our previous studies (14, 16–18).

Statistical Analysis

We evaluated normality for each outcome of interest. As a result, the generalized estimation equation approach was considered, given a lack of normality in the data. We first tested the dose effect on functional recovery by performing analysis of variance with a critical value of 0.05. If a dose response was detected at the 0.05 level, the pairwise comparisons among different dose groups were then conducted at the 0.05 level.

RESULTS

Distribution of MAB1281-positive Cells

No MAB1281-positive cells were observed in the slides of the TBI-positive saline group, which did not receive the injection of hMSCs. In the brains of rats in the hMSC-treated groups, MAB1281-labeled cells were observed in the boundary zone of the injured area, cortex, striatum, and corpus callosum of the ipsilateral hemisphere (*Fig. 1*). The number of MAB1281-positive cells that migrated into the boundary zone

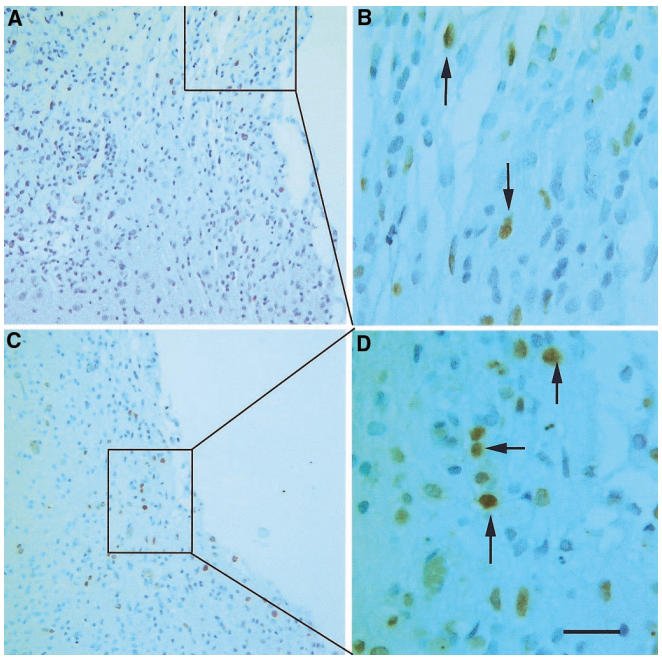

FIGURE 1. *Photomicrographs showing the MAB1281-positive cells in the boundary zone of the lesion area in the rats treated with 1 × 10⁶ hMSCs* (A, B) *and 2 × 10⁶ hMSCs* (C, D). *Injury cavity is visible on the left* (A, C) *with MAB1281-positive cells seen in the boundary area* (inset) *magnified in* B *and* D *to show the cells* (arrow) *more clearly. Sections stained with 3,3′-diaminobenzidine staining technique.* Scale bar *(shown in* D*), 100 μm in* A *and* C *and 50 μm in* B *and* D.

of the ipsilateral hemispheres of the brain was greater in the 2 × 10^6 dose group (*Fig. 2*) than in the 1 × 10^6 dose group (1197 versus 495). The data indicate that hMSCs delivered to brain intravenously selectively migrated into the injured brain tissue in a dose-dependent manner.

Phenotypic Identification of MAB1281-reactive Cells

Double fluorescent staining showed that some MAB1281-positive cells in the brain expressed the immature neuronal marker Tuj1 (*Fig. 3*) and an astrocytic marker, GFAP (*Fig. 4*).

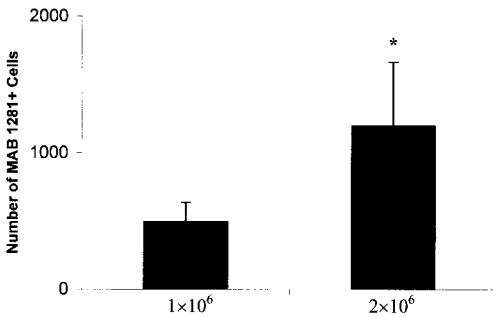

FIGURE 2. *Bar graph showing the number of MAB1281-positive cells in injured brain 28 days after TBI. Significantly more MAB1281-positive cells were measured in the 2 × 10⁶ hMSC-treated group than in the 1 × 10⁶ treated group (* < 0.05).*

The percentage of donor cells expressing these cellular markers was similar in both groups (*Table 1*).

Neurological and Motor Function Evaluation

TBI severity was well balanced before treatment. There was no functional benefit detected with the 1×10^6 dose compared with the control group. A treatment benefit was seen with 2×10^6 hMSCs, with statistically significant improvements in modified neurological severity score observed on Day 7 and in the rotarod test on Day 14. Both tests revealed significant improvement on Day 28 (*Table 2*).

DISCUSSION

Our data show that hMSCs injected intravenously successfully migrate into brain and selectively concentrate around the injury site. Some of the donor hMSCs also express cellular proteins specific for neurons and astrocytes. There was improvement in functional outcome, which was not only maintained but became more significant with time.

This study of hMSCs and previous studies in which rat MSCs were used demonstrate a functional benefit after intracerebral as well as intravenous transplantation of hMSCs (14, 16–18). It is unlikely however, that this finding is due to donor cells replacing damaged neurons. Some of the transplanted cells expressed phenotypic features of neurons and astrocytes, but their number was small, and the probability of their being integrated into functional neural circuitry is low. The functional benefit obtained as a result of hMSC transplantation may be due to the production of neurotrophic growth factors (12). Neurotrophic growth factors are vital for the proliferation and maturation of neurons during embryogenesis. hMSCs can produce growth factors both in vitro and in vivo (2, 12). The expression of several growth factors, including brain-derived neurotrophic factor, nerve growth factor, basic fibroblast growth factor, vascular endothelial growth factor, and hepatocyte growth factor, by hMSCs in vitro was measured by performing enzyme-linked immunosorbent assays with hMSCs cultured alone as well as with TBI tissue extracts of rat brain (2). We found that hMSCs normally secreted brain-derived neurotrophic factor, nerve growth factor, basic fibroblast growth factor, vascular endothelial growth factor and hepatocyte growth factor when cultured in isolation, but this secretion was significantly enhanced when TBI extracts of rat brain were added to the culture media. This finding supports our hypothesis that the functional benefit of hMSC treatment of rats with TBI derives from the induction of neuroprotective factors in the injured brain. Li et al. (12) treated ischemic stroke in rats with intravenous hMSC transplantation and found in vivo production of nerve growth factor and brain-derived neurotrophic factor by hMSCs, which correlated with improvement in functional outcome. Abundant data in the literature suggest that the above-mentioned growth factors, when injected intracerebrally or systemically, can improve outcome in rats after TBI (8, 13, 22, 23); however, these studies have investigated the effects of the exogenous administration of growth factors on neural injury, whereas hMSC transplantation provides an in situ in vivo source of these neurotrophins. This method is better than simple, localized injection of growth factors in many ways, because it provides a distributed, continuous source of neurotrophins. Also, there have been suggestions

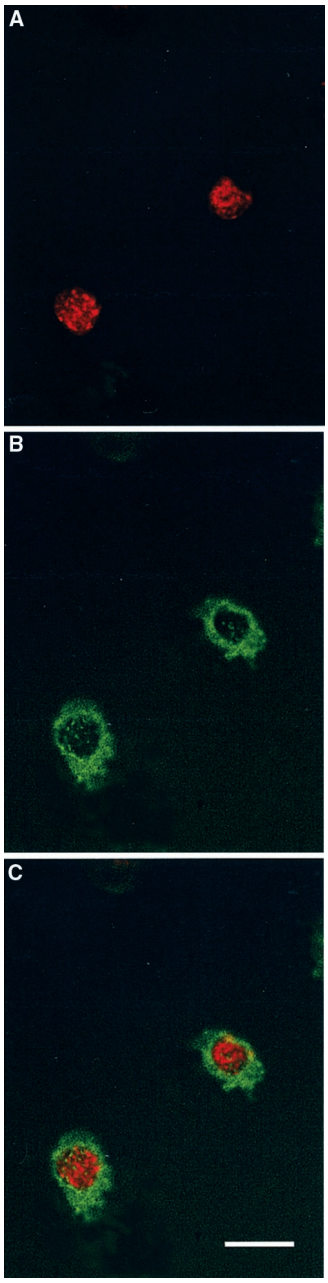

FIGURE 3. *Photomicrographs showing double staining to identify MAB1281-positive cells* (A) *expressing the neuronal marker Tuj1* (B) *in the group treated with* 2×10^6 *hMSCs;* C *is the merged photomicrograph to confirm that* A *and* B *are the same section.* Scale bar *(shown in* C*),* 25 μm *in* A–C.

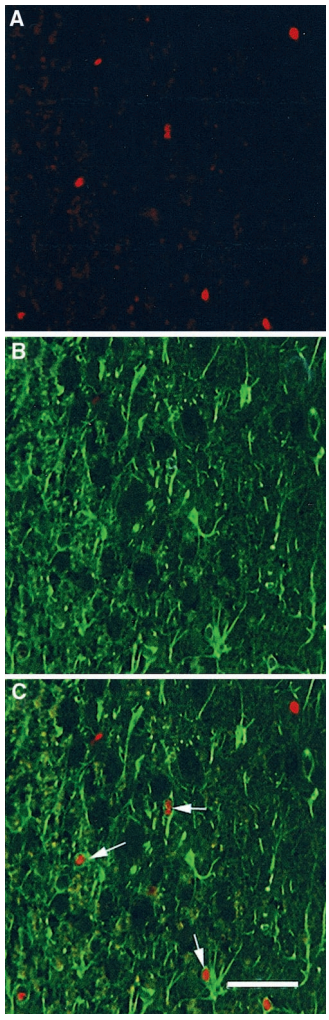

FIGURE 4. *Photomicrographs showing double staining to identify MAB1281-positive cells* (A) *expressing the astrocytic marker GFAP* (B) *in the group treated with* 2×10^6 *hMSCs;* C *is the merged photomicrograph of* A *and* B, *with* arrows *showing these cells.* Scale bar *(shown in* C*),* 75 μm *in* A–C.

TABLE 1. Percentage of Tuj1 and glial fibrillary acidic protein-positive cells among the two groups of rats treated with different doses of human bone marrow stromal cells[a]

Marker	Group 1 (n = 9)	Group 2 (n = 9)
Tuj1/MAB1281	6.0 ± 3.0%	6.3 ± 2.3%
GFAP/MAB1281	12.7 ± 3.5%	13.2 ± 3.8%

[a] GFAP, glial fibrillary acidic protein.

that hMSCs secrete trophic factors that are titrated to the degree of injury and a corresponding disruption of ionic environment (3).

We used hMSCs because the neuroprotective effects of rodent MSCs on neural injury are well documented and because it is important to test whether hMSCs provide therapeutic benefit before they can be used as a potential treatment for TBI in humans. Differences among the donor species regarding their transplantation potential have been recognized, and it has been observed that human fetal brain cells differentiate much more slowly after transplantation compared with fetal rodent brain cells (20); however, in this current study, we found that hMSCs were as effective as the rodent MSCs used in previous studies (14, 16, 18) at improving functional outcome after TBI. Another important question that must be answered in investigations of the administration of hMSCs to treat central nervous system disease is the issue of the number of cells and the optimal dose for transplantation. In the present study, we tested two doses. We found that functional improvement was dose-dependent, with a dose of 2×10^6 cells being significantly more beneficial than 1×10^6 cells. Additional studies are needed to test more dose levels to determine the optimal dose. Also, treatment must be administered at time points other than 24 hours after TBI to determine the therapeutic window available. The control population used in our study consisted of saline-treated rats. A cell control population was not used, because we previously showed that the population of fibroblast cells provides no functional benefit in neural injury models in rats (12).

We are aware of the problem of immunorejection of human cells in rats. There was no significant immune response against hMSCs observed in vivo or in vitro, and such immune rejection interfering with the therapeutic benefit of hMSCs was not a complicating factor. In a related study of hMSCs in cerebral ischemia (12), it was found that intravenous transplantation of hMSCs in rats did not sensitize the rats against hMSCs, as determined by a mixed lymphocyte reaction in vitro. Also, the cytotoxic T cell response that is implicated in graft rejection was not observed after intravenous transplantation of hMSCs in rats subjected to cerebral ischemia. This lack of immune response to hMSCs may be related to the weak immunogenicity of these cells due to the absence or the low expression of major histocompatability complex Classes I and II and costimulatory molecules (CD40, CD80, CD86) (25). There is also the possibility that hMSCs secrete mediators that decrease the immune responses involved in xenograft rejection (1). Although additional studies of the immunogenicity of hMSCs are essential, such cells have been used successfully in rodents (11, 12, 24). hMSCs were shown to decrease myocardial damage in a rat model of cardiac ischemia (11). Zhao et al. (24) transplanted hMSCs into the cortex adjacent

TABLE 2. Neurological functional evaluation results[a]

Days after TBI	Control	1×10^6 hMSCs	2×10^6 hMSCs
Day 1			
MNSS	11.1 ± 1.16	10.7 ± 1.1	11.2 ± 1.3
Rotarod	44.1 ± 16.7	46.7 ± 17.7	42.4 ± 14.0
Day 4			
MNSS	8.7 ± 1.11	8.2 ± 0.9	8.0 ± 1.8
Rotarod	60.8 ± 15.6	66.2 ± 16.8	76.0 ± 23.3
Day 7			
MNSS	6.9 ± 1.3	5.9 ± 1.2	5.2 ± 0.9[b]
Rotarod	97.9 ± 18.8	98.4 ± 20.8	108.0 ± 22.3
Day 14			
MNSS	4.9 ± 1.0	4.6 ± 0.8	3.9 ± 1.2
Rotarod	110.4 ± 14.2	111.4 ± 12.8	128.3 ± 17.8[b,c]
Day 28			
MNSS	4.2 ± 0.6	3.9 ± 0.7	2.3 ± 0.5[b,c]
Rotarod	114.0 ± 10.17	117.2 ± 9.2	136.3 ± 14.7[b,c]

[a] TBI, traumatic brain injury; hMSCs, human bone marrow stromal cells; MNSS, modified neurological severity score.
[b] $P < 0.05$ compared with controls.
[c] $P < 0.05$ compared with 1×10^6 hMSC dose group.

to an area of cerebral infarction and observed improved functional results after transplantation.

Our study clearly shows that the transplantation of hMSCs can be a potential treatment for TBI. This promise is of great significance, because currently no therapy is available to repair biostructural neural damage. hMSCs can be obtained from a patient's own bone marrow, expanded in culture, and used as an autoplastic therapy; however, a patient's own cells may not be able to replicate fast enough to provide enough cells to be used as a treatment in acute stage, even though technology is being developed to amplify the proliferation of hMSCs (10). Also, preliminary data (12) regarding the use of hMSCs to treat stroke and our present work on TBI suggest that allogenic hMSC transplantation is possible. This hypothesis is supported by the observation that hMSCs have a low level of or no major histocompatibility complex receptors (25). Success in the treatment of TBI with hMSC transplantation may eventually be expanded to treat a wide variety of neurological disorders.

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COMMENTS

The authors have performed intravenous transplantation of human bone marrow stromal cells (hMSCs) into rats subjected to percussive traumatic brain injury (TBI). These cells seem to localize preferentially to the site of injury, express phenotypic markers characteristic of neuronal and astrocytic lineages, and promote functional recovery.

The hypothesis, methodology, and results of this study represent a new permutation of investigations previously published by this group using other populations of pluripotent cells (e.g., human umbilical cord cells, rat marrow stromal cells) in various rodent models of central nervous system injury (e.g., stroke, trauma). The demonstrated benefit of hMSCs, which the authors note can lead to autogenous therapies, has more clinical relevance than indicated in the previ-

ous studies. Several issues must be resolved, however, before the promise of these laboratory insights is translated into clinical application. These issues include clarification of the dose-response effect, definition of the therapeutic window, and strategies for permeating the blood-brain barrier and for selective targeting (1). In this study, the breakdown of the blood-brain barrier as a result of TBI probably facilitated the passage of cells from the vascular space into the parenchymal compartment. The fate of these cells that migrated into other organs after intravenous infusion is not reported. Selective intraarterial delivery of genetic and cellular therapeutics is likely to overcome this limitation (1).

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This article concerns the amelioration of functional deficits after TBI in the rat after transplantation of hMSCs. It is a continuation of the innovative approach to neural transplantation and trauma treatment that the authors described previously. In this study, hMSCs instead of murine bone marrow stroma cells were administered intravenously in a head injury model in rats. The injected cells were found in the area of injury, and they improved function within 1 month. These results are intriguing and warrant the continuation of this work. It will be interesting to see the long-term results.

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Mahmood et al. have built on their previous work in investigating the effects of transplanted hMSCs on functional outcome in adult rats with TBI. The hMSCs were harvested from the posterior iliac crests of three human donors and were administered intravenously to adult rats 24 hours after TBI. The outcomes in rats that received 1×10^6 hMSCs did not differ significantly from those of controls on the basis of rotarod test scores and Modified Neurological Severity Scale scores, but rats that received 2×10^6 hMSCs exhibited significantly better results on these tests than either the control group or the group that received 1×10^6 hMSCs. Immunohistochemical analysis revealed that the group that received the higher number of hMSCs had more than twice as many hMSCs in the boundary zone of the injured hemisphere than did the group that received the lower dose of hMSCs.

The major result of this study is that hMSCs injected intravenously seem to be capable of facilitating recovery after TBI. That hMSCs were administered 24 hours after injury suggests that the time window for the administration of this treatment would be clinically feasible. Several important problems must be addressed, however, before this work can be translated to the clinical realm. Perhaps the most obvious of these is the source of donor cells. If hMSCs are to be administered in the acute postinjury period as they were in this study, the use of a patient's own cells probably will not be possible. Another important consideration in the design of a clinical trial is the choice of outcome measures. These need to have direct clinical relevance without being so broad that they become a blueprint for failure.

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Future Meetings—Congress of Neurological Surgeons

The following are the planned sites and dates for future annual meetings of the Congress of Neurological Surgeons:

2003	Denver, CO	October 18–23
2004	San Francisco, CA	October 16–21
2005	Boston, MA	October 8–13
2006	Chicago, IL	October 7–12

Future Meetings—American Association of Neurological Surgeons

The following are the planned sites and dates for future annual meetings of the American Association of Neurological Surgeons:

2004	Orlando, FL	May 1–6
2005	New Orleans, LA	April 16–21
2006	San Francisco, CA	April 22–27
2007	Washington, DC	April 14–19