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Comparative Analysis of Remyelinating Potential of Focal and Intravenous Administration of Autologous Bone Marrow Cells Into the Rat Demyelinated Spinal Cord

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Abstract

The remyelinating potential of autologous bone marrow cells was studied after direct injection and following intravenous injection into rats with a demyelinated lesion in the spinal cord. Both focal and intravenous injections of acutely isolated mononuclear bone marrow cell fractions resulted in varying degrees of remyelination. Suspensions of bone marrow cells collected from the same rat were delivered at varied concentrations (10^2 to 10^5 for direct injection and 10^4 to 10^7 for i.v. injections). The lesions were examined histologically 3 weeks after transplantation. Light microscopic examination revealed remyelination in the dorsal funiculus with both injection protocols, but the extent of remyelination was proportional to the number of injected cells. To attain the same relative density of remyelination achieved by direct injection, intravenous administration of cells required delivery of substantially more cells (two orders of magnitude). However, the availability of autologous bone marrow cells in large number and the potential for systemically delivering cells to target lesion areas without neurosurgical intervention suggest the potential utility of intravenous cell delivery as a prospective therapeutic approach in demyelinating disease.

Keywords

axon; bone marrow; demyelination; regeneration; transplantation

INTRODUCTION

Bone marrow contains a population of pluripotent cells that can differentiate into a variety of cell lineages, including neural cells (Kopen et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Woodbury et al., 2000). Transplantation of an acutely isolated bone marrow mononuclear cell fraction by either direct intraspinal microinjection (Sasaki et al., 2001) or intravenous delivery (Akiyama et al., 2002a) into the demyelinated rat spinal cord results in relatively extensive remyelination. Plastic-adherent cells from bone marrow are referred to as stromal cells (Friedenstein, 1976) and are thought to contain nonhematopoietic stem cells that can differentiate along multiple mesenchymal cell lineages (Prockop, 1997). The mononuclear cell fraction of bone marrow can be readily isolated by density gradient and contains stromal

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cells, hematopoietic and nonhematopoietic stem and precursor cells, and lymphocytes (Azizi et al., 1998; Phinney et al., 1999; Sasaki et al., 2001; Akiyama et al., 2002a). Transplantation of GFP-expressing mouse stromal cells by direct microinjection into the immunosuppressed rat indicates that these cells can form myelin in the demyelinated spinal cord (Akiyama et al., 2002b). When transplanted into a contusive spinal cord injury model, the cells survive and there is significant improvement of gait (Hofstetter et al., 2001). In addition, systemic (i.v.) delivery of the acutely isolated mononuclear bone marrow cell fraction results in remyelination in the adult rat spinal cord (Akiyama et al., 2001).

Bone marrow transplantation has been widely accepted as a powerful practical strategy in the treatment of life-threatening diseases such as leukemia, aplastic anemia, and congenital immunodeficiency (Ikehara, 1998,2001). Precursor cells derived from bone marrow, which could integrate appropriately in the CNS and remyelinate, would be a potentially important cell candidate for transplantation in demyelinating diseases. Although preferential homing of intravenously delivered bone marrow cells to the demyelinated lesion offers the prospect for autologous bone marrow transplantation therapy for the demyelinating diseases, detailed protocol in terms of the administration method and the cell number remains unclear. In the present study, we compared the remyelinating potential of autologous bone marrow transplantation of varying concentrations between direct versus intravenous delivery. The extent of remyelination increased with increasing cell delivery number for both direct and systemic delivery. However, our results indicate that intravenous autologous bone marrow delivery requires significantly more cells for a comparable density of remyelination. These data indicate that significant repair in the EB-X lesion model can be achieved by systemic delivery of cells, thus potentially obviating neurosurgical delivery of cells for cell therapy.

MATERIALS AND METHODS

Induction of Demyelinating Lesions

Adult female Wistar rats were subjected to a standard lumbar spinal cord X-irradiation and ethidium bromide injection (EB-X; Fig. 1A) (Honmou et al., 1996). Briefly, rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) i.p., and a 40 Gray surface dose of X-irradiation was delivered through a 2 × 4 cm opening in a lead shield (4 mm thick) to the spinal cord. The center of the radiation field was positioned just caudal to the 10th thoracic vertebrae (T10) using a Softex M-150 WZ radiotherapy machine (120 kV, 6 mA, 0.5 mm Al filter, SSD 20 cm, dose rate 200 cGy/min). Three days after irradiation, rats were anesthetized as above and sterile laminectomy was performed at T11. The demyelinating lesion was induced by injecting a total of 3 µl of 0.3 mg/ml ethidium bromide (EB) into the dorsal column via a drawn glass micropipette. Three pairs of injections of 0.5 µl each were made at 2 mm intervals along the length of the spinal cord at depths of 0.7 and 0.4 mm. Chari and Blakemore (2002) estimate that repopulation of the EB-X lesion by endogenous progenitor cells within the spinal cord region outside of the irradiation zone can occur at a rate of about 0.5 mm/week during the first month. Our lesion zone induced by EB injections was 6–8 mm in length. Thus, the rostral and caudal margins of the lesions were about 1.6 mm from the edge of the irradiation field, so endogenous progenitors could not reach the lesion zone within the 3-week posttransplantation period.

Preparation of Bone Marrow Cells

Bone marrow was obtained from femoral bones of the demyelinated rats (Fig. 1B). Rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) i.p. A small hole (2 × 3 mm) in the femur was made with an air drill following skin incision (1 cm), and 1 ml of bone marrow was aspirated with a 22 G needle. The samples were diluted in L-15 medium (2 ml) containing 3 ml Ficoll. The cells in the mononuclear layer were collected after centrifugation (2,000 rpm,

for 15 min) and were resuspended in 2 ml serum-free medium [neural progenitor cell maintenance medium (NPMM); Clonetics, San Diego, CA]. After centrifugation (2,000 rpm, for 15 min), the cells were suspended in 1 ml NPMM.

Cell Transplantation

Three days after EB injection, rats were anesthetized as above. The same rats from which bone marrow was obtained were subject later to the demyelinated rats. Autologous bone marrow cells were transplanted into the EB-X rats either locally or intravenously (Fig. 1B). Experiments consist of 10 groups (n = 52). In group 1 (control for i.v. injection), rats were given 1 ml medium alone (without donor cell administration) injected into a left femoral vein (n = 6). In groups 2 to 5, rats were given autologous bone marrow cells of 1×10^4 , 1×10^5 , 1×10^6 , and 1×10^7 , respectively, in 1 ml total fluid volume injected intravenously (n = 5 for each group). In group 6 (control for focal injection), 1 μ l medium alone (without donor cell administration) was directly injected into the middle of the EB-X-induced lesion (n = 6). In groups 7 to 10, suspensions of cells (1×10^2 , 1×10^3 , 1×10^4 , and 1×10^5 cells/ μ l, respectively) in 1 μ l medium was focally injected into the EB-X-induced lesion (n = 5 for each group).

Histological Examination

The rats were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.) and perfused through the heart first with phosphate-buffered solution (PBS) and then with a fixative solution containing 0.5% glutaraldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4. Following in situ fixation for 10 min, the spinal cord was carefully excised, cut into 1 mm segments, and placed into fresh fixative. The tissue was washed several times in Sorensen's buffer, postfixed with 1% OsO₄ for 2 h at 25°C, dehydrated in graded ethanol solutions, passed through propylene oxide, and embedded in EPON. Semithin sections (1 μ m) were cut, counterstained with 0.15% methylene blue, 0.5% azure II, in 0.5% borax, and examined with a light microscope (Olympus BX51).

Both demyelinated and remyelinated lesion areas were measured in the whole dorsal columns near the site of cell injection with Image Analyzer Scion Image Beta 4.0.2. Measurements were obtained from five sections per rat, and five separate rats (n = 5) were analyzed for each experimental condition. The numbers of demyelinated and remyelinated axons were also counted in the whole dorsal columns near the site of cell injection. Counts were obtained from five sections per rat and five separate rats (n = 5) were analyzed for each experimental condition. All variances represent standard error $\pm$ SEM.

RESULTS

The dorsal funiculus of the normal rat spinal cord consists largely of myelinated axons (Fig. 2A and B). To induce a focal region of demyelination, the lumbar spinal cord was first X-irradiated to inhibit mitosis of endogenous glial cells. Subsequently, the central region of the dorsal funiculus was injected with a nucleic acid chelator, ethidium bromide, to kill glia cells, including oligodendrocytes (Honmou et al., 1996). The lesion induced by this procedure is characterized by virtually complete loss of endogenous glial elements (astrocytes and oligodendrocytes) with preservation of axons (Fig. 2C and D). Virtually no endogenous repair by oligodendrocytes or Schwann cells occurs before 6 to 8 weeks. We studied the effects of cell transplantation at about 3 weeks postlesion induction to stay well within the 6–8 week window where endogenous repair does not occur. The higher power light micrograph of the lesion shows compact fields of demyelinated axons in close apposition separated by areas of myelin debris and macrophages (Fig. 2D). The lesion occupies nearly the entire dorsoventral extent of the dorsal columns for 5–7 mm longitudinally. Three days after induction of the EB-X lesion, the autologous bone marrow cell fractions were either microinjected into the dorsal

columns at the center of the lesion through a glass micropipette or injected into the femoral vein. Three weeks after transplantation of bone marrow cells following either direct or i.v. injection, histological analysis was carried out.

Focal injection of 1 μ l medium (without donor cells) into the middle of the EB-X lesion did not promote endogenous remyelination. However, the local transplantation of a suspension of autologous bone marrow cells at a very low concentration (1×10^2 cells/ μ l) in 1 μ l medium exhibited an extremely small but recognizable number of remyelinated axons in the lesion (Figs. 3A and B and 6A). Limited remyelination was also observed very focally near the site of cell injection when cell concentration was increased to 1×10^3 cells/ μ l (Fig. 3C and D). When the cell concentration for direct injection was increased to 1×10^4 cells/ μ l, the remyelinated area expanded from the injected site to the anteroposterior and lateroventral dimension in the demyelinated lesion. A number of presumptive immature myelinated axons with thinner myelin sheaths and promyelinating profiles were observed on the margin of the remyelinated area (Fig. 3E and F). Direct transplantation of 1×10^5 cells/ μ l into the EB-X lesion gave rise to the relatively reliable and extensive remyelination in the lesion zone (Fig. 3G and H). These data are quantitated in Figure 6A.

Intravenous administration of 1×10^4 in 0.5 ml autologous bone marrow cells resulted in minimal remyelination with a few myelin profiles being present in the lesion (Fig. 4A and B), although vehicle alone injected into a vein did not show any evidences of remyelination. When the cell number was increased to 1×10^5 , patchy remyelination was observed in the lesion (Fig. 4C and D). With intravenous delivery of 1×10^6 cells (Fig. 4E and F), more reliable and defined remyelination was observed throughout the lesion. Intravenous transplantation of 1×10^7 cells into EB-X rats resulted in relatively extensive remyelination, which covered over 80% of the demyelinated dorsal funicular lesion zone (Fig. 4G and H). These data are quantitated in Figure 6B. For both direct and systemic delivery, some myelinated axon profiles were associated with large cytoplasmic and nuclear surrounds reminiscent of Schwann cell myelination, e.g., Figures 3H and 4H. We did not characterize these myelinated axons as oligodendrocyte-like or Schwann cell-like by immunostaining in this study. However, previous work has demonstrated both patterns of myelination by bone marrow cell transplantation (Sasaki et al., 2001; Akiyama et al., 2002a, b).

The demyelinated and remyelinated cross-sectional areas following local injection or intravenous administration of autologous bone marrow cells are summarized in Figure 5A and B, respectively. Figure 5C and D represent the calculated percent of remyelination in directly and systemically injected groups, respectively. The numbers of demyelinated and remyelinated axons in the EB-X lesions in each group are displayed in Figure 6. The numbers of the remyelinated axons following bone marrow transplantation were in proportion to those of the transplanted cells in both focally injected and intravenously administrated groups. A similar level of repair was achieved by direct and intravenous delivery, but a significantly larger number of cells (two orders of magnitude) were required for intravenous delivery to achieve comparable repair levels.

DISCUSSION

In the present study, we demonstrated that autologous bone marrow cells isolated by density gradient (mononuclear layer) can remyelinate demyelinated spinal cord axons (EB-X lesion model) after either intravenous administration or direct microinjection into the lesion. The extent of remyelination following intravenous injection of 1×10^7 autologous bone marrow cells was comparable to direct intraspinal injection of 1×10^5 cells, suggesting the feasibility of intravenously transplanted autologous bone marrow cells as a potential cell delivery route.

The lesion model in the present study includes ethidium bromide injection to kill all cells in the lesion zone and focal X-irradiation to block mitosis that delays endogenous repair. Virtually no myelination is observed in the lesion zone for 6–8 weeks, at which time some endogenous myelin repair begins (Honmou et al., 1996). Several lines of evidence indicate that the remyelination was from the transplanted bone marrow cells and not from endogenous host cells. Our studies were carried out within 3 weeks after EB-X lesion induction, which is well within the time at which persistent demyelination is confirmed in a large number of control studies (Blakemore and Crang, 1985; Franklin et al., 1996; Honmou et al., 1996; Kato et al., 2000; Akiyama et al., 2001, 2002a, b; Sasaki et al., 2001). However, the possible facilitation of recruitment of cells outside of the lesion zone from the injection procedure cannot be completely ruled out. Ideally, one would like to use a reliable donor cell-labeling technique to confirm the donor origin of the myelin-forming cells. In this study, we used acutely prepared autologous bone marrow mononuclear cells prepared on density gradient that were not expanded in culture and were therefore not able to incorporate reporter genes into the cells for more definitive assessment of the fate of the injected cells. However, sham injections of medium alone did not result in remyelination. Moreover, the dose-response relationship of myelination with the number of injected cells further suggests that the donor cells were responsible for the remyelination. Future studies with high-efficiency reporter gene incorporation into the endogenous cells will be critical to distinguish between facilitation of endogenous repair vs. homing of injected cells into the lesion. While several lines of evidence suggest that donor cells are indeed responsible for the myelination, we are careful to note that our results do not rule out the possibility of a facilitated endogenous repair mechanism.

Intravenously delivered cells are unlikely to migrate across the blood-brain barrier (BBB) into normal spinal cord tissue, because the BBB would prevent cell access to the parenchyma. Indeed, we did not observe cells in the nonlesion zone after intravenous deliver. Intravenously transplanted bone marrow cells appear to be recruited through the vascular system (Ferrari et al., 1998) and might be allowed to enter the spinal cord lesions because of the partial disruption of BBB in the EB-X lesions. In addition, there may be active targeting mechanisms in the pathological environment of lesion. Expression of chemotactic factors such as monocyte chemoattractant protein 1 is increased in the damaged CNS tissues (Kim, 1996), and injured tissue extract selectively induces chemotaxis of mesenchymal progenitors in vitro (Chen et al., 2001). Adhesion molecules such as intercellular adhesion molecule (Zhang et al., 1995), vascular adhesion molecule 1, and E-selectin are also highly expressed on the endothelial cells in the damaged lesions (Blann et al., 1990; Zhang et al., 1995; Haraldsen et al., 1996; Quesenberry and Becker, 1998). Other unknown molecules may also promote migration of transplanted bone marrow cells into the demyelinated lesions. In addition, migration of bone marrow cells from the hematopoietic environment to the other nonhematopoietic tissues is associated with genetic transformation in part through changes in expression of cell surface adhesion molecules such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and neural cell adhesion molecules (Haraldson et al., 1996; Quesenberry and Becker, 1998; Vermeulen et al., 1998). Thus, the interaction of these molecules may promote the transplanted bone marrow cells to target the spinal cord lesions.

The autologous bone marrow cells injected into the EB-X models rats were acutely prepared from the same demyelinated rats, with cells isolated by centrifugation through a density gradient (Ficoll-Paque: mononuclear cell layer) to remove erythrocytes, platelets, and debris. The recovered mononuclear cell layer consisted of stromal cells, mesenchymal stem cells, hematopoietic and nonhematopoietic stem and precursor cells, and lymphocytes (Azizi et al., 1998). Although the stromal cells and mesenchymal stem cells are highly likely to differentiate into the myelin-forming cells, it still remains unclear which type of cells within the bone marrow cell fraction is responsible for the in vivo differentiation of myelin-forming cells in the present study. Local injection of CD34⁺ cells (hematopoietic stem cells) did not result in

remyelination in our demyelinated model system (Sasaki et al., 2001), suggesting that hematopoietic stem cells are not a candidate cell for remyelination. Bone marrow stromal cells have recently been reported to differentiate into neurons, astrocytes, and myelin-forming cells (Woodbury et al., 2000; Akiyama et al., 2002b), and the stromal or mesenchymal stem cell fraction of the mononuclear cells may be responsible for the myelin repair.

A large proportion of transplanted bone marrow cells differentiated in vivo into myelin-forming cells in the present study, although bone marrow cells predominantly differentiated into astrocytic and neuronal differentiation in normal or ischemic brain (Eglitis et al., 1999). It is well established that stem cells from one region will, when placed to ectopic CNS sites, differentiate with a terminal phenotype appropriate for that ectopic site (Gage et al., 1995; Lundberg and Bjorklund, 1996; Hammang et al., 1997; Snyder et al., 1997; Brustle et al., 1999; Akiyama et al., 2001). The extra-cellular environment into which transplanted stem cells has an important influence on their fate in vivo (Lundberg and Bjorklund, 1996; Hammang et al., 1997; Snyder et al., 1997; Brustle et al., 1999; Akiyama et al., 2001). Pathological CNS tissue is a different environment than intact CNS tissue and markedly alters the terminal differentiated phenotype of transplanted cells (Gage et al., 1995). The lesion into which the cells were placed in the present study was enriched in axons because virtually all glia, including astrocytes and oligodendrocytes, were killed by the lesion protocol (Honmou et al., 1996). This abundance of axon membrane may be an important local environmental signal for bone marrow cell differentiation into myelin-forming cell. In the current demyelinated model, transplanted bone marrow cells retained the capacity to respond to local epigenetic signals in the demyelinated lesions, as they can differentiate into myelin-forming cell phenotypes.

In summary, intravenous delivery of an acutely isolated mononuclear cell fraction of autologous bone marrow cells into the demyelinated model rats resulted in remyelination in a dose-dependent manner with 1×10^7 cells yielding the greatest extent of remyelination. Bone marrow cells are relatively easy to isolate and widely available and currently applied for clinical use for hematopoietic diseases. Future research to determine whether the donor cells are transdifferentiating and forming myelin or if an as yet undefined facilitation of an endogenous repair mechanism is occurring will be important. Moreover, study of more restricted cell types within the mononuclear layer will provide important information on the potential of these cells as candidates for clinical studies in human neurological

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Fig. 1. Schematic representations for the demyelinated model (EB-X). The rats were subjected to a lumbar spinal cord X-irradiation and ethidium bromide (EB) injection (**A**). **B**: Three days after EB injection, autologous bone marrow cells prepared from the femoral bone were transplanted into the EB-X rats either locally or intravenously.

Fig. 2.
Light micrographs of transverse sections through the dorsal column studied at 1 mm intervals through the lesion show normal (**A** and **B**) and demyelinated (**C** and **D**) axons in the dorsal columns. Scale bars: A and C, 250 μ m; B and D, 10 μ m.

Fig. 3.
Light micrographs of the demyelinated lesions of rats given autologous bone marrow cells of 1×10^2 (**A** and **B**), 1×10^3 (**C** and **D**), 1×10^4 (**E** and **F**), 1×10^5 (**G** and **H**) injected focally. Scale bars: A, C, E, and G, 250 μm ; B, D, F, and H, 10 μm .

Fig. 4. Histological analysis of the demyelinated lesions of rats given autologous bone marrow cells of 1×10^4 (**A** and **B**), 1×10^5 (**C** and **D**), 1×10^6 (**E** and **F**), 1×10^7 (**G** and **H**) injected intravenously. Scale bar: A, C, E, and G, 250 μ m; B, D, F, and H, 10 μ m.

Fig. 5. Summary of remyelinated areas in the cross-sections. The lesion area and remyelinated area were simultaneously demonstrated in the directly injected (A) and intravenously transplanted groups (B). The percent remyelinated areas were calculated in the directly injected (C) and intravenously transplanted groups (D).

Fig. 6. Summary of remyelination in the EB-X lesions. Both the demyelinated and remyelinated axons in the lesions were counted in the directly injected (**A**) and intravenously transplanted groups (**B**).