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Research Report

Treatment of rat spinal cord injury with a Rho-kinase inhibitor and bone marrow stromal cell transplantation

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ARTICLE INFO

Article history:
Accepted 25 July 2009
Available online 3 August 2009

Keywords:
Spinal cord injury
Rho-kinase inhibitor
Fasudil
Bone marrow stromal cell (BMSC)
Cell transplantation

ABSTRACT

In light of reports that the administration of fasudil, a Rho-kinase inhibitor, improved rats locomotor abilities following spinal cord injury, we hypothesized that combining fasudil with another type of therapy, such as stem cell transplantation, might further improve the level of locomotor recovery. Bone marrow stromal cells (BMSCs) are readily available for stem cell therapy. In the present study, we examined whether fasudil combined with BMSC transplantation would produce synergistic effects on recovery. Adult female Sprague–Dawley rats were subjected to spinal cord contusion injury at the T10 vertebral level using an IH impactor (200 Kdyn). Immediately after contusion, they were administrated fasudil intrathecally for 4 weeks. GFP rat-derived BMSCs (2.5×10^6) were injected into the lesion site 14 days after contusion. Locomotor recovery was assessed for 9 weeks with BBB scoring. Sensory tests were conducted at 8 weeks. Biotinylated dextran amine (BDA) was injected into the sensory-motor cortex at 9 weeks. In addition to an untreated control group, the study also included a fasudil-only group and a BMSC-only group in order to compare the effects of combined therapy vs. single-agent therapy. Animals were perfused transcardially 11 weeks after contusion, and histological examinations were performed. The combined therapy group showed statistically better locomotor recovery than the untreated control group at 8 and 9 weeks after contusion. Neither of the two single-agent treatments improved open field locomotor function. Sensory tests showed no statistically significant difference by treatment. Histological and immunohistochemical studies provided some supporting evidence for better locomotor recovery following combined therapy. The average area of the cystic cavity was significantly smaller in the fasudil+BMSC group than in the control group. The number of 5-HT nerve fibers was significantly higher in the fasudil+BMSC group than in the control group on the rostral side of the lesion site. BDA-labeled fibers on the caudal side of the lesion epicenter were observed only in the fasudil+BMSC group. On the other hand, only small numbers of GFP-labeled grafted cells remained 9 weeks after transplantation, and these were mainly localized at the site of injection. Double immunofluorescence studies showed no evidence of differentiation of grafted BMSCs into glial cells or neurons. The

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doi:10.1016/j.brainres.2009.07.087

Rho-kinase inhibitor fasudil combined with BMSC transplantation resulted in better locomotor recovery than occurred in the untreated control group. However, the data failed to demonstrate significant synergism from combined therapy compared with the levels of recovery following single-agent treatment.

1. Introduction

Recent efforts to improve treatment for spinal cord injury (SCI) have included stem cell transplantation, drug therapy, and cytokine therapy in various model systems (Ackery et al., 2006; Nishio et al., 2007; Rossignol et al., 2007). However, SCI remains the most devastating type of trauma for patients due to the long lasting disability and the limited response to acute drug administration and efforts at rehabilitation. Although many studies have shown statistically significant recovery following single-agent drug treatment (Baptiste and Fehlings, 2006; Nishio et al., 2007), recovery is generally far from complete. We hypothesized that combined therapies for the appropriate time periods could yield better clinical recovery than a single-agent regimen.

Myelin-associated inhibitors often limit axonal regeneration after SCI (Mueller et al., 2005) as they stimulate intracellular Rho-ROCK kinase activation, leading to neuronal death and growth cone collapse. Targeting Rho-ROCK inhibition is a promising strategy to achieve neuroprotection and axonal regeneration. Fasudil is a ROCK inhibitor, marketed for the treatment of cerebral vasospasm after subarachnoid hemorrhage. Intraperitoneally administered fasudil has significantly improved locomotor recovery after spinal cord injury (Hara et al., 2000). In an experimental spinal cord injury rat study, fasudil significantly improved the BBB score and yielded better outcomes compared with C3 exozyme or Y-27632 (Sung et al., 2003).

Cell transplantation is another approach to neuroprotection and axonal regeneration because it compensates for tissue loss from the SCI. Many cell sources have been used and multiple types of stem cells are now recognized, including the following: ES cells, iPS cells, neural stem cells, adipose tissue-derived stem cells, hematopoietic stem cells, umbilical cord-derived stem cells, and bone marrow-derived stem cells. We previously demonstrated improved locomotion following transplantation of hematopoietic stem cells into mice (Koda et al., 2005; Koshizuka et al., 2004) and bone marrow stromal cell-derived Schwann cells into rats (Kamada et al., 2005).

With respect to stem cells, bone marrow stromal cells (BMSCs) are readily available as they can be collected from the patient and expanded. BMSC transplantation after SCI has resulted in improved locomotor recovery (BBB score) in several previous reports (Chopp et al., 2000; Hofstetter et al., 2002; Ohta et al., 2004; Wu et al., 2003). Some studies have indicated that BMSCs may express trophic factors which improve cell survival (Himes et al., 2006), while others have hypothesized that transplanted BMSCs possess neuroprotective effects (Chen et al., 2005; Chopp and Li, 2002; Neuhuber et al., 2005; Song et al., 2004). Moreover, BMSCs can differentiate to neurons and Schwann cells (Akiyama et al., 2002; Azizi et al., 1998; Chopp and Li, 2002; Deng et al., 2001; Dezawa et al., 2004; Hofstetter et al., 2002; Kim et al., 2002; Kopen et al., 1999; Lee et al., 2004; Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Woodbury et al., 2002). Neurons derived from clonal lines of MSCs have expressed synaptophysin (Woodbury et al., 2002). BMSC-derived neurons have action potentials compatible with those characteristic of functional neurons (Dezawa et al., 2004), suggesting that BMSCs may have the capacity to replace damaged neuronal cells in the spinal cord and form synapses with healthy neurons. On the other hand, transplanted BMSCs do not express neuronal markers (Koda et al., 2005; Lu et al., 2005; Yoshihara et al., 2006), and transplantation does not improve repair or recovery in rats with thoracic contusion injuries (Yoshihara et al., 2006). These discrepancies among spinal cord injury studies will likely require additional studies before they can be resolved.

In the present study, we combined fasudil treatment with BMSC transplantation in a rat spinal cord contusion model. Fasudil administration was started immediately after spinal cord contusion, and BMSCs were injected into the spinal cord 2 weeks later. We assessed the locomotor scale weekly and performed sensory tests 8 weeks after contusion. Histological studies were conducted after 11 weeks to assess tissue preservation and regeneration. In order to detect possible synergistic effects of fasudil and BMSC transplantation, we compared combined (fasudil+BMSC) treatment with single-agent treatments (fasudil alone, BMSCs alone) and with a control group of untreated rats.

2. Results

2.1. *BMSCs showed stem cell-like characteristics in vitro*

Primary cultured BMSCs from GFP-transgenic rats showed fibroblast-like morphology, and their characteristics were maintained over several passages. Because no cell-specific marker for BMSCs has yet been identified, we employed a combination of antibodies in the present study as an alternate approach to detect BMSCs. We based our choice of antibodies on the results of several previous studies (Vimentin and fibronectin, Someya et al., 2008; Nestin, Sauerzweig et al., 2009; CD44, Zhu et al., 2006). Immunocytochemistry showed that primary cultured BMSCs were positive for nestin, fibronectin, vimentin, and CD44 (Fig. 1).

2.2. *Grafted BMSCs survived but did not differentiate into glial cells or neurons in contused spinal cords*

GFP-labeled BMSC grafts survived both in the fasudil+BMSC group and in the BMSC-only group for 9 weeks after transplantation. The majority of the grafted cells remained close to the injection site. The number of donor cells was low in both the fasudil+BMSC group and the BMSC-only group: the mean number of survived transplanted cells was 48.6±44.1 in the Fasudil+BMSC group and 15.4±10.9 in the BMSC-only group. There was no statistically significant difference between these two groups ($p=0.52$). To determine whether grafted rat BMSCs differentiated