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Embryonic stem cells promote motor recovery and affect inflammatory cell infiltration in spinal cord injured mice ☆

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ABSTRACT

The purpose of this study was to determine the fate and the effects of undifferentiated embryonic stem cells (ESCs) in mice after contusive lesion of the spinal cord (SCI). Reproducible traumatic lesion to the cord was performed at T8 level by means of the Infinite Horizon Device, and was followed by intravenous injection of one million of undifferentiated ESCs through the tail vein within 2 h from the lesion.
The ESCs-treated animals showed a significant improvement of the recovery of motor function 28 days after lesion, with an average score of 4.61 ± 0.13 points of the Basso Mouse Scale ($n = 14$), when compared to the average score of vehicle treated mice, 3.58 ± 0.23 ($n = 10$). The number of identified ESCs found at the lesion site was 0.6% of the injected cells at 1 week after transplantation, and further reduced to 0.04% at 1 month. It is, thus, apparent that the promoted hind-limb recovery cannot be correlated to a substitution of the lost tissue performed by the exogenous ESC. The extensive evaluation of production of several neuroprotective and inflammatory cytokines did not reveal any effect by ESC-treatment, but unexpectedly the number of invading macrophages and neutrophils was greatly reduced. This may explain the improved preservation of lesion site ventral myelin, at both 1 week ($29 \pm 11\%$) and 1 month ($106 \pm 14\%$) after injury. No teratoma formation was observed, although an inappropriate colonization of the sacral cord by differentiated nestin- and β-tubulin III-positive ESCs was detected.

Introduction

A mechanical force applied to the spinal cord (SCI) results in a complex damage that causes neurons, astrocytes and oligodendrocytes death. The mechanical injury is followed by a secondary degeneration that is the complex result of a process comprising inflammation, progressive hemorrhagic necrosis, edema, demyelination, excitotoxicity and excessive intracellular accumulation of calcium. This structural destruction proceeds for several days and extends far beyond the original site of the trauma (Bethea, 2000; Gorio et al., 2005, 2007; Gris et al., 2004; Hall, 1992; Popovich and Jones, 2003). Several pharmacological approaches have been tested in the past 20 years, most of them aimed at counteracting specific components of the mechanisms underlying secondary degeneration (Gorio et al., 2002, 2007; Klussmann and Martin-Villalba, 2005; Popovich et al., 1999). Very recently it was also demonstrated that ED-1 cells are directly responsible for retraction of injured axons through physical cell–cell interactions and the depletion of infiltrating macrophages resulted in a significant reduction in axonal retraction (Horn et al., 2008). Several reports have shown that the regenerative capacity of the injured cord may be improved trough the blockade of substances such as chondroitin sulfate and myelin proteins, that inhibit or abort sprouting and regeneration of lesioned axons (Agudo et al., 2008; Barritt et al., 2006; Buss et al., 2005; Fouad et al., 2004; Gonzenbach and Schwab, 2008; Simonen et al., 2003; Tom and Houle, 2008), and by transplanting fetal nervous tissue with the aim of ameliorating the lesion environment (Horvat, 1991; Houle and Reier, 1988; Tessler, 1991).

More recently stem cells have represented an innovative tool (Puceat and Ballis, 2007; Reier, 2004; Schultz, 2005) for neurodegenerative diseases treatments. Stem cells can be divided into two broad categories, ESCs and adult somatic stem cells (Tiedemann et al., 2001; Trounson, 2005; Vats et al., 2005). Different types of stem cells have been implanted in rat and mouse models of spinal cord injury with variable outcomes; some examples include olfactory ensheathing cells (Lavdas et al., 2008; Marshall et al., 2006), spinal cord stem cells (Klein and Svendsen, 2005), bone marrow derived stem cells (Ankeny et al., 2004), dermis derived stem cells

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(Biernaskie et al., 2007; Gorio et al., 2004; Kyung et al., 2007), and neural stem cells (Bottai et al., 2008; Snyder and Macklis, 1995; Snyder et al., 1997). The results of these treatments were quite heterogeneous, even when the same type of cells was used. Overall these numerous reports support the requirement for further investigation to establish fate and effects of stem cell application to the injured spinal cord.

The plasticity and potentiality of ESCs have been known since their discovery in mouse (Evans and Kaufman, 1981; Kleinsmith and Pierce, 1964) and in humans (Thomson et al., 1998), and a whole mouse was generated starting from ESCs (Nagy et al., 1993). Unlike normal somatic cells, ESCs do not undergo senescence and can be grown in virtually unlimited quantities retaining high telomerase activity and normal cell cycle signaling. With proper culture techniques, these cells do not go through the genomic, mitochondrial and epigenetic changes that lead to transformation (Zeng and Rao, 2007). Rodent ESCs can be directed in their differentiation to neuronal (Conti et al., 2005, 2006; Finley et al., 1996) or glial fate (Liu et al., 2000); moreover, ESC-derived motor-neurons can survive, integrate and help restore function following transplantation into paralyzed rats (Deshpande et al., 2006). Recently, ESC derived oligodendrocytes (Sharp and Keirstead, 2007), survived in the injured cord and improved recovery (Hatch et al., 2009). On the other hand, the use of undifferentiated ESCs raises great concerns about the formation of tumors, even when such risk decreases with their progressive cellular differentiation (i.e. reduced multipotency). Transplanted ESCs may form teratomas, which are constituted by cells from endodermal, mesodermal and ectodermal lineages (Nussbaum et al., 2007; Reubinoff et al., 2000).

In this study the acute intravenous (i.v.) administration of undifferentiated mouse ESCs is shown to affect secondary degeneration, to block the invasion of inflammatory cells, to preserve ventral myelin and to promote recovery of function after acute SCI. A colonization of the sacral portion of the cord, where ESCs differentiated in nestin and β-tubulin III-positive cells, was also observed.

Materials and methods

Animals

Adult male CD1 mice (weighing 28–30 g) were used in this study. Animals were housed under standard conditions (22 ± 2 °C, humidity 65%, and artificial light between 08.00 am and 08.00 pm). Food and water were available *ad libitum*.

All animal procedures were approved by the Review Committee of the University of Milan and met the Italian guidelines for laboratory animals, which conform to the European Communities Directive of November 1986 (86/609/EEC).

ESCs culture

The ESCs line was derived from 129/Sv mice as already described elsewhere (Alvarez et al., 2004), and was stably transfected with the pCX-(β-act)-eGFP expression vector (Arnhold et al., 2000). ESCs were passaged every 2 days on gelatin-coated T75 or T175 cm^2 flasks in the presence of Glasgow minimum essential medium (GMEM) (Sigma) supplemented with 0.1 mM non-essential aminoacids (Invitrogen), 40 mM glutamine (Invitrogen), 2 mM sodium pyruvate (Gibco), 0.05 mM 2-mercaptoethanol (Invitrogen), 10% fetal bovine serum (FBS) (PAA) and 10 ng/ml leukemia inhibitory factor (LIF) (Chemicon). The cells were incubated in 5% CO_2 at 37 °C and passaged when confluence was reached (every 3–4 days). At each passage, the cells were detached with 0.1% trypsin, centrifuged at 1000 rpm for 10 min and re-plated at the same density after assessing their viability by Trypan Blue exclusion.

Isolation and culture of fibroblasts

After euthanization by lethal dose of 4% chloral hydrate, fibroblast were isolated from mouse skin and cultivated as described elsewhere (Kim et al., 2005). Briefly, the skin was disinfected with 70% ethanol and the hair was shaved off. The dermis was separated by scratching the outer surface of the skin with a razor blade and washed with PBS. The tissue was minced and digested in PBS containing dispase 1.5 mg/ml (Sigma) and collagenase 0.15 mg/ml (Sigma) at 37 °C until complete disgregation.

The disgregated tissue was then centrifuged at 400 × *g* for 15 min, re-suspended in DMEM containing 10% FBS and incubated at 37 °C with 5% CO_2 until fibroblasts covered the surface of the flask almost completely.

Spinal cord injury and post-surgical care

Moderate spinal cord injury was induced using an Infinite Horizon (IH) Impactor (Precision Systems and Instrumentation). The surgical procedures were performed as described previously (Gorio et al., 2002, 2005). Briefly, mice were anesthetized with 4% chloral hydrate. A laminectomy was performed at T8 level and the exposed dorsal surface of the cord was subjected to a moderate contusion injury with a force of 50 kdyn for 1 s. After injury, the muscles were sutured and the skin was closed with surgical clips. Antibiotics (penicillin/streptomycin, Invitrogen) and lactate Ringer's solution were administered for 7 days. Manual expression of the urinary bladder was performed daily until bladder reflex recover.

Experimental groups

Thirty injured animals were randomly divided into two groups for behavioral analysis, before assessing any motor performance; the first group underwent ESCs i.v. injection, whereas the second group was injected with 100 μl of sterile phosphate buffer saline (PBS). Within those groups three animals (one from group 1 and two from group 2) were discarded the first day after transplantation because they had a BMS score greater than 3 (plantar placement with or without weight support, indicating that the injury was too mild). Within the next week three animals died due to urinary problems (zero from group 1 and three from group 2) and those were discarded from the behavioral analysis. At the end of the behavioral analysis period these mice were processed for immunohistochemical study. The animals that were analyzed 1 week after the transplantation were also divided in two groups of four animal each (ESCs and PBS treated) (one of each group was discarded the first day after transplantation because had a BMS score greater than 3. The operators that assessed the score did not know which kind of treatment had been received by the mouse under analysis. To study cytokine expression with Real-Time PCR, for each time point (1, 2 and 7 days) we randomly choose five mice from each of the following five groups: (1) non-operated animals (control group); (2) animals with T8 laminectomy without injury (sham-operated control group); (3) animals subjected to SCI and injected with 100 μl of sterile PBS; (4) animals subjected to SCI and injected with 1×10^6 fibroblasts (fibroblasts-transplanted control group); and (5) animals subjected to SCI and injected with 1×10^6 ESCs (ESCs-transplanted group). In these groups no animal were discarded because the lesion resulted milder than expected.

Labeling and preparation for transplantation of ESCs

ESCs were labeled with Hoechst 33258 or with Quantum dots (Q-dot), a novel Invitrogen fluorescent marker (Chan et al., 2005; Dahan et al., 2003) or alternatively with green fluorescent protein (GFP) (transduced with GFP containing vector). For Hoechst staining, ESCs were incubated for 30 min at 37 °C with Hoechst 33258 (2 μg/ml final