Research Article

Behavioral Improvement By Mouse Embryonic Stem Cell Transplantation After Spinal Cord Injury In Rats

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Summary

Objectives: We transplanted mouse embryonic stem cells to improve functional loss in a rat model of clip-compression spinal cord injury (SCI). The mouse embryonic stem cells (mESCs) were transplanted to injured cord 7 days after injury.

Methods: We have treated undifferentiated mouse embryonic stem (ES) cells to induce differentiation in vitro into neuron-like cells with good cell viability for use a graft. In this study, we induced spinal cord injury (SCI) in rats using clip-compression, and infused mES cells after SCI. Rats were examined behaviorally using motor and sensory test with neurological assessment.

Results: Motor function of the recipients was gradually improved, whereas little improvement was observed in control rats. This result may suggest that the grafted cells have synaptic connection in the recipient brain. Our study revealed that stem cell transplantation can have a positive effect on behavioral recovery and reduction of infarct size in focal ischemic rats.

Conclusion: We review the application of stem cell transplantation to the spinal cord, emphasizing the use of embryonic stem cells for reconstruction of spinal cord injury. Thus, this study provides strong evidence to support that transplantation of mESC could improve functional recovery after SCI.

Key words: Clip-compression, Functional recovery, Mouse embryonic stem cells, Spinal cord injury, Transplantation

Siçanlarda Spinal Kord Yaralanması Sonrası Fare Embryonik Kök Hücre Transplantasyonu ile Davranışsal Gelişme

Özet

Amaç: Klip-kompresyon spinal kord yaralanması (SKY) siçan modelinde işlevsel kazanım için fare embryonik kök hücre transplantasyonu uyguladı. Fare embryonik kök hücreleri (fEKH) yaralanmadan 7 gün sonra nakledildiler.


Sonuç: Alıcılarda motor işlevler kademeli olarak düzeltken control siçanlarında çok az gelişme gözlandı. Bu sonuçlar aşılanı hücrelerin alıcının beyinde sinaptik bağlantılara olabileceğini düşündürdü. Bu çalışma hücre naklinin davranışsal iyileşmede ve iskemik siçanların infarkt sahalarında azalma ile oluşumu etkilerde bulunabileceğini ortaya çıkardı.

Yargı: Spinal korda kök hücre naklini gecikerek özellikle spinal kord yaralanmasının rekontrüksiyonunda embryonik kök hücrelerin kullanılmasına vurgu yapıldı. Böylece bu...
INTRODUCTION

Numerous pathophysiological mechanisms of the secondary spinal cord injury (SCI) have been postulated. Though the mechanism of this progressive damage is not fully clarified, several attempts have been made to inhibit this phenomenon after lesioning. This study reviews the role of transplantation, focusing on stem cells and peripheral nerve transplantation and transfer. Neural stem/progenitor cells (NSPCs) have previously been identified in both the mammalian brain and spinal cord. They have the ability to self-renew and are multipotential for both neurons and glia. Because of these qualities, they have been useful for repair of the spinal cord by generating new cells and an environment that would promote axonal regeneration. However, this regenerative ability of endogenous stem/progenitor cells in mammals appears to be limited as proliferation and differentiation cease within a few days of trauma, providing only small numbers of new cells. It has been reported that poor NSPC survival, even under optimized conditions for timing and location of transplantation, with large cavities and only a small number of surviving NSPCs located near healthier tissue. Therefore, a potential alternative source of NSCs is from blastocyst-derived cells, which are expanded as totipotent embryonic stem (ES) cells. Induction of ES cells into committed precursors can yield purified populations of NSCs, precursors, or differentiated neural cell types. The resulting stem cells can be expanded in culture as neurospheres which may then be used for transplantation. Grafting of neural differentiated mouse ES cells into a rat thoracic spinal cord clip-compression injury resulted in the survival, migration, differentiation into astrocytes, oligodendrocytes and neurons, and improved locomotor function. The objectives in the present experiments were to examine functional recovery at 35 days after transplantation of spinal cord derived mESCs into the injured adult rat spinal cord (35 g injury) with and without prior transplantation. In the future experiment, cells from transgenic rats expressing the gene for enhanced green fluorescent protein (eGFP) would be used to identify the transplanted cells. Most previous studies agreed that stem cell therapy is an attractive or promising candidate for functional repair in cases of brain damage. Thus, the priming strategy tested in stem cell transplantation after brain ischemia may represent a clinically feasible manipulation of cell preparation for more effective transplantation therapy.

MATERIAL AND METHODS

Cell culture

ES cell cultures were prepared from stocks of an EK1 cell line (TC-1 derived from 129S6) maintained in our laboratory. Not more than 40 passages were used for experiments. The passage procedure of undifferentiated ES cells was performed every 2 days on gelatin-coated T25 flasks in the presence of 1000 U/ml of leukemia inhibitory factor from Chemicon International (LIF) (LIF2010, Temecula, CA) and high-glucose dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, Germany) with 15% FBS (Hyclone), 0.1 mM mercaptoethanol, 1 uM sodium pyruvate, 1x non-essential amino acids and 1 mM L-glutamine (GibcoBRL). Briefly, ES cells were harvested from T25 flasks by trypsinization with 0.25% trypsin and placed into a standard 100-mm bacterial Petri dish in ESIM without adding LIF or β-mercaptoethanol. Medium was removed
and cells were resuspended in modified Sato medium. Cells were then plated on poly-D-lysine (PDL) and laminin coated 35-mm glassbottom dishes for imaging studies or 24-well plates in preparation for serum deprivation (SD) experiments.

We established EGFP labeled mouse embryonic stem cells that could be easily detected by their fluorescence expression in frozen tissue sections. The established embryonic stem cells expressed EGFP even after differentiated into various types of cells. They also could form EGFP expressing transplant cells when they were injected into spinal cord injury region. The injected cells were found mainly in the injury site including injury peripheral area. These cells could be detected by fluorescence microscopy without any treatment on the cells. Accordingly, the embryonic stem cells labeled with EGFP might be very useful in analyzing cells derived from embryonic stem cells in mixed cell culture in vitro, and also very helpful in analyzing transplanted cells derived from differentiated embryonic stem cells.

Animal spinal cord injury model

Male Sprague-Dawley rats were used for the experiments (8 weeks old at time of injury, 200-240g, n=35). This study was approved by the animal care and use committee of Namseoul University in accordance with the policies established in the guide to the care and use of experimental animals of the Canadian council on animal care and use of experimental animal of the American veterinary medical association guideline. The transplant group (n=25) received both SCI and cell transplantation and the transplant control group (n=10) got SCI and PBS injection. However, there was no behavioral change in SCI rats in relation to the injection procedures. Acute clip-compression injury of the thoracic spinal cord was conducted with a 35g clip. After laminectomy, the exposed spinal cord was compressed at the T10 level by a 35-g clip. Cell transplantation was performed 7 days after injury. A small longitudinal incision was made in the exposed dura overlaying the dorsal surface of the spinal cord. A microinjection system was used to transplant 10µl of cell suspension(1x 10^5 cells) into the injury site using a 30-gauge
needle on a 25µl Hamilton syringe held on a micromanipulator.

**Locomotor performance**

In order to explore functional benefits of the transplanted mES cells, we assessed neurological and behavioral activities up to 35 days after transplantation. Locomotor activity was evaluated using the BBB locomotor rating scale for 4 min. Two independent blinded examiners observed and video recorded hind-limb movements and assessed the animal's locomotor function. Motor subscores were also determined according to the method of following locomotor elements independently of forelimb-hindlimb coordination: toe clearance, paw positioning, instability of the trunk and tail position. Ladder-walk analysis with the difficulty of the task was modified by varying the position of the metal rungs. For the regular arrangement, the rungs were spaced at 2cm intervals. For the irregular pattern, the distance of the rungs varied from 1 to 5cm. Five templates of irregular rung patterns were used, so that the same patterns were applied to all animals to standardize the difficulty of the test and enhance comparability of the outcome. Rats were trained for 1 week prior to injury to traverse a horizontal ladder. Recordings were analyzed in slow motion and the number of footfalls for each hind limb was recorded and the average was calculated for each rat, each day.

**Histological Assessment**

All animals were transcardially perfused with 4 % paraformaldehyde in 0.1 M phosphate buffer after being anesthetized. Rats were sacrificed by performing decapitation method in according AVMA guideline. The spinal cord was dissected and postfixed for 24 hrs in the same fixative, then placed in 30 % sucrose PBS solution for another 24hrs. The portions of the spinal cord corresponding to the area of the injury site and transplant region were frozen. 10 µm thick serial longitudinal sections were obtained and stained with hematoxylin and eosin. The entire area of injury was visualized and stained to determine the true epicenter of the injury. The sections at every 30 µm in the rostral and caudal directions of the injury epicenter were examined under at 40x, 100x and 200x magnification using bright field and fluorescence microscopy with stained H-E for a visual analysis of cavity size.

**Statistical Analysis**

The experimental results were expressed as mean±S.E.M. A one-way analysis of variance (ANOVA) was used for multiple comparison followed by Dunnett. Differences with p<0.05 were considered statistically significant.

**RESULTS**

**Histological determination of infarction lesion**

Non-transplant animals with BBB scores less than 7 showed the formation of large cavities (Fig.2). The spinal cords of mESC-transplant animals had cavities much smaller than those of non-transplant animals (Fig.3). These results suggested that mESC-transplant reduced the formation of cavities after injury in the SCI model.

**Motor behavioral index**

Locomotor performance evaluated daily by BBB scoring showed a significant improvement in rats receiving mESCs only (Fig. 4) compared with control group. This improvement, compared with the medium only control group, reached significance at 2 weeks, although an early trend was noted. Locomotor performance was also evaluated daily by motor subscoring. After the transplant of mESC, transplant animals showed a significant functional improvement of BBB scores as compared to non-transplant animals at all time point examined.
**Fig 1:** Differentiation of EK1 cells with EGFP fluorescence expression. All the cells with bright EGFP showed their specific morphology of the differentiated cells at 40th passage. Most of mESCs have appearance with specific morphology of the differentiated cells with projections, round form and polygonal cells. (X 200 magnification). The cells have shown a wider diameter, larger nuclei, and multiple processes.

**Fig 2:** Longitudinal section of non-transplant after spinal cord injury. The host spinal cord tissue was stained hematoxylin & eosin. Non-transplant animals with BBB scores less than 7 showed the formation of large cavities. Large cavitation was seen in the rats receiving PBS compared with rats receiving transplanted cells. A large volume of spinal cord epicenter cavity was shown from non-transplant group.

**Fig 3:** A significant reduction in the percentage cavitation was seen in the rats receiving mESCs compared with rats receiving medium only. The spinal cords of mESC-transplant animals had cavities much smaller than those of non-transplant animals.
DISCUSSION

Stem cell transplantation can restore formation in rodent models of spinal cord injury by stimulating the production of neuronal cells and formation of synaptic plasticity. Several researches reported that transplantation of stem cell into rat spinal cord following experimental injury reduced infarct volume and improved behavioral outcome. Treatment of SCI remains a challenging work in basic and clinical researches. With advances in the pathophysiology of SCI, it is well known that there is almost always a varying amount of residual tissue intact traversing the lesioned segment after SCI. We found that transplanted mESCs produced significant functional improvement after clip-compressive SCI. Indeed, all functional tests showed significant improvement in rats receiving mESCs compared with control group. It is of interest that most studies reporting functional improvement after cell transplantation showed early onset of improvement within the first 2–3 weeks after injury which suggests a neuroprotective rather than a regenerative mechanism.\(^{(2,16,17)}\) However, it is also notable that many transplanted rats had very few transplanted cells surviving after several weeks, suggesting that the cells do not need to survive long term to produce functional recovery.\(^{(5,6,10)}\)

Thus, transplantation of mESCs and earlier cell environment produced a major improvement in function after SCI. It is most likely that these salutary effects are manifestations of neuroprotection, although it is unknown whether this is due to growth factors or other agents elaborated by the transplanted cells. Also, although we may suggest playing in an important role by an early effect of mESCs, this does not rule out a regenerative component to account for further functional recovery. Thus regeneration remains a possibility to be examined in future studies with transplanted spinal cord–derived mESCs.
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