Transplantation of mouse embryonic stem cell after middle cerebral artery occlusion[1]

Transplante de células-tronco embrionárias de camundongo após a oclusão da artéria cerebral média

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ABSTRACT

PURPOSE: Stem cell transplantation has been extensively studied as individual therapies for ischemic stroke. The present investigation is an initial effort to combine these methods to achieve increased therapeutic effects after brain ischemia. Cell transplantation may recover massive neuronal loss by replacing damaged brain cells.

METHODS: Undifferentiated mouse embryonic stem (mES) cells were used to induce differentiation in vitro into neuron-like cells with good cell viability for use a graft. In this study, middle cerebral artery occlusion (MCAO) was induced in rats using intra-luminal vascular occlusion, and infused mES cells after MCAO. The animals 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. Consequently after euthanasia, rats were histochemically investigated to explore graft survival with green fluorescent protein (GFP).

CONCLUSION: The mouse embryonic stem cells may have advantage for use as a donor source in various neurological disorders including motor dysfunction.

Key words: Ischemia. Embryonic Stem Cells. Middle Cerebral Artery. Transplantation. Mice.

RESUMO

OBJETIVO: O transplante de células-tronco tem sido extensivamente estudado como terapias individuais para o AVC isquêmico. A presente investigação é um esforço inicial para combinar estes métodos para alcançar aumento de efeitos terapêuticos após a isquemia cerebral. O transplante de células pode recuperar a perda neuronal intensa, substituindo as células do cérebro danificado.

MÉTODOS: Células tronco embrionárias indiferenciadas de camundongo foram utilizadas para induzir *in vitro* a diferenciação de células como neurônio com boa viabilidade para utilizar como enxerto. Neste estudo foi induzida a oclusão da artéria cerebral média em camundongos, usando a oclusão vascular intraluminal e células embrionárias infundidas. Os animais foram examinados comportamentalmente utilizando motor e teste sensorial com avaliação neurológica.

RESULTADOS: A função motora dos receptores melhorou gradualmente, ao passo que pouca melhora foi observada nos animais controle. Este resultado pode sugerir que as células enxertadas têm conexão sináptica no cérebro receptor. Nosso estudo revelou que o transplante de células-tronco pode ter um efeito positivo na recuperação do comportamento e na redução do tamanho do infarto na isquêmica focal em camundongos. Após a eutanásia foi realizada análise histoquímica para avaliar a sobrevida do enxerto com proteína fluorescente verde (GFP).

CONCLUSÃO: As células embrionárias de camundongo podem ser utilizadas como enxerto em várias desordens neurológicas, incluindo disfunção motora.

Descritores: Isquemia. Células-Tronco Embrionárias. Artéria Cerebral Média. Transplante. Camundongos.

Introduction

Cell therapy using stem cells is awaited by stroke patients with impaired movement and cognitive functions. Middle cerebral artery occlusion (MCAO) induces a massive unilateral loss of neuron, and it causes behavioral dysfunction in rats[1]. It is well known that the degree of disability does not simply reflect the severity or distribution of the impaired blood supply, and populations of adjacent cells in the brain can display dramatically different vulnerabilities to equivalent degrees of ischemia. The continued expansion of ischemic infarction is not caused directly by the reduction in local blood flow, but by secondary processes[2]. The proper process of functional recovery in stroke patients being the main issue, this gradual enlargement of infarction can be limited by a variety of interventions that do not interfere with cerebral blood flow. Therefore, we performed an analysis to investigate the evolution of infarct after MCAO. We performed this analysis in rats, a model system that has been less well characterized. In order to analyze the changes following transient MCAO, several different histochemical methodologies can be utilized. 2,3,5-Triphenyltetrazolium chloride (TTC) is one of the most common histochemical stains used to assess cerebral injury. In ischemic tissue, lack of TTC staining is considered "infarcted" and defined as core and viable tissue is stained red[3]. Although widely accepted and used, TTC staining has received criticism as TTC is a marker of tissue dehydrogenase and mitochondrial dysfunction and may not represent irreversible cell death, therefore this method may overestimate infarct size. Despite this criticism, TTC is still a reliable, rapid, and inexpensive method for analyzing enzymatically dysfunctional cells, most of which will eventually degenerate[4]. We examined the effects of the transplantation of mouse ES cells on behavioral function induced by focal ischemia in rats. Stem cell transplantation has established as a potential effective therapy for CNS disorders such as ischemic stroke and spinal cord injury. Embryonic stem (ES) cells are capable of proliferating and differentiation into neural progenitor cells with the use of induction protocols leading to the development of functionally mature neurons and glial cells[5]. Using stem cells including ES cells as grafts has provided hope for tissue repair and functional restoration after CNS injury. Self-renewing, totipotent embryonic stem (ES) cells may become a virtually unlimited donor source for tissue transplantation[5-6]. ES cells have been shown to differentiate preferentially into neuronal cells, when cultured under the conditions that favor the differentiation, survival and enrichment of neuronal cells[6]. We have focused on determining the appropriate culture condition to induce neural cell differentiation of ES cells with good cell viability. Thereafter, we have transplanted the ES cell derived neuron-like cells into rats with motor cortex injury as recipients. And we have evaluated adaptation of the graft with GFP-expressing fluorescence and recovery of motor function of rats transplanted with ES cell-derived neuron-like cells. A cell based-therapy may have the advantage of exerting multiple therapeutic effects at various sites and times within the lesion as the cells respond to a particular pathological microenvironment. Although a single injection of mESCs several hours after ischemia onset can reduce infarction size and improve functional outcome in rodent cerebral ischemia models[7]. To this end, infusion of growth factors has been shown to promote endogenous progenitor proliferation in response to ischemia and subsequently migrate into the hippocampus to regenerate new neurons[8]. Although endogenous neurogenesis and migration of precursor cells may help to replace some lost neurons in brain structures such as striatum[9], transplantation of exogenous stem cells remains to be the most liable way to repair the massive damage in the cerebral cortex after ischemic stroke. There have been many reports that embryonic or neural stem cell graft reduces the infarct size with functional improvement in the experimental models. Most previous studies agreed that stem cell therapy is an attractive or promising candidate for functional repair in cases of brain damage[10]. 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.

Methods

Experimental design

Twenty four transient ischemic middle cerebral artery occlusion (MCAO) rat models were prepared. After the MCAO procedures, all of the rats were randomly assigned to one of two groups (n=24): infarct with PBS-only injection (group A, n=10), infarct with mESC transplantation (group B, n=14).

Mouse embryonic stem 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.

Middle cerebral artery occlusion model

The rat MCAO model was used as a stroke model. This study was approved by the animal care and use committee of Namseoul University, and all procedures were carried out in accordance with institutional guidelines. We induced permanent MCAO by using a previously described method of intraluminal vascular occlusion.[11] Adult male Sprague-Dawley rats (n=24) weighing 250–300g were anesthetized with an intrapenitoneal (i.p.) injection of ketamine (75 mg/kg) and xylazine (10 mg/kg). We induced transient left middle cerebral artery occlusion (MCAo) for 90 min as previously described[10-11]. Briefly, a 4-0 nylon monofilament coated with silicone was inserted from the left common carotid artery (CCA) via the internal carotid artery to the base of the left MCA. After the occlusion for 20 minutes, the filament was withdrawn.

Cell transplantation and TTC staining

The rats received one injection of mES cells along the anterior-posterior axis of the ipsilateral cortex targeting to the striatum by using a predetermined stereotactic frame (David Kopf Instrument, Tujunga, CA, USA) three hours after MCAO. After anesthesia with an intraperitoneal injection of ketamine hydrochloride (90 mg/kg) or urethane anesthetized (1.5g/kg, 30% aqueous solution, ip), the rats were given 25.0 µl deposits of suspended cells (3 x 10^5 cells per µl) along the anterior-posterior axis into the target brain at these coordinates: from the bregma, 1.5 mm laterally and to 3 mm depth. The deposits were delivered by an infusion pump at 0.5 µl/min. Cells were injected while withdrawing the pipette in 100 µm increments. A 2 min waiting period at the end of injection allowed the ES cells to settle before needle removal (the total procedure needed about 50 min). To suppress rejection of mouse ES cells, rat hosts of the ESC transplanted group were administered cyclosporine A (15 mg/kg/day s.c.; Sandoz Pharmaceutical Corp. NJ) diluted in extra virgin oil, starting with a double-dose 1 day before surgery. Ten days after transplantation, the dosage was reduced to 10 mg/kg/day until the day before sacrifice. Rats were euthanized at 15 days of reperfusion. The brains were chilled at −80ºC for 4min to slightly harden the tissue. Five, 2-mm coronal sections were made from the olfactory bulb to the cerebellum and then stained with 1.5% TTC (Sigma, St. Louise, MO). The stained brain sections were captured with Magnifier digital camera.

GFP-gene transfection to mouse ESCs with vector

GFP was transferred for the labeling of transplanted cells using chicken actin-pEGFP-1 vector. Mouse ESCs were exposed to the infectious viral particles in 40 ml of the culture media at 37°C for 12 h. Cells were infected with recombinant pEGFP-1 vector(Clontech Laboratories, Inc., Palo Alto, CA) carrying chicken actin promoter. GFP-expression was confirmed at five days after transfection before transplantation. Finally, the GFP labeled cell concentration was adjusted to 3.0×10^5cells/µl using PBS just before transplantation.

15 days after transplantation, GFP-expressing cells were detected in vivo. Brains of the deeply-anesthetized rats were removed, fixed in 4% paraformaldehyde in phosphatebuffer, dehydrated with 30% sucrose in 0.1 M PBS for overnight, and frozen in powdered dry ice. Coronal cryostat sections (10 µm) were processed. To excite the GFP fluorescence, a 488-nm laser line generated by an argon laser was used. Confocal images were obtained using a Zeiss laser scanning confocal microscope with the use of Zeiss software.

Statistical analysis

Quantitative data were expressed as mean ± SEM. Two-way ANOVA and Student's *t* test with the Bonferroni correction for multiple pair-wise comparisons were used for statistical analysis. *p* values <0.05 were considered significant.

Results

Morphological characteristics of mESC

Primary mESCs cultured as plastic adherent cells could be maintained in culture. Figure 1 shows the morphological features of these cells. Characteristic doom like-shaped cells can be recognized. The *in vitro* neural differentiation from mouse ES to ES-N cells was performed as described previously.

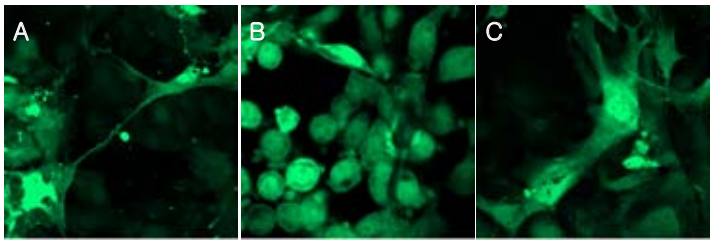

FIGURE 1 - Differentiation of EK1 cells with EGFP fluorescence expression. All the cells with bright EGFP showed their specific morphology of the differentiated cells at 40th passage. **A**, cells with projections; **B**, cells with round form; **C**, polygonal cells (200x).

Histological determination of infarction lesion

15 days after transplantation, the rats were perfused and stained with TTC to obtain a second independent measure of infarction volume. Normal brain (gray matter) tissue typically stains with TTC, but infarcted lesions show no or reduced staining. TTC staining obtained 2 weeks after MCAO without cell transplantation is shown in Figure A. Figure B showed that the reduced staining on the lesion side primarily in the corpus striatum. There was a progressive reduction in infarction size with mESC treatment. Intracerebral delivery of mESCs resulted in very substantial reduction in infarct lesion as estimated from TTC staining. Cell transplantation reduces MCAO-induced brain infarction. Representative TTC stained brain sections are shown where rats were injected with PBS (A; n=10) or were transplanted with mESC (B; n=14) after MCAO. Animals were killed 24 h later and the brains were sliced into 2 mm sections and stained with 2,3,5-triphenyltetrazolium chloride (TTC). Infarct volumes in brains from PBS and mESC treated animals are shown in the Figure 2 A, B. Values are presented as mean±SD.

FIGURE 2 - Brain slices stained with TTC to visualize lesions. TTC-stained brain slices from PBS injection (**A**) and following intracerebral delivery of mESC after MCAO are shown in B, respectively.

Cell survival and Identification of donor cells in vivo

In the model of focal cerebral ischemia that we employed, which involves occlusion of the MCA at its origin, the striatum is the site of the most pronounced ischemic damage and neuronal loss. Therefore, the first approach we undertook was to transplant cultured neural precursor cells directly into the ischemic region of the rat striatum (Figure 3). This was done 24h after the onset of ischemia, and rats were killed 15 days later. After focal ischemia, however, transplanted cells survived and migrated into the ischemic striatum (Figure 3). The surviving cells of transplanted GFP-positive mESC were mainly resided in the transplanted site in all rats receiving mESCs. The transplanted cells tended to correlate to infarct volumes in each rat.

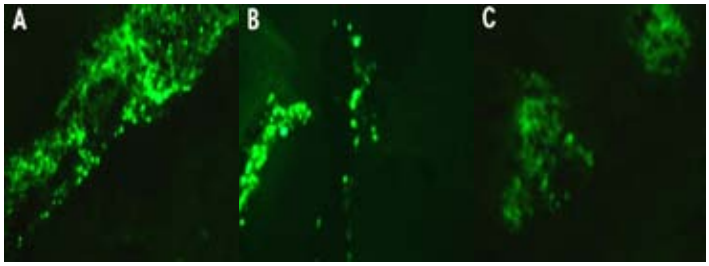

FIGURE 3 - Migration of implanted mESCs after focal ischemia. GFP-positive cells were transplanted into the lateral ventricle (LV) on the side of the ischemic after MCAO. Transplanted cells were found in the ischemic region (**A,B,C**).

Motor behavioral index

In order to explore functional benefits of the transplanted mES cells, we assessed neurological and behavioral activities up to 15 days after transplantation. Neurological severity scores (NSS) were calculated based on a series of motor, sensory, reflex, and balance tests[12] between control and cell-transplanted, respectively. During 15 days after transplantation, rats that received PBS show a little behavioral improvement, while transplantation of mESC-transplanted already displayed an early beneficial effect on NSS function and performed significantly better than the control group (Figure 4A, B, C). Thus, cell-transplanted group conferred accelerated recovery in a number of sensorimotor activities. By 15 days after transplantation, group that received mESCs performed significantly better compared to control rats. To assess the motor function more specifically, animals were trained on the rotarod three days prior to ischemic insult (five days before transplantation). Transplantation of mESC improved motor function on the rotarod test 15 days after transplantation. Behavioral activities longer than 15 days after transplantation were not tested in this investigation.

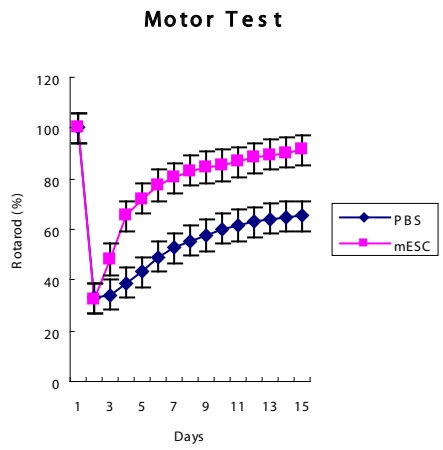

FIGURE 4A - Motor functions were examined between control group and mESC-transplanted group. Cell transplantation enhanced motor ability after ischemia. Scores were compared to baseline data generated before stroke and are represented as % of control. P<0.05 compared with PBS (n=10) only; P<0.05 compared with cell transplanted group (n=14).

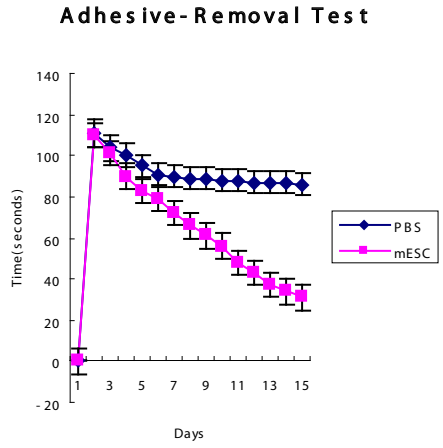

FIGURE 4B - Somatosensory function was examined between control group and mESC-transplanted group. Improved somatosensory function was shown in transplanted group. P<0.05 compared with PBS (n=10) only; P<0.05 compared with cell transplanted group (n=14).

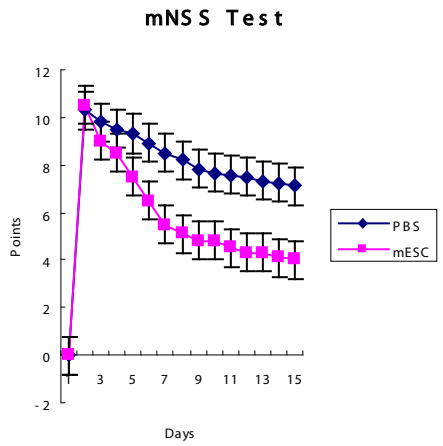

FIGURE 4C - Neurological dysfunctions were examined between control group and mESC-transplanted group. Impaired neurological behaviors were significantly recovered in transplanted group. P<0.05 compared with PBS (n=10) only; P<0.05 compared with cell transplanted group (n=14).

Discussion

The present study revealed several findings that are important for investigators that utilize murine models of stroke. Firstly, the transient MCAO model, which is widely used in stroke research, induces a peak volume of injury as delineated by TTC staining by 24h and remains unchanged through day 7 of reperfusion. A spatiotemporal evolution of core and penumbra was also seen[6]; at earlier time points the histological infarct core, as measured by TTC is in the striatum, and the viable tissue was around this core, and included the cortex. Subsequently the TTC-defined core expands to involve most of the cortex supplied by the MCA[4]. Stem cell transplantation can restore function in rodent models of diabetes, immunodeficiency, and myocardial infarction, by stimulating the production of pancreatic b-cells, lymphocytes, or endothelial cells from endogenous precursors[13]. Several researches reported previously that transplantation of stem cell into rat brain following experimental stroke reduced infarct volume and improved behavioral outcome. In the present study, we found that transplantation also stimulated neurogenesis in the ipsilateral striatum to stroke. Graft placement relative to the lesion was a critical determinant of cell survival; cells deposited close to the lesion edge did not survive well, but transplants survived robustly when located farther from the lesion. This finding suggests that mES cells can survive if they are transplanted into nonischemic tissue. Injected embryonic cells homed to sites of damage in stroke brain[14]. It has been reported previously that the infarct area of the brain massive macrophage infiltration, in a scavenger role, was noted[15].

Because the magnitude of the inflammatory response and its harmful effects as well as the types of released cytokines change with time after ischemia[16], the timing of the transplant could significantly influence graft survival, and longer survival could be predicted if cells are transplanted once inflammation has subsided. Moreover, in mESC-transplanted group, significant reduction of apoptotic cells in the ischemic core and infarct volume was observed. Even in a focal stroke model, it was suggested that greater than 80% of newly-formed neurons, which occurs in the subventricular zone of lateral ventricle or in the dentate gyrus of the hippocampus in the adult brain, died, most likely because of unfavorable environmental condition including lack of trophic support and exposure to toxic products from damaged tissues[17].

The present study shows that transplantation of mESC not only increased early survival of transplanted cells but also accelerated behavioral recovery following stroke. This is consistent with previous report that grafted embryonic stem cells develop

into functional neurons and could integrate into host cortical circuitry[18]. The functional recovery 15 days after transplantation suggests the therapeutic advantage of accelerated repair processes and functional restoration.

Although the functional improvement may be regarded as mild, such benefits could be of clinically significance. The promotion of early repair may have two-fold benefits: it may allow patients to regain lost functions faster than transplantation with regular cells; the time saved by the early recovery may be critical for the therapeutic window of function repair in certain tissues[5]. The motor functional benefit, however, was more persistent in mESC transplantation, showing much better performance than control animals at 15 days after MCAO. More long-term investigation may be needed to verify the persistence of the morphological and functional benefits of the transplantation strategy. The pro-survival effect combined with possible stimulation of endogenous regeneration mechanisms should provide an optimal environment for tissue repair in the damaged brain and contribute to successful functional recovery after ischemic stroke[5-6]. In our study, the infarct size was significantly reduced in cell-transplanted group compared with the control-operated group. Although the basis for the propagation of injury is unclear, it is well known that an ischemic brain infarct progresses over time[19]. Therefore, we postulate that the reduction of the infarct area in the transplanted group, compared with the control operated group was induced by reduction of secondary damage in the penumbra area initiated by ischemia and cell death. In addition, cell therapy, rather than direct replacement of the defect through cell transplantation, might trigger certain positive developments in the plasticity and functional recovery of the brain tissue by endogenous cell mediated effect[20-21]. We demonstrated that intracerebral transplanted mES cells survived, migrated into the infarct area from injection site. A systemically delivered cell based-therapy may have the advantage of exerting multiple therapeutic effects at various sites and times within the lesion.

The cell transplantation therapy represents a novel approach that may enhance the efficacy and effectiveness of stem cell transplantation after ischemic stroke without compromise of long-term safety concerns. This strategy harnesses the innate ability of cells to intrinsically for increased cell survival and repair capability. We propose that cell transplanted strategy presents a unique and beneficial method for improving clinical potential. Further study may prove that, in addition to ischemic stroke, the strategy is applicable to cell therapies for other CNS and non-CNS disorders.

Conclusion

The mouse embryonic stem cells may have advantage for use as a donor source in various neurological disorders including motor dysfunction.

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