行政院國家科學委員會專題研究計畫　成果報告

神經幹細胞移植合併膠質神經滋養素基因治療對脊髓損傷之神經再生療效

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The Regenerative Effect of Neural Stem Cell Transplantation with GDNF Gene Therapy on Spinal Cord Injury in Rats

執行期間：93 年 8 月 1 日至 94 年 7 月 31 日

計畫主持人：王有智

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中文摘要

脊髓損傷在全世界的年發生率約為百萬分之十五到四十，而在台灣，每年也約有三百至五百名病患。這些脊髓傷害患者的神經功能受損，導致四肢或下半身癱瘓而不能行走，對個人、家庭與社會均造成重大的損失。傳統的醫療方法，包括脊髓神經減壓、脊髓神經修復、脊椎復位與內固定等手術，以及藥物或物理治療等，但其療效對於嚴重的脊髓損傷仍相當有限。因此，我們必須採取新的治療策略才行。

最近國內外動物研究均顯示：將神經幹細胞移植到受損的腦或脊髓中，可分化並彌補死亡的神經細胞，使動物的部分神經功能恢復。另外，基因治療應用於神經疾病亦前景可期。近年來，已有多項研究利用病毒載體攜帶膠質神經滋養素（GDNF）基因來治療巴金森氏症及腦中風，具有不錯的效果；而 GDNF 也被報告可以改善脊髓損傷鼠的神經功能。因此，結合 GDNF 基因治療與幹細胞移植，對脊髓損傷之治療應更具潛力。

本實驗室最近已從大鼠胚腦中成功培養出神經幹細胞，並且也建立了大鼠脊髓截斷損傷模式。另外，三總神經外科馬辛一博士亦已成功將 GDNF 基因接入腺相關病毒載體（簡稱 AAV-GDNF）中；故本計劃希望藉由馬博士所提供的 AAV-GDNF 來研究神經幹細胞移植與 GDNF 基因治療對大鼠脊髓損傷之療效。

我們將 SD 鼠第八至第九胸椎處（簡稱 T8-9）的脊髓完全截斷，以造成老鼠的後肢癱瘓，然後立即以異體的肋間神經片段橋接於截斷處，再將攜帶 AAV-GDNF 或 AAV-eGFP（一種綠色螢光基因）的神經幹細胞植入神經橋接處，對照組則僅在橋接處注射生理食鹽

水。幹細胞移植後，每週以 BBB scale 評估老鼠後肢的運動功能。四個月後，我們將老鼠犧牲並取下脊髓進行免疫螢光染色，以觀察神經幹細胞在體內生長與分化的情形。

結果發現：未做神經橋接與幹細胞移植的脊髓損傷鼠其後肢在脊髓截斷 8 週後仍幾近癱瘓，平均 BBB scale 僅 1 分，而有神經橋接的動物其後肢的髖、膝及踝關節於治療 8 週後已有顯著收縮，BBB scale 在生理食鹽水組、eGFP 組及 GDNF 組平均分別為 6.2、6.8 與 7.3 分，其中 GDNF 組動物的後肢尚發現有間歇性的踏步。然而，在治療 8-9 週後，生理食鹽水組和 eGFP 組的後肢運動功能開始變差，至 16 週時 BBB scale 僅剩 1-2 分；相反地，GDNF 組在 16 週時仍維持 8 分左右。

免疫螢光染色顯示：在 GDNF 組的脊髓損傷處附近及其近端脊髓，可發現有許多幹細胞、神經元、星狀細胞及 GDNF 陽性細胞，而遠端脊髓則看不到這些細胞，表示遠端組織環境仍無法讓神經細胞存活下來，這應是老鼠後肢在治療後 4 個月仍無法站立或行走的原因。在 eGFP 組，僅見少數的神經元和星狀細胞於脊髓損傷處的附近或近端脊髓，而無幹細胞或 GDNF 陽性細胞，這表示在缺乏 GDNF 的作用下，植入脊髓的幹細胞無法存活，同時也印證了這組動物的後肢運動功能在治療 8 週後又開始惡化的現象。然而，在 GDNF 組所看到的神經元及星狀細胞是否由移植的幹細胞分化而來，以及 GDNF 陽性細胞究竟是植入的幹細胞或已分化的神經細胞，本實驗未加證實，這一點有待未來進一步釐清。

總結，本研究已初步證明了神經幹細胞移植合併 GDNF 基因治療對於脊髓損傷的療效，未來將延長動物治療後的觀察期，看看這種治療方式是否能夠使脊髓損傷鼠的後肢站立行走；另外也要以神經電生理測量及多重免疫染色技術進一步確認 GDNF 的治療效果與作用機轉。

關鍵詞：腺相關病毒載體、基因療法、膠質神經滋養素、神經幹細胞、脊髓受傷

Abstract

Spinal cord injury (SCI) occurs over the world with an annual incidence of 15 to 40 cases per million. In Taiwan, approximately 300 to 500 persons sustain SCI each year. Quadriplegia or paraplegia resulting from SCI causes devastating damage to the patient’s life quality, his or her family, and the society. Traditional surgical procedures for SCI include neurosurgical decompression, repair of the spinal cord and nerves, and spinal reduction with internal fixation of the vertebral structures. The other treating modalities consist of pharmacological therapy, physical therapy and nursing care. However, all the above treatments are of limited efficacy for serious SCI. It indicates that new treating strategies must be sought for.

Recently, animal studies have demonstrated that neural stem cells (NSCs) can differentiate and replenish the lost neural

cells after being transplanted into the impaired brains or spinal cords, leading to partial recovery of neurological function. Additionally, gene therapy is also a promising strategy for nervous diseases. In the recent year, many studies found that injection of viral vectors carrying glial cell line-derived neurotrophic factor (GDNF) gene showed beneficial effects on Parkinson's disease and ischemic stroke. It has also been reported that GDNF could ameliorate neurological deficits in rats with SCI. Therefore, combination of GDNF gene transfer with NSC transplantation is of more potential for SCI.

In our laboratory, we have successfully isolated and cultured NSCs from the rat fetal brain and established a rat model of transectional spinal cord injury. As well, Dr. Ma in the Department of Neurosurgery, Tri-Service General Hospital (TSGH) has successfully incorporated GDNF gene into the adeno-associated viral (AAV) vector (AAV-GDNF). Therefore, in this study, we intended to investigate the effect of combined NSC transplantation and AAV vector-mediated GDNF gene therapy, the AAV-GDNF needed in the latter being provided by Dr. Ma, on spinal cord injury in rats.

Adult female Sprague-Dawley rats were subjected to complete spinal cord transection at T8-9 level. Then the two cord stumps were bridged with segments of heterogenous intercostal nerve. Immediately after nerve bridging, NSCs transfected with AAV-GDNF or AAV encoding enhanced green fluorescent protein (AAV-eGFP) were injected into the space between the nerve grafts. The control rats were injected with Ringer's saline. The hindlimb motor function of the animals was evaluated with BBB scale weekly after NSC transplantation. Four months later, the animal were sacrificed and the spinal cord was removed for immunofluorescence study.

Animals with spinal cord transection only (without intercostal nerve bridging) demonstrated rare joint movement in the hindlimbs even 8 weeks after transection (mean BBB scale: 1.0). By contrast, those with intercostal nerve bridging, either injected with Ringer's saline or transplanted with NSC-AAV-eGFP or NSC-AAV-GDNF, showed remarkable movement of hip, knee, and ankle joints in the hindlimbs 8 weeks after transection. Intermittent stepping of the hindlimbs was also observed in animals with GDNF gene therapy. The mean BBB scales were 6.2, 6.8 and 7.3 for the saline, eGFP and GDNF groups, respectively, all significantly higher than the transection only group (p <0.001). However, the BBB scales of the saline and eGFP groups went downhill from 8-9 weeks to 1.0-2.0 at 16 weeks after spinal cord transection. Instead, the BBB scale of the GDNF group maintained at significantly higher level (8.0) than the former two groups 16 weeks after transection (p <0.01).

In NSC-AAV-GDNF-treated rats, many NSCs, neurons, astrocytes and GDNF-positive cells were found in the spinal cord rostral to and around, but not caudal to, the injured site. It indicates that the environment distal to the lesion was unfavorable to survival or regeneration of neural cells. This may be responsible for the inability of the hindlimbs to support weight and walk 4 months after NSC transplantation in the GDNF group. In the eGFP group, only few neurons and astrocytes were seen in the spinal cord rostral to and around the injured site but NSCs and GDNF-positive cells were not present. It indicates that the transplanted

NSCs could not survive at lack of GDNF, corresponding to the observation that the hindlimb motor function in this group deteriorated from 8 weeks after NSC transplantation. Whether the neurons and astrocytes found in the GDNF group were differentiated from the transplanted NSCs, or whether the GDNF-positive cells were transplanted NSCs or differentiated neural cells is unclear from this study due to lack in evidence of double immunostaining.

In summary, we preliminarily demonstrated the beneficial effect of combined NSC transplantation and AAV-mediated GDNF gene therapy on spinal cord injury. This type of treatment partially improved the hindlimb motor function of animals with SCI. However, it is still far away from recovery of steady standing and gait with coordination. The mechanisms exerted by NSC transplantation and GDNF gene therapy deserve further investigations.

Key words: adeno-associated viral (AAV) vector, gene therapy, glial cell line-derived neurotrophic factor (GDNF), neural stem cell, spinal cord injury

Introduction

Spinal cord injury (SCI) occurs over the world with an annual incidence of 15 to 40 cases per million. Each year approximately 10,000 persons in America and 300 to 500 persons in Taiwan sustain SCI. The major causes of SCI range from traffic accidents, community violence to workplace-related and sport injuries (1). Quadriplegia or paraplegia resulting from SCI causes devastating damage to the patient's life quality, his or her family, and the society. Microscopically, SCI leads to axonal degeneration, demyelination, activation of microglias and astrocytes, and death of neurons and oligodendrocytes (2). The general treatments for SCI include surgical, pharmacological and physical therapies (3). Traditionally, neurosurgeons treat the patients with surgical decompression to renormalize the cord anatomy, by repairing the spinal cord or nerves, and by spinal reduction with internal fixation to restabilize the vertebral structures (4). Administration of methylprednisolone was recommended as a pharmacological therapy on the basis of the National Acute Spinal Cord Injury Studies, but has proved only slightly improving the neurological function of patients (5). Physical therapy contributes to re-build up the neurological function, especially the muscular status (6). However, severe SCI with cord ischemia and cell death may still result in permanent neurological deficits, such as ventilator dependence, quadriplegia, paraplegia and myeloradiculopathy. Therefore, we have to seek for new ways to treat this devastating disease in addition to the traditional modalities.

Current strategies employed by investigators to enhance regeneration in the injured spinal cord include neutralization of potential growth inhibitory molecules, transplantation of cells or tissue that support axonal elongation, and delivery of neurotrophic factors to promote axonal growth (7-9). Although transplantation of fetal tissue has resulted in functional recovery from Parkinson's disease and Huntington's disease (10), clinical use of fetal cells is limited by the availability of

donor tissue as well as logistic, immunological and ethical consideration. Neural stem cells (NSCs) as a source of multipotent graft may be an alternative to fetally derived cells. NSCs can be isolated from embryonic or adult brain tissue, as well as from embryonic stem (ES) cells (11). These cells are able to proliferate *in vitro* through many passages without losing their multipotentiality. Furthermore, NSCs can be induced to differentiate into enriched populations of glial or neuronal progenitors (11). Some animal experiments have also demonstrated that NSCs can differentiate into neurons and glial cells after being transplanted into the impaired brains or spinal cords (12-15). However, when pluripotent NSCs are transplanted, the engrafted cells predominantly differentiate into glial cells, failing to replenish the lost neurons caused by brain or spinal cord injury (11). It indicates that further differentiated neuronal- and glial-restricted precursor cells may be more effective than pluripotent NSCs for specific neurological diseases. On the other hand, co-administration of NSCs and neurotrophic factors may guide a more appropriate differentiation and neural regeneration for these diseases.

Glial cell line-derived neurotrophic factor (GDNF) has been reported neuroprotective for cerebral ischemia, Parkinson's disease and SCI (9, 16-18). Its effects on the injured neural tissues can be prolonged when GDNF gene is incorporated into virus vectors that transfect either the endogenous or transplanted cells (19-23), the so-called gene therapy. The viral vectors usually employed for gene therapy include retrovirus, herpes simplex virus, adenovirus and adeno-associated virus (AAV) vectors (23), among which AAV vector is relatively less toxic and longer-term effective (22). AAV is a DNA virus that is non-pathogenic to humans and can integate into the host genome. It needs additional genes provided by a helper virus, such as adenovirus, to replicate. This increases the risk of cytotoxicity caused by the helper viruses when used with AAVs to co-transfect the cells. The dependence on helper viruses was overcome by Xiao et al. who made a recombinant AAV (rAAV) vector possessing the helper genes but without the structural and replication genes of adenovirus (24).

Now gene therapy using rAAV vectors has been applied to several disease models, including Parkinson's disease, demyelinating disease, cerebral ischemia, SCI and brain tumors (19, 20, 21, 25, 26), with promising results. In a model of SCI, Ruitenberg et al. reported that AAV-mediated brain-derived neurotrophic factor (BDNF) gene transfer completely reversed lesion-induced atrophy of rubrospinal neurons that are important in the control of motor function (27). In the above researches, the investigators administered specific gene-carrying AAV vectors directly to the animals. Up to the present, only a few studies have been reported using AAV vectors to transduce neuronal or neural progenitor cells (28, 29). Neural stem cells modified with rAAV-mediated GDNF (rAAV-GDNF) gene transfer for SCI has almost not been

investigated. Therefore, this study is intended to explore whether the strategy of rAAV-GDNF modified NSCs transplantation is effective for SCI in an animal model.

In our laboratory, we have successfully isolated and cultured epidermal growth factor (EGF)-responsive neural progenitor cells as neurospheres from 14-day-old fetal rat brains (Fig.1). The EGF-responsive cells were confirmed as NSCs (30-32). The neurospheres have been modified by viral vector-mediated GDNF gene transfer to assist the regeneration of the transplanted dopaminergic neurons for parkinsonian animals (21). Dr. Chiang, one of the component project investigators in this integrative research, and his colleagues in Tri-service General Hospital (TSGH) have also successfully utilized EGF-responsive neurospheres to guide the axonal growth of the transplanted fetal mesencephalic cells in rats with Parkinson's disease (unpublished data). These results encourage us to test the EGF-responsive neurospheres in SCI. On the other hand, Dr. Ma, another component project investigator of this integrative research, has developed a double-stranded rAAV (dsAAV) vector and incorporated GDNF gene into it. This newly systhesized dsAAV vectors have markedly higher transducing efficiency than the traditional single-stranded AAV (ssAAV). Furthermore, dsAAV exhibits higher in vivo DNA stability and more circularization than ssAAV, leading to faster, stronger and prolonged transgene expression (data unpublished). Therefore, in this study, we modified the NSCs with Dr. Ma's rAAV-GDNF and transplanted them into rats subjected to complete spinal cord transaction to explore the effect of combined NSC transplantation and gene therapy.

Materials and methods

Preparation of Neural Stem Cells (NSCs)

The rat NSCs were prepared according to Chiang et al. (32). Pregnant Sprague-Dawley rats with 14- or 15-day-old fetuses were deeply anesthetized by ether. The fetuses were removed from the uterus and put into cold Hank's Balanced Salt Solution (HBSS) (GIBCO, NY). The brain areas of cerebral cortex, striatum and mesencephalon were dissected carefully. These tissues were cut into small pieces and then washed with HBSS twice before being put into 1-2 ml of protease-papain-DNase solution (PPD), which consists of 10 mg papain, 100 mg protease, 10 mg DNase 1, and 12.4 μl 1M $MgSO_4$ in 100 ml HBSS. After 30 min incubation at 37℃, gentle trituration with a Pasteur pipet was performed 10 times. The entire procedure was repeated once and then the PPD was replaced by Dulbecco's modified Eagle's medium (DMEM)/F12 nutrient (1:1, GIBCO, NY) containing DNase 1 (1%, w/v). After a 15-min incubation, gentle trituration was performed another 10 times. The dissociated single cells were collected by centrifugation at 1500 *g* for 5 min. The cells were resuspended in

DMEM/F12 medium containing N2 supplement (25 mg/ml insulin, 100 mg/ml transferrin 20 nM progesterone, 60 mM putrescine, and 30 nM selenium chloride; Sigma) and cell counting was performed with a hemacytometer. Viability was checked by trypan blue exclusion method.

A plating density of 50,000 cells/cm^2 was used to culture epidermal growth factor (EGF)- responsive neurospheres in non-coated 75 cm^2 flasks with N2 defined medium containing 20 ng/ml EGF (GIBCO, NY). EGF was added every 4 days and the medium was changed every 2 weeks. No passage was performed on these neurospheres.

Preparation of rAAV-GDNF

The rAAV-GDNF was prepared by Dr. Ma's laboratory, according to the procedure described by Tsai et al. (19). Rat GDNF cDNA was obtained by a reverse transcriptase-polymerase chain reaction (RT-PCR) from RNA prepared from rat embryonic brain tissues. The forward primer used in the PCR reaction was 5'-Tagcggccgc ATGAAGTTATTG-3', which not only spanned the region covering the initiation codon (underlined) and its flanking sequences, but also reacheed a *Not*I site (the lowercase letters) at the 5' end. The reverse primer was 5'-AagcggccgcTCAGATACATCC-3', which spanned not only the stop codon (underlined) and its flanking sequences but also a *Not*I site (the lowercase letters) at the 5' end. The GDNF cDNA was cloned between the two *Not*I sites of pXX-UF-1 to replace the enhanced green fluorescent protein (eGFP) gene just downstream from the human cytomegalovirus immediate early promoter. The recombinant AAV encoding GDNF (rAAV-GDNF), pXX2 and pXX6 were cotransfected into 293 cells for viral vector production. The virus was purified twice by cesium chloride ultracentrifugation. The titer of rAAV-GDNF was determined by a dot blot assay (24).

Transfection of NSCs with rAAV-GDNF

Whole neurospheres were exposed to either rAAV-GDNF or rAAV encoding enhanced green fluorescent protein (rAAV-eGFP) in 5 ml of growth medium in poly-HEMA (12%)-coated T25 flasks for 18 h. The neurospheres were then resuspended in fresh growth medium for 7 days. Successful transduction was observed by visualizing GFP expression using a fluorescence microscope. To estimate the transduction efficiency, some of the rAAV-eGFP-transfected neurospheres were dissociated using trypsin, and the cells were plated onto poly-D-lysine (0.01%) and laminin (0.001%) coated chamber slides (30,000 cells per well) in 0.5 ml of serum-free neurobasal medium supplemented with L-glutamine (0.5 mM) and B27 (2%, v/v). Cells were fixed for 1 h, and the proportion expressing GFP was counted under a fluorescence microscope using a counting grid. To assay for GDNF secretion, The neurospheres transfected with rAAV-GDNF or rAAV-eGFP or non-transfected neurospheres were plated onto poly-L-lysine/laminin-coated chamber slides in 0.5 ml of serum-free neurobasal medium supplemented with L-glutamine (0.5 mM) and B27 (2%, v/v). The neurospheres were maintained in culture for 18 days. The plating medium was replenished every 48 hr, and 0.5 ml sample aliquots were stored at -70

℃ for later analysis. Measurement of GDNF in the sampled medium was performed using a commercially available GDNF ELISA kit.

Spinal Cord Injury and Transplantation of NSCs with rAAV-GDNF

Adult female Sprague-Dawley rats (250-300 g) were anesthetized with 4% halothane and placed in a prone position. Anesthesia was maintained with 2% halothane. The body temperature was monitored and kept at 37°C by a temperature controller. Using aseptic surgical techniques, an incision extending from the mid to low thoracic regions on the dorsal side was made. Laminectomy of the caudal portion of the T8 and all of the T9 spine was performed under an operative microscope to expose the spinal cord. The spinal cord was completely severed at T8-9 level with microscissors and gentle aspiration to produce a gap of 3 mm between the two stumps of the cord. The vertebrae (T7-10) around the lesion were fixed with Ti-cron to the proximal ribs (T7-10), and transverse fixation of the T7 and T10 vertebrae to the corresponding ribs was also made. Then about 18 segments of intercostal nerves were excised from another rat and bridged in to the gap of the cord. 10 μl of NSCs (50,000 cells/μl) transfected with rAAV-GDNF or rAAV-eGFP were injected into the space between the nerve bridges. The lesion cavity was covered serially with fibrin glue and a piece of gelfoam.. The surgical site was sutured layer-by-layer and the animal was allowed to awake. Postoperative nursing care included manual bladder expression 2-3 times per day, injection of lactated Ringer's saline for dehydration, giving of food supplement for weight loss, administration of antibiotics for bacterial injection, and visual inspection for skin irritation or decubitus ulcers.

Assessment of Hindlimb Motor Function

The hindlimb motor function was assessed with Basso, Beattie and Bresnahan (BBB) locamotor rating scale as described previously (32), before and weekly till 4 months after spinal cord transection. With this 21-point scale, animals achieving scores of 1-8 were capable of hindlimb joint movements without weight support. Those in ratings of 9-13 demonstrated various degrees of hindlimb weight support and forelimb-hindlimb coordination, and those achieving scores of 14-21 show improvement in paw and tail position, toe clearance, and trunk stability during a fully supported and coordinated gait.

Immunohistochemistry of the Spinal Cord

Four months after spinal cord transection, the animals were sacrificed and transcardially perfused with cold normal saline. The spinal cord was removed and dissected by cutting 1-cm segments, around and rostral and caudal to the lesion center. These 1-cm tissue segments were embedded in Tissue-Tek OCT medium and cryosectioned at 10-μm thickness. After rinsed with PBS, the frozen sections were blocked with 5% skim milk in PBS for 30 min. Then they were incubated overnight at 4°C with primary antibodies against (1) nestin for neuroepithelial stem cells; (2) neuronal nuclear antigen (Neu-N) for neurons; (3) glial fibrillary acidic protein (GFAP) for astrocytes; (4) GFP for tracing the transplanted NSCs and (5) GDNF for GDNF expression of the transplanted NSCs.

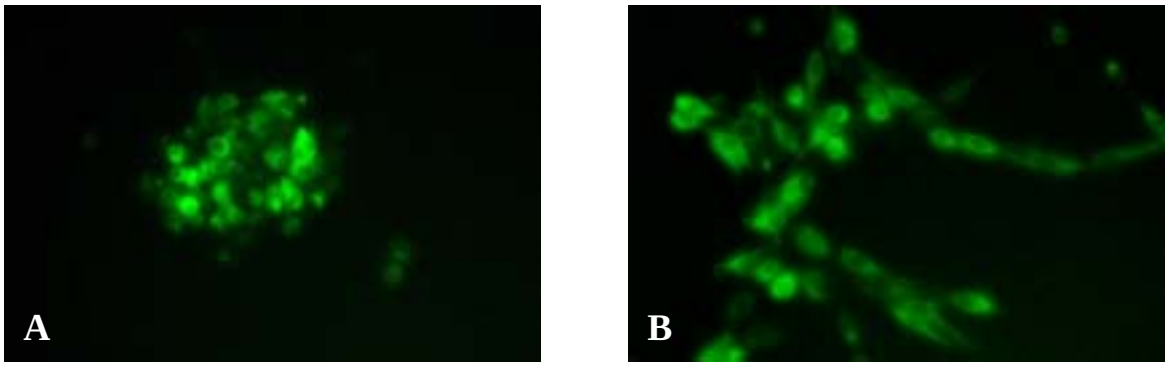

Fig. 1. Epidermal growth factor-responsive neural stem cells cultured from 14-day-old fetal brain. The cells were positively immunostained for nestin which was revealed by FITC-conjugated secondary antibodies. (**A**) a neurosphere suspending in the medium; (**B**) cells attached on the bottom of the culture flask.

Subsequently, the sections were rinsed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h at room temperature. The immunofluorescence was examined under a fluorescenc microscope.

Data analysis

All values were expressed as mean ± SEM. Differences among groups were analyzed by one-way analysis of variance (ANOVA) and Duncan's test. $p<0.05$ was considered statistically significant.

Results

Figure 2 shows GFP expression in NSCs transfected with AAV-eGFP. Only trace amount of GFP was observed in the neurospheres one day after AAV-eGFP transfection (Fig. 2A). The neurosphere was slightly green 2 days (Fig. 2B) and brightly green 3 days (Fig. 2C) after AAV-eGFP transfection. The strong green fluorescence sustained for 7 days when the newly divided neural stem cells also looked green (Fig. 2D). Then the fluorescence began to fade away. One month after AAV-eGFP transfection, GFP was almost not seen in the neurospheres (picture not shown). The average transduction efficiency of AAV-eGFP for NSCs was about 50%.

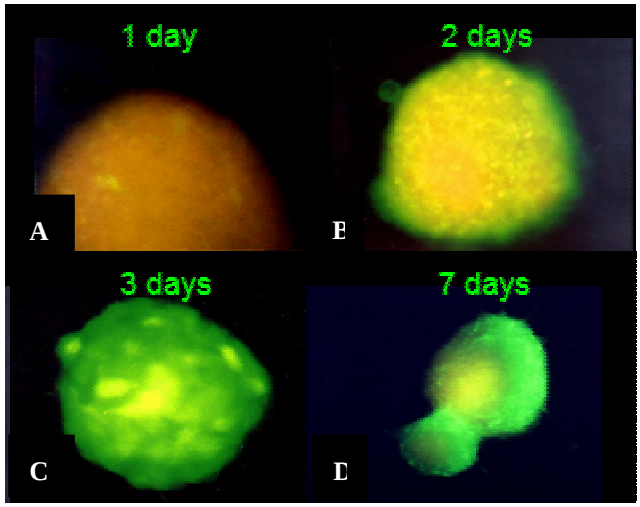

Fig.2. Rat neurospheres transfected with double-stranded type 2 adeno-associated viral (AAV) vectors carrying enhanced green fluorescent protein (eGFP) gene. The neurospheres were examined under a fluorescent microscope. One day after AAV-eGFP transfection, only trace amount of GFP expression was observed in the neurosphere (A). The neurosphere was slightly green 2 days (B) and brightly green 3 days (C) after AAV-eGFP transfection. The strong green fluorescence sustained for 7 days when the newly divided neural stem cells also looked green (D). Then the fluorescence began to fade away.

The contents of GDNF in the culture medium and cell lysate of NSCs transfected with AAV-GDNF were shown in Figure 3. The concentrations of GDNF in the medium and cell lysate increased and culminated in one day after AAV-GDNF transfection. The peak GDNF expression lasted until day 7 then the GDNF concentration became decreased. On day 14, GDNF was not detectable in the medium but was still expressed at a half-peak level in the NSCs.

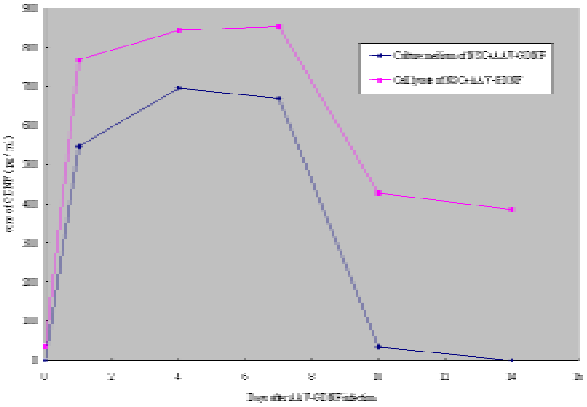

Fig. 3. Contents of glial cell line-derived neurotrophic factor (GDNF) in the culture medium and cell lysate of neural stem cells (NSCs) transfected with double-stranded type 2 adeno-associated viral (AAV) vectors encoding GDNF (AAV-GDNF). Contents of GDNF were measured with an ELISA kit. The concentrations of GDNF in the medium and cell lysate increased and culminated in one day after AAV-GDNF transfection. The peak GDNF expression lasted until day 7 then the concentration became decreased. On day 14, GDNF was not detectable in the medium but was still expressed at a half-peak level in the NSCs.

after transection (mean BBB scale: 1.0). By contrast, those with intercostal nerve bridging, either injected with Ringer's saline or transplanted with NSC-AAV-eGFP or NSC-AAV- GDNF, showed remarkable movement of hip, knee, and ankle joints in the hindlimbs 8 weeks after transection. The mean BBB scales were 6.2, 6.8 and 7.3 for the saline, eGFP and GDNF groups, respectively, all significantly higher than the transection only group (p <0.001). On the other hand, animals treated with NSC-AAV-GDNF had significantly better hindlimb motor function than those injected with Ringer's saline during 7-8 weeks after transection (p <0.05). The BBB scales of the saline and eGFP groups went downhill from 8-9 weeks to 1.0-2.0 at 16 weeks after spinal cord transection. Instead, the BBB scale of the GDNF group maintained at significantly higher level (7.0-8.0) than the former two groups from 9 to 16 weeks after transection (p <0.01).

Four months after spinal cord transection and transplantation of NSCs, many nestin-positive cells were found in the spinal cord 1 cm rostral to (Fig. 5B) and around (Fig. 5D) the lesion site in the GDNF group, indicating that the transplanted NSCs were still alive. The processes of NSCs were better incorporated into the host tissue in the rostral than in the injured segment. Nestin-positive cells were not seen in the caudal segment of the spinal cord in the NSC-AAV-GDNF-treated rats (Fig. 5F) and in any segment of the NSC-AAV- eGFP-treated spinal cord (Fig. 5A, 5C, 5E).

Many neurons were seen in the NSC-AAV-GDNF-treated spinal cord at 1 cm rostral to (Fig. 6B) and around (Fig. 6D) the lesion site, but not in the caudal segment (Fig. 6F). The neuronal nuclei around the lesion site were larger with weaker fluorescence than those resided in the rostral region. Only few neurons were found in the rostral (Fig. 6A) and injured (Fig. 6C) segments of the NSC-AAV-eGFP-treated spinal cord. Similarly, no neurons were observed in the caudal segment (Fig. 6E).

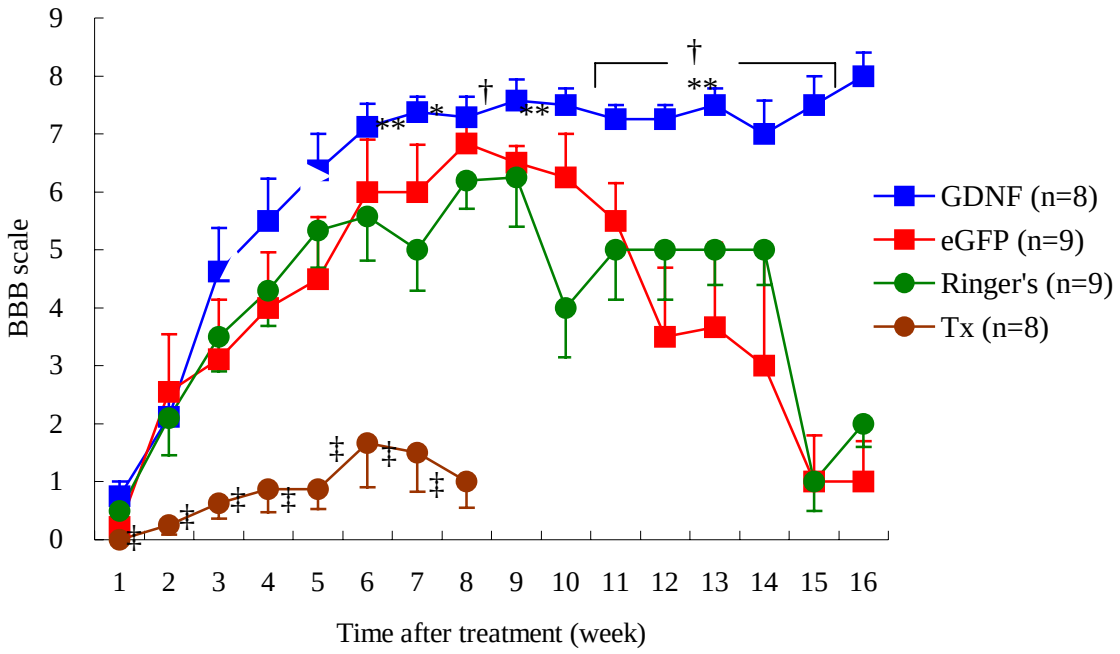

Fig. 4. Hindlimb motor function of rats subjected to complete spinal cord transection at T8-9 level. The two cord stumps were bridged with segments of intercostal nerves. Then neural stem cells modified with GDNF or eGFP gene transfer were injected into the nerve bridges. Rats treated with nerve bridging plus injection of Ringer's saline and with cord transection only (Tx) were regarded as controls. The BBB scale is a 21-point evaluation system, with higher point indicating better hindlimb function. Statistical significance: *$p < 0.05$ and **$p < 0.01$ compared to the Ringer's group; †$p < 0.01$ compared to the eGFP group; ‡$p < 0.001$ compared to the other three groups.

There were many astrocytes found in the spinal cord 1 cm rostral to (Fig. 7B) and around (Fig. 7D) the lesion site in the GDNF group. The dendrites of these astrocytes densely interlaced in the rostral segment but those in the injured segment did not. In the NSC-AAV-eGFP-treated spinal cord, there were less astrocytes in the rostral segment (Fig. 7A) and even fewer in the injured segment (Fig. 7C). In the caudal segment of the spinal cord, astrocytes were not seen in both GDNF and eGFP groups.

Many GDNF-positive cells were distributed in the rostral and injured segments of the NSC-AAV-GDNF-treated spinal cord (Fig. 8B, 8D). Instead, only few GDNF-positive cells were seen in the caudal segment (Fig. 8F). The spinal cord was negatively stained for GDNF in the eGFP group, as expected (Fig. 8A, 8C, 8E). On the other hand, GFP was not stained by immunofluorescence in the spinal cords of both the eGFP and GDNF groups.

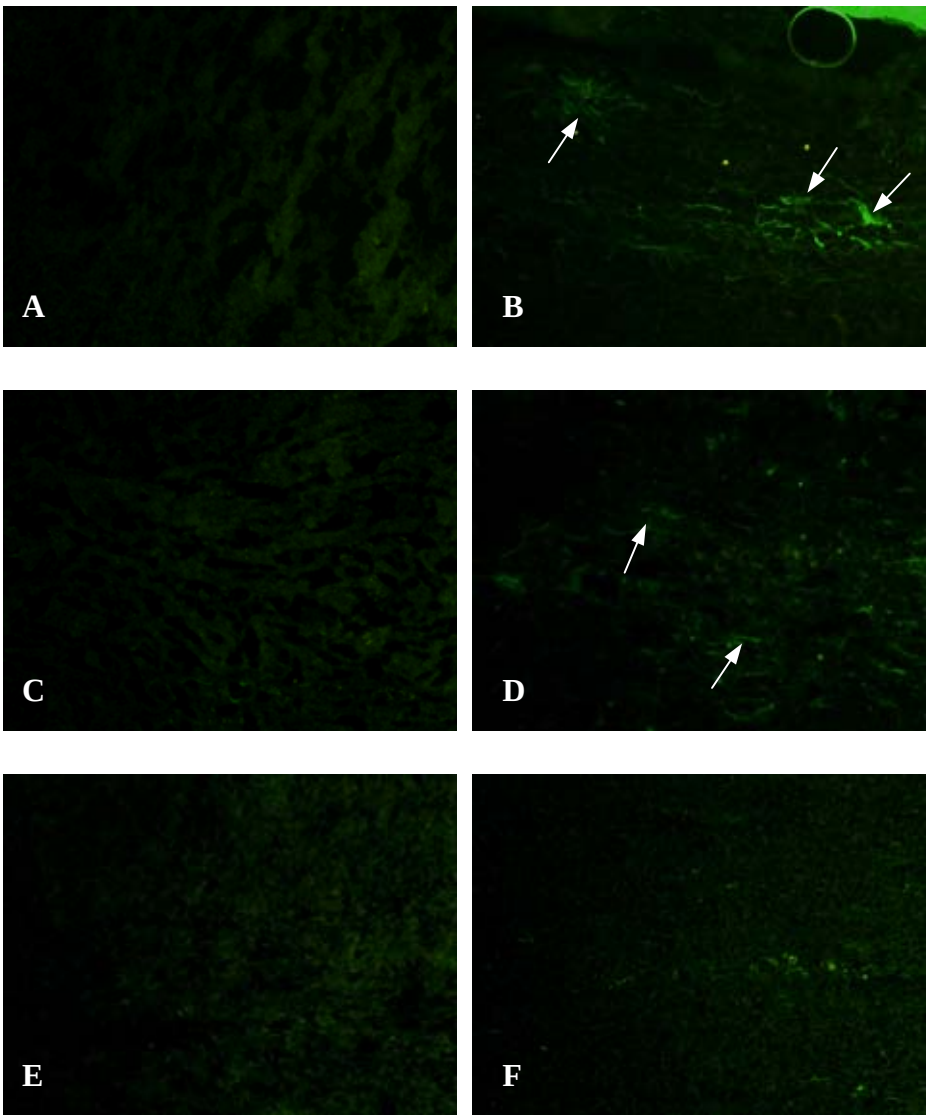

Fig. 5. Photomicrographs (200X) of the rat spinal cord showing the immunofluorescence for nestin, a marker of neural stem cell (NSC). Rats were subjected to complete spinal cord transection and transplantation of NSCs between the two cord stumps. The NSCs were modified with adeno-associated viral (AAV) vectors carrying enhanced green fluorescent protein (eGFP) or glial cell line-derived neurotrophic factor (GDNF) gene. The animal was sacrificed 4 months after treatment and the spinal cord segments 1 cm rostral to (A, B), around (C, D), and caudal to (E, F) the lesion were subjected to cryosectioning and immunofluorescence staining. Nestin-positive cells (arrows) were found in the rostral (B) and injured (D), but not caudal (F) segments of the NSC-AAV-GDNF-treated rats. The green fluorescence was stronger in the rostral than the injured segment. By contrast, nestin-positive cells were not seen in any segment of spinal cord in the NSC-AAV-eGFP-treated rats (A, C, E).

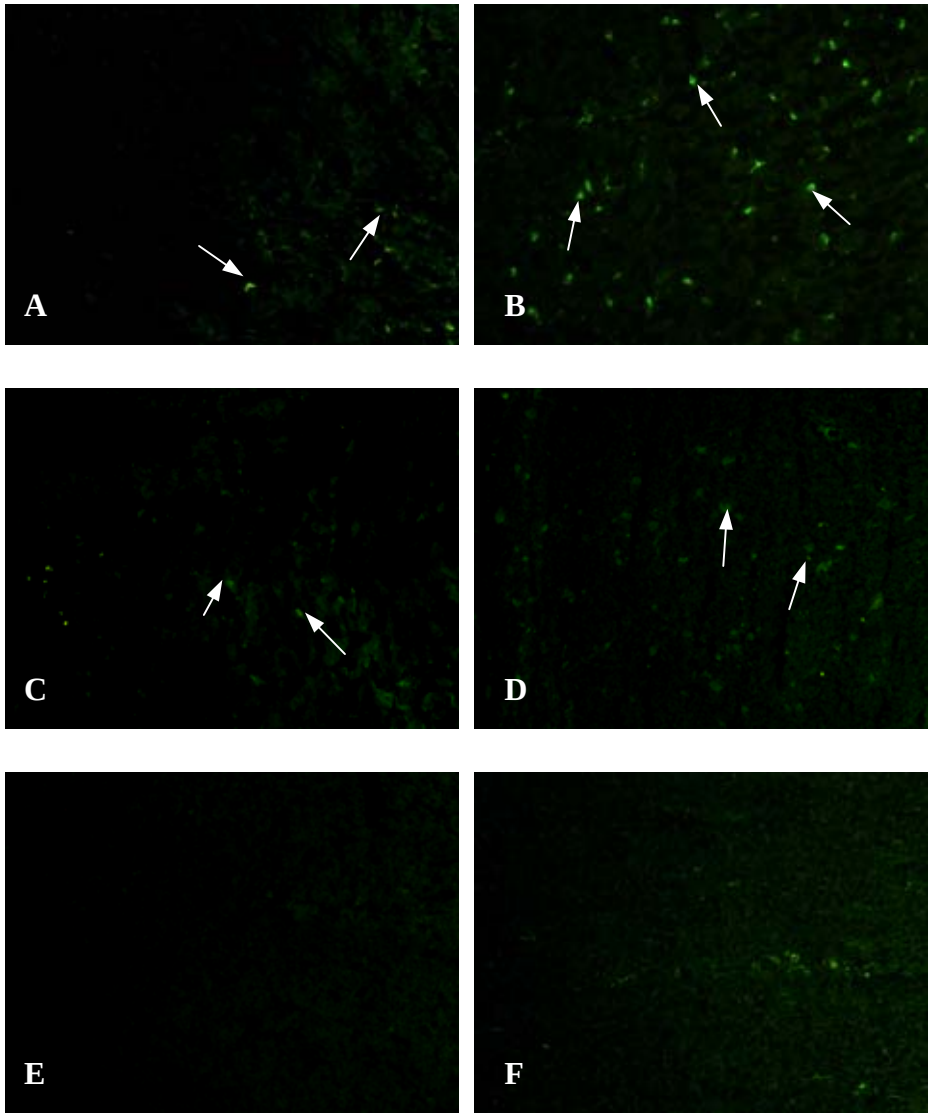

Fig. 6. Photomicrographs (200X) of the rat spinal cord showing the immunofluorescence for neuronal nuclear antigen (Neu-N). Rats were subjected to complete spinal cord transection and transplantation of neural stem cells (NSCs) between the two cord stumps. The NSCs were modified with adeno-associated viral (AAV) vectors carrying enhanced green fluorescent protein (eGFP) or glial cell line-derived neurotrophic factor (GDNF) gene. The animal was sacrificed 4 months after treatment and the spinal cord segments 1 cm rostral to (A, B), around (C, D), and caudal to (E, F) the lesion were subjected to cryosectioning and immunofluorescence staining. Many neurons (arrows) were found in the rostral (B) and injured (D), but not caudal (F) segments of the NSC-AAV-GDNF-treated rats. The green fluorescence was stronger in the rostral than the injured segment. Only few neurons were seen in the rostral (A) and injured (C), but not caudal (E) segment of spinal cord in the NSC-AAV-eGFP-treated rats.

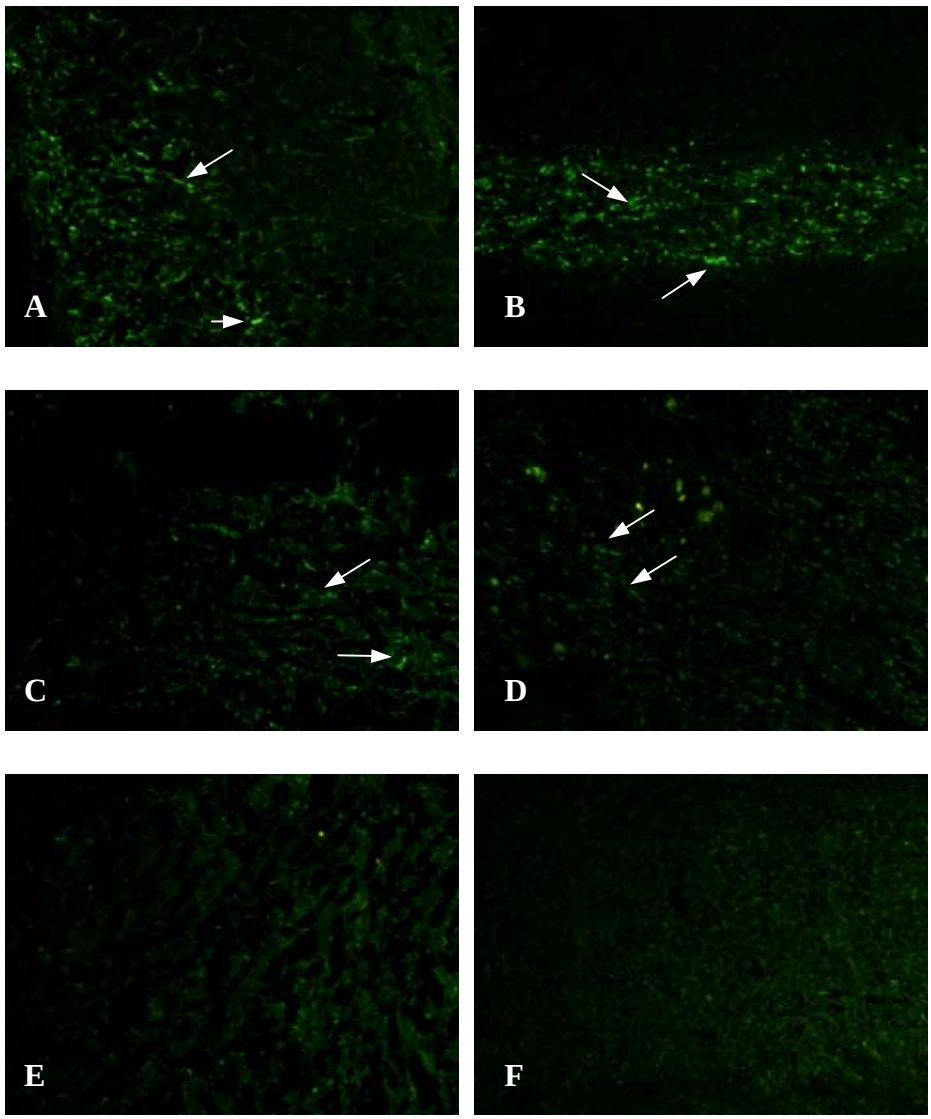

Fig. 7. Photomicrographs (200X) of the rat spinal cord showing the immunofluorescence for glial fibrillary acidic protein (GFAP), a marker of astrocyte. Rats were subjected to complete spinal cord transection and transplantation of neural stem cells (NSCs) between the two cord stumps. The NSCs were modified with adeno-associated viral (AAV) vectors carrying enhanced green fluorescent protein (eGFP) or glial cell line-derived neurotrophic factor (GDNF) gene. The animal was sacrificed 4 months after treatment and the spinal cord segments 1 cm rostral to (A, B), around (C, D), and caudal to (E, F) the lesion were subjected to cryosectioning and immunofluorescence staining. Many GFAP-positive cells (arrows) were found in the rostral (B) and injured (D), but not caudal (F) segments of the NSC-AAV-GDNF-treated rats. The green fluorescence was stronger in the rostral than the injured segment. Less GFAP-positive cells in the rostral (A) segment but few in the injured (C) and none in the caudal (E) segment of spinal cord were seen in the NSC-AAV-eGFP-treated rats.

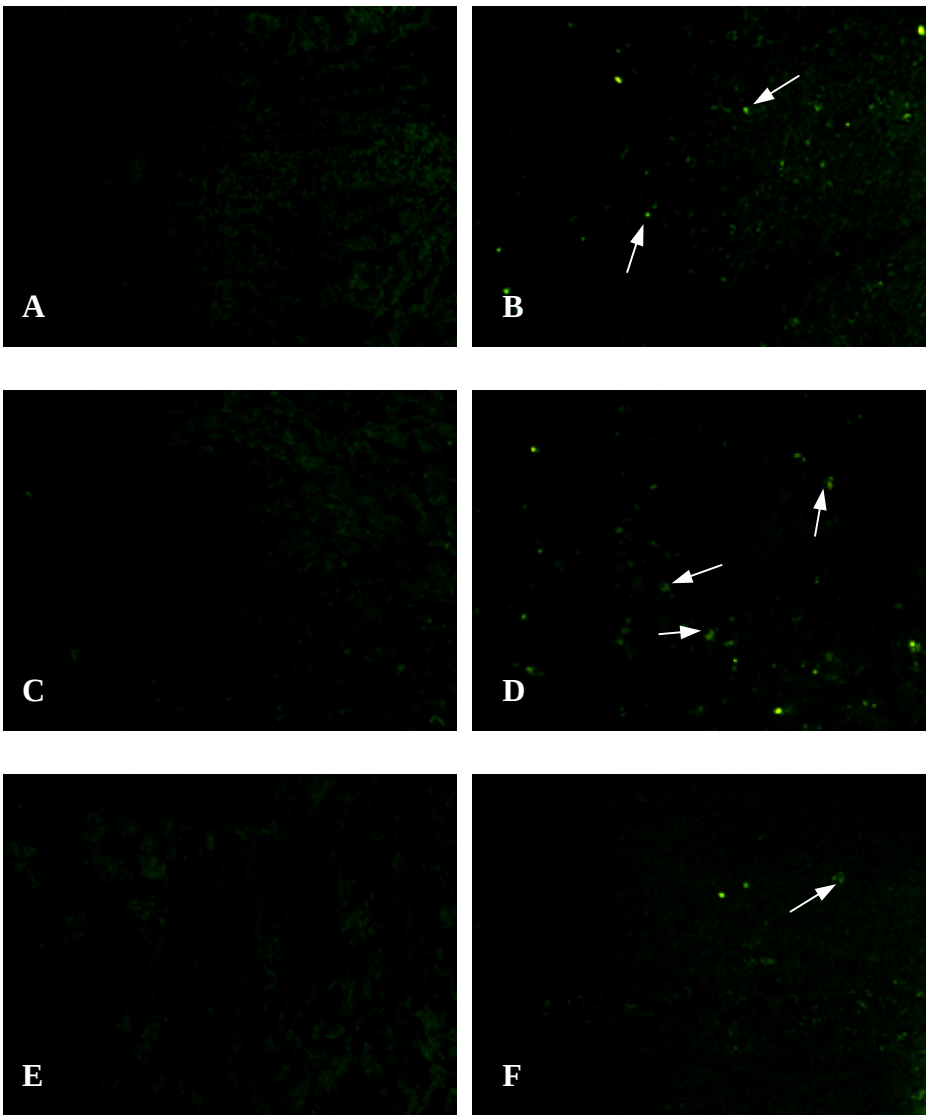

Fig. 8. Photomicrographs (200X) of the rat spinal cord showing the immunofluorescence for glial cell line-derived neurotrophic factor (GDNF). Rats were subjected to complete spinal cord transection and transplantation of neural stem cells (NSCs) between the two cord stumps. The NSCs were modified with adeno-associated viral (AAV) vectors carrying enhanced green fluorescent protein (eGFP) or (GDNF) gene. The animal was sacrificed 4 months after treatment and the spinal cord segments 1 cm rostral to (A, B), around (C, D), and caudal to (E, F) the lesion were subjected to cryosectioning and immunofluorescence staining. Many GDNF-positive cells (arrows) were found in the rostral (B) and injured (D) segments, but only few in the caudal segment (F), of the NSC-AAV-GDNF-treated rats. By contrast, GDNF-positive cells were not seen in any segment of spinal cord in the NSC-AAV-eGFP-treated rats (A, C, E).

Discussion

In this study, we used a strategy of combined NSC transplantation and AAV vector-mediated GDNF gene therapy to treat spinal cord injury in rats, which was never reported by previous studies. The results demonstrated that the hindlimbs of rats receiving NSC transplantation with GDNF gene therapy in the lesion site of the spinal cord significantly contracted, though not

supported, from 7 weeks after treatment. This effect lasted at least for 9 weeks, i.e., till 4 months after treatment. On the contrary, in rats receiving transplantation of NSCs with eGFP gene transfer or nerve bridging with saline injection, marked hindlimb contraction was only seen during 7-10 weeks after treatment, but became to abolish thereafter. It indicates that GDNF expressed by the transplanted NSCs may augment and extend the regenerative effect of the NSCs themselves or the intercostal nerve bridge. However, the animals still could not support their weight with the hindlimbs during our observation period. Longer time will be needed to observe the effect of such combined treating strategy in the future studies.

Four months after NSC transplantation and GDNF gene therapy, many NSCs, neurons and astrocytes were found in the spinal cord rostral to and around, but not caudal to the lesion and transplantation site. The above cells were better incorporated and organized in the rostral than the injured tissue. Similarly, many GDNF-postitive cells were also seen in the rostral and injured segments, but only few in the caudal segment of the NSC-AAV-GDNF-treated spinal cord. These findings indicate that the environment, at least in terms of the GDNF supply, was still unfavorable to neural regeneration in regions distal to the NSC transplantation. This may explain why the hindlimbs of the animal still could not support its weight at that time. In the NSC-AAV-eGFP-treated animals, no NSCs and only few neurons and astrocytes were seen in the corresponding segments of the spinal cord, suggesting that the transplanted NSCs could not survive this period without the supply of GDNF. This was why the hindlimb motor function deteriorated from 8-9 weeks after NSC transplantation in the eGFP group. Whether the neurons and astrocytes found in the NSC-AAV-GDNF-treated spinal cord were differentiated from the transplanted or endogenous NSCs was unclear in this study, since the transplanted NSCs did not simultaneously carry eGFP gene for tracing. Similarly, whether the GDNF-positive cells were transplanted or endogenous NSCs or differentiated neural cells was uncertain due to lack in evidence of double immunostaining for GDNF and specific neural cell markers.

In the culture medium, the quantities of intracellular and extracellular GDNF produced by

NSCs transfected with AAV-GDNF reached a plateau in one day after transfection. This characteristic was favorable to the injured spinal cord since early expression of GDNF by the transplanted NSCs may exempt more neural cells from apoptosis or death in the acute stage. The secreted GDNF disappeared while the intracellular GDNF decreased to a half-peak level 14 days after transfection. This phenomenon seemed unfavorable for long-term effect of GDNF gene therapy. Nevertheless, GDNF-positive cells were present in the spinal cord around the lesion and NSC transplantation site even 4 months after transplantation. Although the extracellular GDNF contents of the spinal cord were not measured, it is very likely that the much more survival and differentiation of neurons and astrocytes in the NSC-AAV-GDNF-treated spinal cord is due to the effect of long-term released GDNF. However, quantification of the tissue GDNF contents of the spinal cord is needed to confirm this possibility.

Whether the joint movement of the

hindlimbs in the NSC-AAV-GDNF-treated animals was due to reconnection of the central and peripheral nerve fibers or local neural circuits remains to be investigated by neurophysiological studies or nerve tracing. On the other hand, the effect of NSC-AAV-GDNF on regeneration of oligodendrocytes should also be studied in the future, because the latter is important to axonal regeneration in the injured spinal cord. Additionally, the dose, site and timing for NSC transplantation need to be further explored to find the optimal mode for administration to spinal cord injury.

In summary, we preliminarily demonstrated the beneficial effect of combined NSC transplantation and AAV-mediated GDNF gene therapy on spinal cord injury. This type of treatment partially improved the hindlimb motor function of animals with SCI. However, it is still far away from recovery of steady standing and gait with coordination. The mechanisms exerted by NSC transplantation and GDNF gene therapy deserve further investigations.

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