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Human periodontal ligament stem cells repair mental nerve injury*

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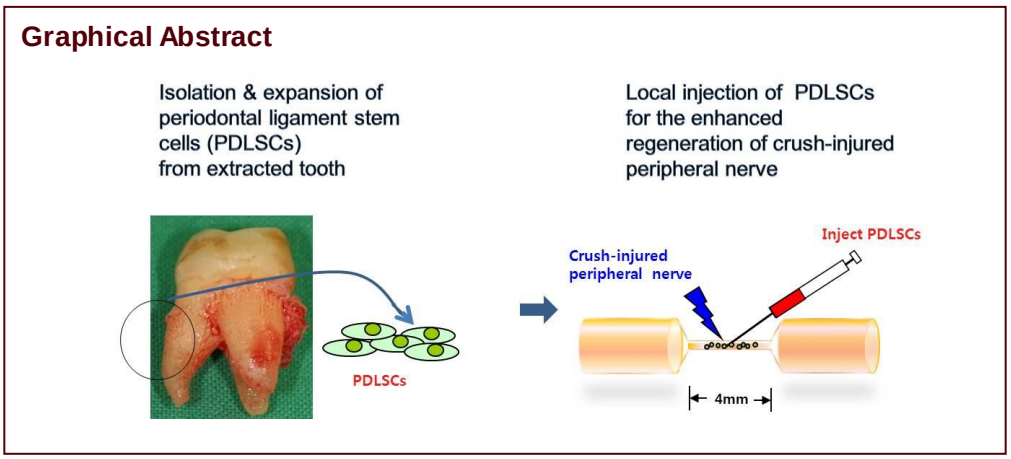
Graphical Abstract



Abstract

Human periodontal ligament stem cells are easily accessible and can differentiate into Schwann cells. We hypothesized that human periodontal ligament stem cells can be used as an alternative source for the autologous Schwann cells in promoting the regeneration of injured peripheral nerve. To validate this hypothesis, human periodontal ligament stem cells (1×10^6) were injected into the crush-injured left mental nerve in rats. Simultaneously, autologous Schwann cells (1×10^6) and PBS were also injected as controls. Real-time reverse transcriptase polymerase chain reaction showed that at 5 days after injection, mRNA expression of low affinity nerve growth factor receptor was significantaly increased in the left trigeminal ganglion of rats with mental nerve injury. Sensory tests, histomorphometric evaluation and retrograde labeling demonstrated that at 2 and 4 weeks after injection, sensory function was significantly improved, the numbers of retrograde labeled sensory neurons and myelinated axons were significantly increased, and human periodontal ligament stem cells and autologous Schwann cells exhibited similar therapeutic effects. These findings suggest that transplantation of human periodontal ligament stem cells show a potential value in repair of mental nerve injury.

Key Words

neural regeneration; peripheral nerve injury; stem cells; periodontal ligament stem cells; mental nerve; Schwann cells; cell transplantation; sensory nerve; neurotrophic factor; neuroregeneration

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Conflicts of interest: None declared.

Ethical approval: Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Laboratory Animal Resources of Seoul National University in Korea.

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INTRODUCTION

The mental nerve is often injured during dentoalveolar, orthognathic, or tumor surgery[1-8]. As a consequence, a large proportion of patients with mental nerve injury experience various forms of permanent sensory disturbances, including persistent paresthesia, severe dysesthesia and pain[8-10]. Numerous therapeutic interventions, including surgery and pharmacotherapy, have been used to enhance the functional recovery of nerve injuries[11-12]. However, the clinical effect is usually unsatisfactory, especially after severe injury[13-15].

In peripheral axonal regeneration, Schwann cells play an integral role[16-17] and Schwann cells transplantation has been shown to enhance axonal outgrowth both *in vitro* and *in vivo* [18-21]. However, harvesting autologous Schwann cells may result in defects to the peripheral nerves of the donor. Furthermore, isolation and expansion of Schwann cells to obtain a sufficient amount within a reasonable time period is technically difficult. So finding a favorable isolation and expansion profiles of autologous stem cells, or improving the possibility of differentiating these cells into Schwann cell phenotypes is the reason of an increased interest in use of stem cells for regenerative medicine and tissue engineering[22-23]. Many kinds of mesenchymal stem cells transplanted into lesions in the peripheral nervous system can then integrate into axonal pathways to regenerate and re-myelinate injured axons[19-20]. Mesenchymal stem cells from bone marrow and umbilical cord were reported to differentiate sufficiently into cells with Schwann cell properties to support limited neurite outgrowth *in vitro* and axonal regeneration equivalent to that of Schwann cells *in vitro* and *in vivo* [22, 24-25]. In our previous study, it was reported that co-transplantation of umbilical cord mesenchymal stem cells and Schwann cells in a sciatic 5-mm axotomy defect noticeably augmented nerve regeneration[26-27]. Human umbilical cord blood-derived mesenchymal stem cells injected into the crush-injured rat sciatic nerves also promoted nerve regeneration[28]. If stem cells are transplanted at the time of injury, they can reduce the time needed to achieve nerve repair by creating a population of glial cells that are ready to support and enhance nerve regeneration when neurons switch to regenerative metabolism[25]. Such approaches may also make surgical repair of nerve injury sites easier. Specifically, the application of stem cells at the time of primary surgical wound inspection may increase the clinical applicability of stem cell-based therapies.

Recently, periodontal ligament stem cells have provided an appropriate source of cells for periodontal tissue regeneration[29-30]. Human periodontal ligament stem cells have a number of advantageous properties, including the fact that they are easily accessible, making them attractive as potential sources for cell therapies[30]. In addition, periodontal ligament stem cells are associated with fewer ethical problems with respect to their use, so long as informed consent is obtained from donors. Likewise, periodontal ligament stem cells are able to differentiate into other cell types and subsequently proliferate. Widera *et al* [31] in 2007 reported periodontal ligament stem cells can be differentiated into neuronal and glial cell lineages. Noticeably, periodontal ligament stem cells can be harvested donor-specifically and autologously during routine periodontal surgery or from wisdom and premolar teeth intended for extraction due to therapeutic reasons, a major practical advantage with respect to widened clinical use[30-31]. Thus, periodontal ligament stem cells can be a promising alternative source of autologous Schwann cells for the repair of peripheral nerve damage. The purpose of the study was to evaluate the effects of transplantation of human periodontal ligament stem cells on the regeneration of rat mental nerve following a crush injury.

RESULTS

Quantitative analysis of experimental animals

A total of 54 rats were initially included and

then randomly and equally divided into three groups (n = 18): PBS, Schwann cell, and periodontal ligament stem cell groups. Rats in these three groups were given injection of PBS, Schwann cells, and periodontal ligament stem cells to injury sites, respectively, after nerve injury and included in the final analysis.

Six rats in each group were used for sensory tests and histomorphometric evaluation, six rats were used for retrograde labeling, and the remaining six rats were allocated for real-time reverse transcriptase polymerase chain reaction (RT-PCR) of the trigeminal ganglion.

Nerve growth factor (NGF), p75^{NTR}, and trkA mRNA expression in the trigeminal ganglions

Quantitative RT-PCR of left side (affected) trigeminal ganglions showed that the highest mRNA expression of NGF, p75^{NTR}, and trkA in the Schwann cell group appeared on the 5th day following treatment. Furthermore, although mRNA expression of NGF, p75^{NTR}, and trkA in the periodontal ligament stem cell group was higher compared with the PBS group, only p75^{NTR} expression was increased significantly. The mRNA expression of NGF and p75^{NTR} in the periodontal ligament stem cell group was similar to the Schwann cell group (Figure 1).

Functional recovery of rats

Sensory tests were conducted to assess the functional recovery of mental nerve. The difference score decreased in all three groups during the observation period. Similar to the Schwann cell group, the periodontal ligament stem cell group exhibited a significant reduction in the post-treatment mean difference score compared with the PBS group after 4 weeks. However, no significant difference was found between the Schwann cell and periodontal ligament stem cell groups (Figure 2A). The scale of the difference gap showed a significant decrease in the periodontal ligament stem cell group during the 1st and 2nd weeks compared with the PBS group; however, no significant difference was observed when compared with the Schwann cell group (Figure 2B).

Quantification of labeled sensory neurons

Representative photomicrographs of retrograde labeling at the trigeminal ganglions are illustrated in Figure 3 A–C. The number of labeled sensory neurons counted in the periodontal ligament stem cell and Schwann cell groups was significantly higher than that in the PBS group (P < 0.05) (Figure 3D). The difference between Schwann cell group and periodontal ligament stem cell group was not statistically different.

Quantification of myelinated axons

Sections distal to the crush injury site stained with toluidine blue revealed an appearance typical of regenerating nerves (Figure 4A–C), characterized by the presence of myelinated fibers of small and medium size, small clusters of fascicles, and an enlarged area of connective matrix. Nerves in the periodontal ligament stem cell group showed a markedly higher axon number compared to the PBS group, but not significantly different from the Schwann cell group (Figure 4D).

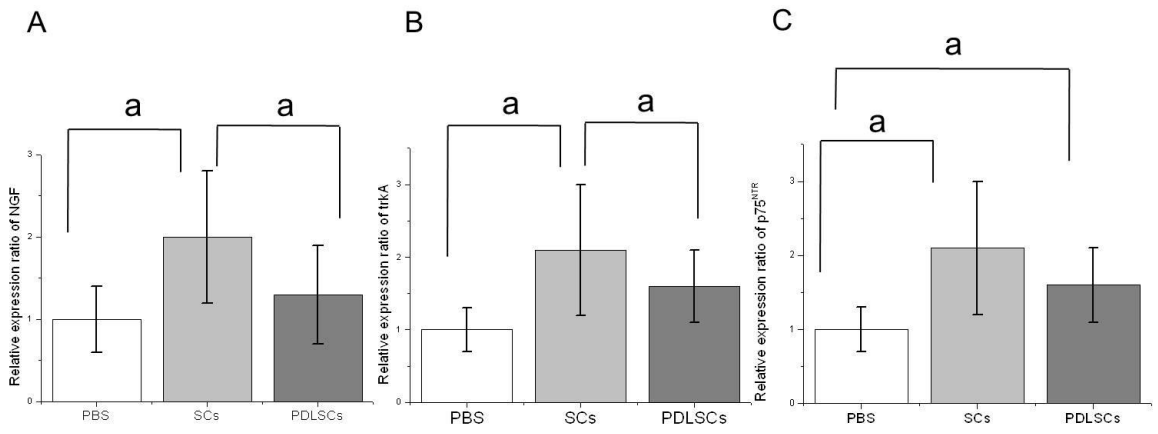

Figure 1 Quantification of mRNA expression of nerve growth factor (NGF), trkA and p75^{NTR} at injury site 5 days after treatment (real-time reverse transcription-PCR).

Data are presented as mean ± SD of six rats per group and analyzed by one-way analysis of variance followed by *post hoc* least significant difference test (LSD). ^{a}P < 0.05. SCs: Schwann cells; PDLSCs: periodontal ligament stem cells.

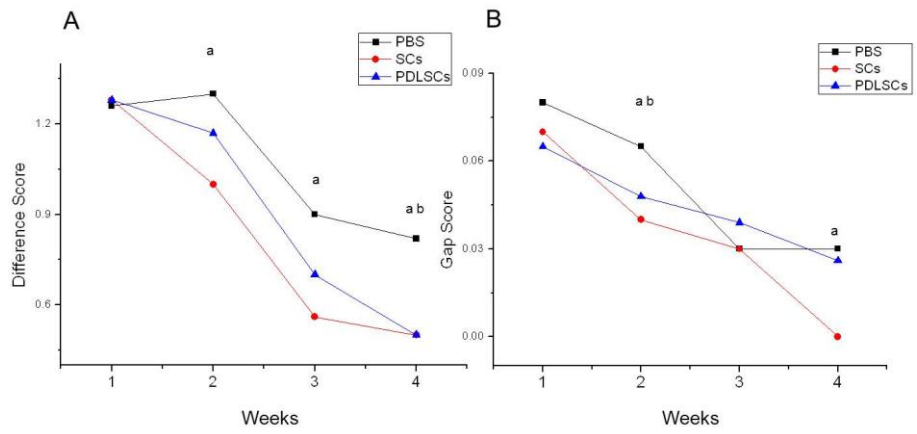

Figure 2 Sensory function of rats treated with periodontal ligament stem cells or Schwann cells.

Sensory function was assessed by sensory tests. One-way analysis of variance followed by least significant difference test (LSD) was used to compare sensory test values between groups at each time interval. Data are presented as mean ± SD of six rats per group. (A) Difference score. [a]$P < 0.05$, *vs*. Schwann cell group (SCs) at 2, 3 and 4 weeks; [b]$P < 0.05$, *vs*. periodontal ligament stem cell group (PDLSCs) at 4 weeks. (B) Gap score. [a]$P < 0.05$, *vs*. SCs at 2 and 4 weeks; [b]$P < 0.05$, *vs*. PDLSCs at 1 and 2 weeks.

DISCUSSION

Initially, we used the inferior alveolar nerve as an injury model to simulate a dental-implant-associated crush injury, but the animal suffered too much surgical trauma from bone removal to access the nerve. Furthermore, the inferior alveolar nerve was damaged during the nerve-isolating procedure. However, as a sensory nerve model, the mental nerve was very easy to locate and expose an approximately 2-cm length for treatment and evaluation[32]. Also, most of the sensory neurons from the inferior nerve are localized in the trigeminal ganglion, and most of its axons (65–70%) are distributed to the mental nerve in rats[33].

The rationale of using nerve crush model instead of nerve defect or transection model is as follows: We sometimes use other models such as nerve transection or nerve defect model. However, the problem of these models is lack of reproducibility because different levels of microsurgical technique (microsuture and micro-handling) resulted in variable degrees of nerve regeneration. Furthermore, the injection can be leaked out through the cut window or the gap approximated by microsutures in cases of cut model[34]. Besides this model, we have transection nerve model and nerve stretching model. However, in case of installing dental implantation, the trigeminal nerve is generally crushed, not transected nor stretched.

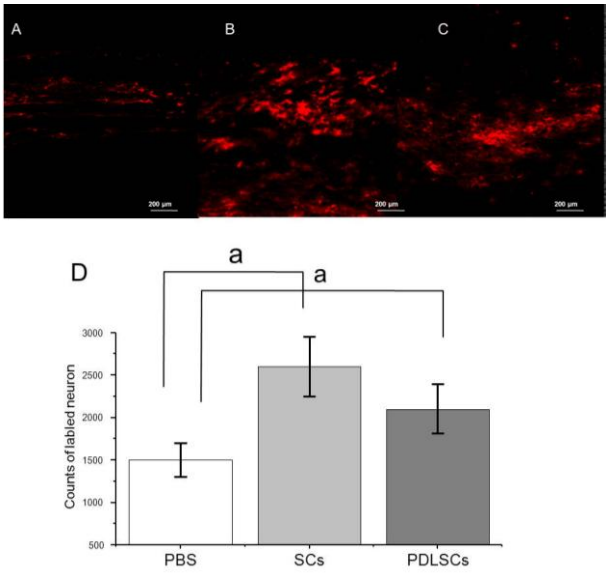

Figure 3 Representative photomicrographs of retrograded trigeminal ganglion after 5 day labeling with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI).

(A–C) PBS, SC, and PDLSC groups. Scale bars: 200 μm. (D) Bar graphs of the total number of labeled sensory neurons. Data are presented as mean ± SD of six rats per group. [a]$P < 0.05$. One-way analysis of variance followed by post hoc least significant difference test (LSD) was used to compare DiI retrograde tracing results. The positive trigeminal ganglion neuron was labeled with red color. There were more positive trigeminal ganglion neurons in the Schwann cell (SC) and periodontal ligament stem cell groups (PDLSC) than the PBS group.

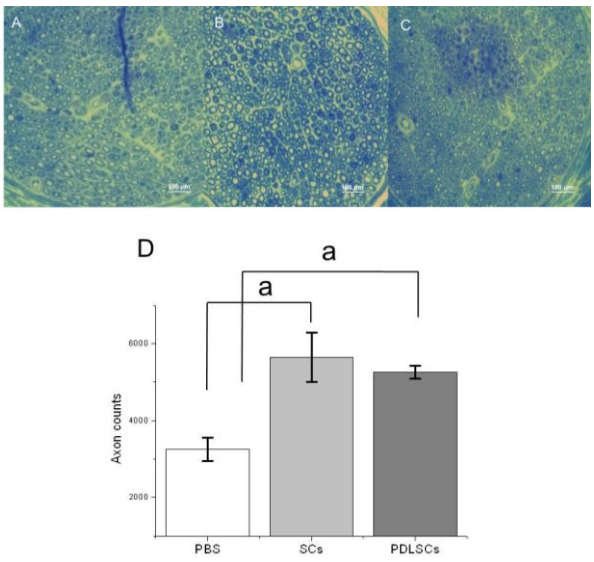

Figure 4 Photomicrographs of histologic features in semithin sections obtained distal to the crush injury site (4 weeks postoperatively).

The total fiber number (N) was then calculated by multiplying the mean fiber density by the total cross-sectional area of the whole nerve cross section. (A–C) PBS, Schwann cell (SC) and periodontal ligament stem cell (PDLSC) groups, respectively. Scale bars: 100 μm. (D) Myelinated axon number. Data are presented as mean ± SD of six rats per group. The number of myelinated axons in the periodontal ligament stem cell group (arrow) was significantly greater than in the PBS group ($^aP < 0.05$). One-way analysis of variance followed by *post hoc* least significant difference (LSD) was used. The difference between SC and PDLSC groups was not significant.

One of the distressing complications of dental implant installation is damage to the inferior alveolar nerve, where the nerve is generally crushed by the fixtures, not transected. That is the second reason why we chose mental nerve crush model. This model is more close to the clinical situation in our field. Although the crush model has drawbacks like self-regeneration with certain time without any intervention, it is more precise and reproducible to assess subtle changes after treatment.

We measured the level of NGF/p75^{NTR}/trkA mRNA in trigeminal ganglions. NGF is a prototypical neurotrophin family member believed to enhance peripheral nerve regeneration. Elevated NGF levels in culture medium of explanted chick embryo dorsal root ganglia induces neurite outgrowth from ganglial cell bodies[35]. In addition, a model of transected sciatic nerve in adult, male, Wistar rats demonstrated that 4-methylcatechol stimulated synthesis of NGF (and/or NGF-related molecules) increased peripheral nerve regeneration[36]. NGF has been shown to directly increase Schwann cell survival and migration, as well as enhance axonal regeneration and indirectly prevent death of axotomized sensory neurons[37]. Hence, the increased NGF and its neurotrophin receptor p75NTR and trkA in the trigeminal ganglion can be an indicator of nerve regeneration. Our finding that the expression of NGF and trkA in the trigeminal ganglion sensory neurons was higher by the fifth postoperative day in the periodontal ligament stem cell group than PBS group may reflect a higher regeneration activity induced by periodontal ligament stem cell injection to the crush injury site.

Periodontal ligament stem cells and Schwann cells were successfully injected into the crush-injury site without leakage. The fate of the transplanted periodontal ligament stem cells in our study remains unclear. We previously showed that stem cells (human umbilical cord mesenchymal stem cells and umbilical cord blood mesenchymal stem cells) injected into rat sciatic nerves are viable over a period of 4 weeks, but that cell numbers generally decrease with time[27-28]. To the best of our knowledge, there is currently no data concerning the survival of periodontal ligament stem cells *in vivo*; however, other studies have reported that 0.5–38% of the remaining transplanted cell types are viable[38-39]. Although periodontal ligament stem cells might exert their supportive effect by providing an initial regenerative boost while also facilitating the sub-acute phase of nerve regeneration, new strategies need to be developed to improve stem cell survival and improve PDLSC-mediated nerve regeneration. Specifically, the ability of nutrients to diffuse into the nerve conduits as well as the attachment of stem cells to the biomaterial constituting the conduit is essential and must be optimized, especially for peripheral nerve regeneration. Concomitant delivery of trophic factors such as granulocyte-colony stimulating factor and vascular endothelial growth factor is another promising approach to improve stem cell survival[22]. It is also important to determine the number of transplanted stem cells required to achieve the desired regenerative effect. In our study, we chose an injection concentration of 1×10^6/cells per rat, which was the same as Erba and colleagues[38], who reported the regeneration potential and survival of transplanted undifferentiated adipose tissue-derived stem cells in peripheral nerve conduits. This approach was different from that of Matsuse *et al*[22], who used transpermeable tubes filled with human umbilical cord stromal cells ($1–2 \times 10^7$/mL per person) for nerve regeneration.

In this experiment, we did not measure the protein level

of NGF/p75^{NTR}/trkA, instead their mRNA expression level was checked. In our previous unpublished data, periodontal ligament stem cells were confirmed to produce NGF. Although the amount of NGF secreted by the transplanted periodontal ligament stem cells *in vivo* was not measured in this experiment, periodontal ligament stem cells transplanted into the distal end of trigeminal ganglions can increase their expression in trigeminal ganglions via distal neurotrophic influence of periodontal ligament stem cells. The hypothesis of periodontal ligament stem cell transplantation to the injury site of nerve is that the transplanted periodontal ligament stem cells can be differentiated into Schwann-like cells if they are alive or periodontal ligament stem cells can be a source of positive material for inducing axonal regeneration if they are dying. There are reports that neuronal progenitor cells grafted into peripheral nerve gaps may differentiate into Schwann cell-like supportive cells[40]. According to Cuevas *et al* [41], 5% of transplanted stem cells become Schwann cells. Zhang *et al* [42] assessed the ability of mesenchymal stem cells to differentiate into Schwann cells in mechanically injured rat sciatic nerves, and found that mesenchymal stem cells had been incorporated around the injured nerves and demonstrated partial expression of GFAP, S-100, and P75. In our previous experiment, umbilical cord mesenchymal stem cells and umbilical cord blood mesenchymal stem cells enhanced peripheral nerve regeneration functionally, electrophysiologically, and histomorphometrically, suggesting that undifferentiated stem cells can differentiate into Schwann cells *in vivo* [28].

It is generally known that the stem cells are less immunoreactive. Henning *et al* [43-44] demonstrated that direct injection of human umbilical cord blood cells is an effective treatment for acute myocardial infarction model in rats without any immnunosuppressive drugs. Kim *et al* [45] also reported successful human umbilical cord blood-derived multipotent stem cells therapy for Buerger's disease at animal limb model without any immunosuppression. Those are the rationale for xenotransplantation without immune suppression. However, in some experiments, cyclosporin or FK506 has been still used to evade immune reaction for xenotranplantaion research model. In fact, there have been numerous reports of positive nerve regeneration in animals and humans undergoing immunosuppression by FK506[46-58]. Although previous studies found that FK506 is maximally effective when administered at high doses (5–10 mg/kg per day) during the entire regeneration period in a rat sciatic nerve model[58], prolonged systemic immunosuppression might not be justified for ensuring the success of nerve regeneration. Wang and colleagues[59] examined the effect of low-dose FK506 on nerve regeneration in a model more applicable to the severe peripheral nerve injuries observed in a clinical setting, and showed that it was feasible to reduce the dose of FK506. In the present study, a dose of 0.5 mg/kg per day was selected based on observed side effects and nerve regeneration[59-60]. In this experiment, an animal group which received only FK506 was not established. But comparison between periodontal ligament stem cells + FK506 group and FK506 group is necessary to check pure effect of locally injected periodontal ligament stem cells on the regeneration of crushed mental nerve.

Transplantation of periodontal ligament stem cells into a crushed mental nerve enhanced the recovery of sensory function and axonal regeneration. Notably, there was a significant improvement in sensory recovery, especially by the 4th week after transplantation, supporting the therapeutic effects of transplanted periodontal ligament stem cells on a crushed mental nerve injury. Furthermore, the effectiveness of periodontal ligament stem cells for axonal regeneration was comparable to that of Schwann cells.

Taken together, local injection of periodontal ligament stem cells to an injury site is a potential strategy for peripheral nerve regeneration following crush injury at the mental nerve. The periodontal ligament stem cells injection approach resulted in nerve regeneration similar to the level obtained after administration of autologous Schwann cells.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

Experiments were performed at the Department of Oral and Maxillofacial Surgery, Seoul National University Dental Hospital, Seoul, Korea, between January 2008 and May 2010.

Materials

A total of 54 male Sprague-Dawley rats, aged 6 weeks and weighing approximately 200–250 g, were purchased from an animal supplier (Samtaco Co., Korea). Surgeries on the animals were performed 1 week after housing adaptation. All animal surgeries and experimental procedures were carried out in accordance with the care

guidelines of the Laboratory Animal Resources of Seoul National University, Korea.

Methods

Isolation and culture of autologous Schwann cells

Autologous Schwann cells were harvested and isolated using a modified method reported by Jirsova *et al*[61]. In order to prepare autologous Schwann cells, each rat was identified with number not to confuse the donor animal. A portion of the left sciatic nerve approximately 2.5 cm in length was harvested and kept in Dulbecco's modified Eagle's medium plus glutamax (DMEM; Invitrogen, Carlsbad, CA, USA) containing 1% penicillin streptomycin. After the perineural tissue and epineurium were stripped off by microscissors and microforceps following nerve dissection into segments 1 cm in length, nerve fascicles were isolated and washed with the same medium in non-coated wells of a 6-well plate. Nerve fascicles underwent pre-degeneration in DMEM containing 2 μmol/L forskolin (Merck Co., Germany), 10 ng/mL fibroblast growth factor (Invitrogen), 50 ng/mL glia growth factor (Acorda Therapeutics, Hawthorne, New York, USA), and 5 μg/mL bovine pituitary extract (Invitrogen) for 2 weeks. The medium was changed 2 to 3 times per week.

Pre-degenerated nerve fascicles were then enzymatically dissociated by incubating the cells with dissociation solution containing 10% fetal bovine serum (FBS), 1% pen/strep, 0.125% collagenase (Type IV; Sigma, St. Louis, MO, USA), and 1.25 U/mL dispase (Roche Diagnostics Co., Germany) for 24 hours at 37°C and 5% CO_2. Dissociation solution and tissue residues were then transferred into new tubes, to which an equivalent amount of Hank's Balanced Salt Solution (HBSS; Sigma) was added to quench enzymatic activity. Tissue residues were thoroughly processed using Pasteur glass pipettes until a homogeneous solution was obtained, after which samples were centrifuged at 800 r/min for 5 minutes at room temperature. The supernatant was removed and the pellet was re-suspended in 10% FBS DMEM. Centrifugation was performed once more as described above, and primary peripheral nerve cells were plated at a density of 1 x 10^6 living (trypan blue-negative) cells per well on a laminin-ornithine- coated 6-well plate (35 mm^2). DMEM supplemented with 10 ng/mL heregulin (R&D Systems Co., Germany), 2 μmol/L forskolin, 10 ng/mL fibroblast growth factor, 50 ng/mL glial growth factor, and 5 μg/mL bovine pituitary extract served as a growth medium, to which 1% bovine serum albumin was added after 24 hours to enhance seeding efficiency. On the second day, the media was changed to growth medium without bovine serum albumin to flush out any residues so that only primary Schwann cells were left for continuing culture. Cultured cells were subsequently maintained in DMEM supplemented with 10% FBS, 2 μL forskolin, and 2 ng/mL heregulin (Sigma) to stimulate Schwann cell proliferation.

The detachment of Schwann cells, grown with the ground fibroblasts layer, was conducted with ice-cold growth medium by means of a 1 mL blue tip and monitored by phase-contrast microscopy. The suspension of floating cells, mainly Schwann cells, was transferred into newly laminin-ornithine coated 6-well plates and cultured at 37°C and 5% CO_2. Cold jet technique was repeatedly applied up to three times if fibroblast contamination was observed again. It could achieve a stably high cell yield from human nerves with final purity above 99% within 5 days.

Isolation and culture of periodontal ligament stem cells

Human PDLSCs from the wisdom teeth were kindly prepared by the Laboratory of Department of Periodontology, College of Dentistry, Yonsei University, Korea. The multipotency of culture-expanded human periodontal ligament stem cells was maintained, and the cells were confirmed to be immunopositive for early mesenchymal progenitor marker STRO-1.8[62].

Passage 0 (P0) periodontal ligament stem cells were seeded at a density of 1.0 × 10^4 cells in a 100-mm dish with 2 mL of serum-free α-MEM at 37°C for 60 min. Periodontal ligament stem cells were then cultured in α-MEM (Gibco BRL Life Technologies, Gaithersburg, MD, USA) supplemented with 15% fetal bovine serum (Gibco BRL), 2 mmol/L L-glutamine (Gibco BRL Life Technologies), 100 μmol/L L-ascorbate- 2-phosphate (Sigma), and 1% antibioticantimycotic (Gibco BRL Life Technologies,). Cells were incubated at 37°C in 95% humidified air and 5% CO_2.

Establishment of models of mental nerve injury and stem cell transplantation

Animals were anesthetized with an intraperitoneal injection of a 4:1 mixture of ketamine HCl and xylazine hydrochloride. Next, a submandibular skin incision was made and the left mental nerve was exposed to the mental foramen. A standard surgical needle holder engaged to the second clip of both arms was then used to create a crushing injury at a distance approximately 10 mm distal to the mental foramen. The nerve was clamped for 30 seconds to produce a 4-mm-wide crush

injury. To allow for later identification, the injury site was marked by introducing a single 9-0 Nylon (Ethicon®, Gargrave, UK) epineural stitch under 16 × magnification using a surgical microscope (OPMI Carl Ziess, Oberkochen, Germany) at the distal limit of the injury.

The PBS group received PBS (6 μL/rat) immediately after injury, while each rat in the Schwann cells and periodontal ligament stem cell groups received a single injection of 6 μL of PBS containing 1 x 10^6 Schwann cells or periodontal ligament stem cells to the crushed site. To alleviate the anticipated immune response following initiation of human periodontal ligament stem cell xenografts, FK506 was subcutaneously administered in the periodontal ligament stem cell group for 7 days (0.5 mg/kg per day). Cells were injected at the crush site immediately after the crush injury using a Hamilton syringe with a 33-gauge needle.

Quantification of NGF, p75^{NTR}, and trkA mRNA expression by real time RT-PCR

Five days after surgery, six animals in each group were anesthetized and the trigeminal ganglions on the left side were harvested. RNA was extracted using Trizol reagent (Invitrogen). Purified RNA was obtained using a RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). mRNA encoding NGF, p75^{NTR}, and trkA was reverse transcribed to cDNA using a first-strand synthesis kit (Invitrogen) and the amount of cDNA was quantified using real-time PCR[63]. The following primers were used to amplify specific cDNA regions of the transcripts of interest: NGF (GeneBank Reference Sequence No. XM¬_227523.3), p75NTR (GeneBank Reference Sequence No. X05137.1), trkA (GeneBank Reference Sequence No. M85214.1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GeneBank Reference Sequence No. NM¬_017008.3). GAPDH quantification was used as a normalization control. The percentage differences in the mRNA levels over control values were calculated using the ΔCt method as described previously (Applied Biosystems Manual, Foster City, CA, USA) by Chen *et al* [63]. PCR reactions were repeated at least twice.

Sensory tests

Sensory tests were conducted and scored as described previously by Seino *et al* [64] and were used to compare the behavioral response sensitivities to mechanical stimulation. A series of von Frey filaments (Semmes-Weinstein Monofilaments, North Coast Medical, Inc., CA, USA) were used to determine touch sensitivity to mechanical stimulation. Von Frey filaments (bending force: 0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, and 6.0 g) were delivered from above to the center of the whisker pad. Head withdrawal and touching or scratching of the facial regions upon application of a von Frey filament was considered a positive response. A negative response was defined as a lack of two sequential withdrawal responses elicited by three sequential stimulations. The different score was defined as the difference between the mechanical touch thresholds (grams) of the ipsilateral and contralateral sides of the injury, and calculated as the value of the ipsilateral mental area (b) minus the value of the contralateral area (d). The gap score was defined as the difference between the mechanical touch thresholds of the medial and distal parts of the mental nerve, and calculated as the value of the ipsilateral lip area (a) minus the value in the vicinity of the mental foramen (b). By definition, higher scores indicate a poorer recovery; as a damaged nerve recovers functionally, its score approaches zero. Statistical comparisons of differences in the mean scores at post-operative weeks 1, 2, 3, and 4 within each group were performed.

Retrograde labeling and quantification of sensory neurons

Trigeminal ganglion neurons were labeled retrogradely with the fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) to count the regenerated nerve fibers. Mental nerves were cut distal to the crush injury lesion at 4 weeks postoperatively, and DiI was placed onto the proximal end of transected mental nerve. After wound closure, the rats were allowed to recover. Five days later, the animals were deeply anesthetized and perfused with saline and paraformaldehyde solutions as described by Savignat *et al* [65]. After performing a craniotomy, the ipsilateral trigeminal ganglia were removed and post-fixed overnight with a solution of paraformaldehyde. The ganglia were immersed in a 20% sucrose solution for 2 days, then embedded in Tissue Tek (Sakura, Japan) and frozen in liquid nitrogen. Serial 35 μm longitudinal sections were generated at –20°C in a cryostat microtome (Leica CM30505 Cryostat, Italy). Sections were then observed under a fluorescence microscope equipped with a rhodamine filter (Olympus FV-300, Japan). The sections obtained from the top and bottom of the trigeminal ganglion on the left side, as well as those showing signs of technical or processing errors, were excluded from counting. The labeled neurons in each trigeminal ganglion section were randomly selected and their area (neuronal soma size) was measured and averaged with computer software (OPTIMAS version 6.5) according to

the technique of Savignat *et al* [65].

Histomorphometric evaluation

At the end of a 4-week follow-up period, six rats from each group were anesthetized. Mental nerves were then re-exposed and the nerve segment, including the crush-injury site, was harvested. The nerves were immediately immersed into a fixation solution containing 2.5% glutaraldehyde in PBS (pH 7.4) at 4°C for 24 hours. Only the distal portion (5 mm distal to the injury) was used for histomorphometric evaluation. The nerve segment was post-fixed with 2% osmium tetroxide for 2 hours. Next, it was washed with a solution of PBS (pH 7.4) and routinely processed and embedded in Epon 812 (Nisshin EM, Tokyo, Japan). Serial transverse semi-thin sections 1 μm in thickness were cut with a microtome (LEICA, Ultracut, UCT, Austria) and stained with 1% toluidine blue for light microscopy examination (Olympus, BX41, TF, Japan). Images were captured using a specialized system, a SPOT RTTM-KE color mosaic (Diagnostic Instruments, Inc., Sterling Heights, MI, USA), and digitized by SPOT software Ver. 4.6 (Diagnostic Instruments, Inc., USA). To simplify myelinated axon counting, the total cross-sectional area of nerves was measured at 40 × (Olympus, BX41, TF, Japan), and three sampling fields were then randomly selected at 200 × magnification as previously reported[47-51]. Mean fiber density was calculated by dividing the total number of nerve fibers within a sampling field by its area (N/mm^2). The total fiber number (N) was then estimated by multiplying the mean fiber density by the total cross-sectional area of the whole nerve cross section, which assumed a uniform distribution of nerve fibers across the entire section.

Statistical analysis

Data analysis was performed using StatView software (Version 5.0.1, SAS Institute, Cary, NC, USA). All data were presented as mean ± SD. One-way analysis of variance followed by least significant difference test (LSD) was used to compare the results of the sensory tests between groups at each time interval, NGF, trkA, and $p75^{NTR}$ mRNA expression, as well as the number of DiI retrograde-labeled neurons and axon numbers between groups postoperatively. Values of $P < 0.05$ were considered statistically significant.

Research background: Many studies have confirmed that transplantation of Schwann cells exhibits therapeutic effects in the treatment of peripheral nerve injury. However, there are several problems in the clinical use of this technique, for example, difficulties in harvesting, isolating and expanding autologous Schwann cells.

Research frontiers: After induced by chemical inducer or co-cultured with Schwann cells, stem cells can differentiate into Schwann cell-like cells. We had tried to induce various stem cells to differentiate into Schwann cell-like cells. We found that under the condition of induction without special inducers, periodontal ligament stem cells can express Schwann cell marker S100. However, there are few reports describing that stem cells can differentiate into mature Schwann cells.

Clinical significance: Results from this study provide theoretical evidence that periodontal ligament stem cells can be used as an alternative source for the autologous Schwann cells in the treatment of peripheral nerve injury.

Academic terminology: Periodontal ligament stem cells are the cells harvested from the periodontal ligament and located between tooth root surface and the surrounding bone.

Peer review: It is interesting that potential periodontal ligament stem cells can be used as autologous Schwann cells in the treatment of peripheral nerve injury. An animal model of facial nerve crush injury without loss of Schwann cells should be considered. In addition, it seems more meaningful to establish an animal model of facial nerve transaction.

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