



Differentiated adipose-derived stem cells promote peripheral nerve regeneration.

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Differentiated adipose-derived stem cells promote peripheral nerve regeneration

Abstract

Introduction: Many reports have indicated that adipose-derived stem cells (ADSCs) are effective for nerve regeneration. We investigated nerve regeneration by combining a polyglycolic acid-collagen (PGA-c) tube, which is approved for clinical use, and Schwann cell-like differentiated ADSCs (dADSCs).

Methods: 15-mm-long gaps in the sciatic nerve of rats were bridged in each group with tubes (group I), with tubes injected with dADSCs (group II), or by resected nerve (group III).

Results: Axonal outgrowth was greater in group II than in group I. Tibialis anterior muscle weight revealed recovery only in group III. Latency in nerve conduction studies was equivalent in group II and III, but action potential was lower in group II. Transplanted dADSCs maintained Schwann cell marker expression. ATF3 expression level in dorsal root ganglia was equivalent in groups II and III.

Discussion: dADSCs maintained their differentiated state in the tubes and are believed to have contributed to nerve regeneration.

Key words: adipose-derived stem cells, tubulization, critical gap, differentiation, peripheral nerve, regeneration

Introduction

Currently, autologous nerve grafting is often the first choice for primary, irreparable peripheral nerve defects that form as a result of trauma or malignant tumor resection. There is no doubt that autologous nerve grafting is the best procedure in terms of early regeneration of damaged nerves, but it also leads to complaints of new pain and numbness at the nerve donor site, which is its greatest disadvantage. Therefore, Lundborg¹ *et al.* reported the novel concept of tubulization, which induces axonal regeneration by bridging the nerve deficit area with a conduit, as a noninvasive method of nerve grafting; this has become the foundation of current artificial nerve conduit research. The superior performance of autologous nerve grafting may be due to the use of the basement membrane as a scaffold and the abundance of live Schwann cells as a cellular component. Therefore, research on artificial nerve conduits has progressed in two areas: the tube material and its shape as scaffolding for nerve regeneration, and the encapsulation of transplanted cells and growth factors as substitutes for Schwann cells.

Cultured Schwann cells were initially used as transplanted cells, but research using stem cells has flourished recently. Mesenchymal stem cells, such as bone marrow-derived stem cells and adipose-derived stem cells (ADSCs), have been the focus of recent research, in addition to induced pluripotent stem cells and embryonic stem cells. This technology has already been applied *in vivo*, hence relatively few obstacles to clinical use are believed to exist. By encapsulation of a stromal vascular fraction, a cell group that does not undergo differentiation or culturing, into a silicon tube, Suganuma *et al.*² concluded that axonal regeneration is promoted by the trophic effects of secreted humoral factors. There have also been many reports from various investigators that ADSCs promote nerve regeneration²⁻¹¹. Thus, ADSCs could be considered effective grafted cells for nerve regeneration.

Regarding the scaffolding, the initial tube material was composed of silicon, but highly biocompatible materials, such as collagen, poly-glycolic acid, and poly(L-lactide-co-ε-caprolactone), are now used for the tubes. A large number of nerve regeneration induction tubes have been approved and are sold in each country. Currently in Japan, a polyglycolic acid-collagen (PGA-c) tube, comprised of a poly-glycolic acid tube filled with collagen fiber (Nerbridge™, TOYOBO, Osaka, Japan), and a

collagen tube filled with collagen fiber (Renerve™, NIPRO, Osaka, Japan) have received regulatory approval as nerve regeneration tubes, and they are now available as options for clinical use. Building on these principles, in the current study, we investigated peripheral nerve regeneration using an experimental model that combined a PGA-c tube and differentiated ADSCs (dADSCs), which are ADSCs induced to differentiate into Schwann cell-like cells. The aim of this study was to facilitate further nerve regeneration with material that can be used readily in clinical practice based on the results of previous research using ADSCs.

Methods

Animals

This research was approved by the Advanced Science Research Center, Kanazawa University (Approval Number: AP-153584). The experiments used 12–14 week-old, 200–250 g female Wistar rats. After pretreatment with diethyl ether (Wako, Osaka, Japan) inhalation, the rats were anesthetized by intraperitoneal administration of 1% pentobarbital (Kyoritsu, Tokyo, Japan) (2 mg/kg). At study end, the rats were euthanized by intraperitoneal injection of a lethal dose of 10% pentobarbital (2 ml).

Collection and differentiation of ADSCs

Subcutaneous fatty tissue was collected from the inguinal region of the rats, minced with a scalpel, and treated for 45 min in 0.12% Type 1 collagenase (Wako). This mixture was then filtered through a 70-µm filter and centrifuged at 1,300 rpm for 6 min. The supernatant was discarded, the cell pellet was suspended in Dulbecco's Modified Eagle Medium (Wako) containing 10% fetal bovine serum and 1% penicillin/streptomycin and the cells were cultured. The cells were subcultured and then differentiated.

Differentiation was performed in accordance with the report by Kingham *et al.*¹². Passage 2 cells were cultured for 24 h in medium supplemented with 1 mM β-mercaptoethanol (Sigma-Aldrich, Missouri, U.S.A.), and then cultured for 72 h in medium supplemented with 35 ng/ml all-trans retinoic acid (Wako). Then, the cells were cultured for 12 days in differentiation medium (Minimum Essential Medium Eagle- Alpha Modification, supplemented with 5 ng/ml platelet-derived growth factor (Peprotech, New Jersey, U.S.A.), 10 ng/ml basic fibroblast growth factor (Peprotech), 14 µM forskolin (Wako), and 252 ng/ml glial growth factor 2 (Peprotech)). The medium was exchanged every 3 days.

Surgical procedure and cell injection method

After performing general anesthesia using the aforementioned method, an incision was made in the lateral side of the left thigh of the rats to expose the sciatic nerve. A 15-mm length of nerve was resected to create a nerve gap. Three groups were created as follows: The control group (group I),

where the gap was bridged with a 16-mm-long PGA-c tube filled with collagen fiber and injected with physiological saline; the dADSC group (group II), where the gap was bridged with a 16-mm-long PGA-c tube injected with dADSCs suspended in 200 μ l of physiological saline ($1-5 \times 10^6$ cells/ml); and the autograft group (group III), where the resected sciatic nerve was reversed and sutured in place. In groups I and II, the nerve stumps at either end of the tube were stretched by 0.5 mm to create a 15-mm gap inside the tube. There were 16 rats in each group, and 4 rats per group were evaluated at 2, 4, 8, and 12 weeks after surgery, respectively.

Endpoints

Confirmation of Schwann cell markers in dADSCs - immunofluorescence staining

The cells were fixed in 4% paraformaldehyde (Wako), washed with PBS, and treated with Tris-buffered saline with 0.1% (v/v) Tween-20 (TBS-T) for 20 min for membrane permeabilization. Blocking buffer (Agilent, California, U.S.A.) was added and the cells were incubated at room temperature for 60 min. Rabbit-derived anti-S100 antibody (1/100, Abcam, ab52642, Cambridge, England) and mouse-derived anti-glial fibrillary acidic protein (GFAP) antibody (1/100, Abcam, ab10062) were each diluted in dilution buffer (Agilent) as primary antibodies and added to the cells. The cells were incubated overnight at 4 °C, and washed with phosphate-buffered saline (PBS). Alexa Fluor 488-labeled goat-derived anti-rabbit antibody (1/1000, Abcam, ab150077) or Alexa Fluor 594-labeled goat-derived anti-mouse antibody (1/1000, Abcam, ab150116) was added as a secondary antibody and the cells were incubated at room temperature for 1 h. The cells were then washed with PBS and sealed with mounting medium containing 4',6-diamidino-2-phenylindole; DAPI (Thermo Fisher Scientific, Massachusetts, U.S.A.).

Confirmation of Schwann cell markers in dADSC - western blotting

The cells were washed twice with PBS and lysed in CellLytic™ MT Cell Lysis Reagent (Sigma-Aldrich) containing 10 μ g/ml protease inhibitor cocktail. The cells were collected with a cell scraper and incubated for 15 min on ice. The lysate was centrifuged at 4 °C, for 15 min at 13,000 \times g, the

supernatant was recovered, and the protein concentration was determined using a protein assay (Bio-Rad Laboratories, California, U.S.A.). Laemmli Sample Buffer (Bio-Rad Laboratories) supplemented with β -mercaptoethanol was added to the sample, boiled at 100 °C for 5 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresed samples were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). After blocking in 3% skim milk in TBS-T, the membrane was incubated overnight at 4 °C with primary antibodies for S100 (1/5000, Abcam, ab52642), GFAP (1/5000, Abcam, ab10062), and β -actin (1/5000, Sigma-Aldrich, A5441). Then, after the membrane was washed 3 times in TBS-T, it was incubated for one hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. The membrane was then washed with TBS-T, immersed in chemiluminescence reagent for western blots (ImmunoStar® Zeta, Fujifilm, Osaka Japan), and the signal was detected with a luminescence image analyzer (LAS4000, Fujifilm).

Axonal staining and measurement

In the 2-, 4-, and 8-week models of group I and II, the nerve bridges were extracted with 5 mm of nerve at each end. After fixing in 10% formalin, the samples were embedded in paraffin and long-axis sections of the nerve bridge were created. After deparaffinization, the sample was treated with antigen activation solution (Polysciences, Pennsylvania, U.S.A.) and then blocked for 10 min with blocking buffer (Agilent). Rabbit-derived anti- β III-tubulin antibody (1/100, Abcam, ab52623) was added and incubated overnight at 4 °C. Then, the sections were incubated with Alexa Fluor 488-labeled goat-derived anti-rabbit antibody (1/1000, Abcam, ab150077) at room temperature for 45 min, washed, and mounted. A BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) and image analysis software (BZ Analyzer) were used for image acquisition and measurement. Three randomly selected sections were used for each rat, and the maximum value of the 3 sections was adopted as the axonal outgrowth distance.

Wet muscle weight ratio

In the 2-, 4-, 8-, and 12-week rats, the tibialis anterior muscle was collected from both lower legs, the mass of each muscle was measured, and the operated side/non-operated side ratio was calculated.

Nerve conduction study (NCS)

The NCS was performed 12 weeks after transplantation. The distal latency and compound muscle action potential (CMAP) were measured in the sciatic nerve, stimulating 5 mm proximal to the proximal nerve suture, and recording at the tibialis anterior muscle belly, located 5 mm distal to the inferior edge of the patella.

Quantitative RT-PCR

In the 2- and 4-week models, the lumbar spine was accessed with a midline incision in the lumbar region, laminectomy was performed from L3 to S1, and L4–6 dorsal root ganglia (DRG) were collected under microscopic examination. The DRG were placed in RNAlater™ (Sigma-Aldrich) immediately after collection, incubated for 24 h at 4 °C, and then stored at –20 °C. After RNA was extracted from the stored samples using NucleoSpin RNA™ (Takara Bio, Shiga, Japan), reverse transcription was performed using PrimeScript RT Master Mix™ (Takara Bio). Activating transcription factor 3 (ATF3) mRNA expression was then measured with quantitative RT-PCR using the Applied Biosystems StepOne Real-Time PCR System. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a housekeeping gene. DRG from the non-operated side were used as controls and gene expression was quantified using the delta-delta CT method. The primer sequences used were as follows: ATF3, forward 5'-3' GCTGCTGCCAAGTGTCGAA and reverse 5'-3' CGGTGCAGGTTGAGCATGTA; and Gapdh, forward 5'-3' GGCACAGTCAAGGCTGAGAATG and reverse 5'-3' ATGGTGGTGAAGACGCCAGTA.

Cell tracing

DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) labeling was used for cell tracing. dADSCs were labeled with DiI(Thermo Fisher Scientific) in medium at 37 °C for 15 min and

then transplanted into the PGA-c tubes for the 2- and 4-week models. The excised nerve bridge was fixed in 4% formalin and a frozen long-axis section was created. Immunostaining for S100 was performed using the aforementioned method and then the sample was observed by fluorescence microscopy.

Statistical methods

SPSS Statistics ver. 24 (IBM, New York, U.S.A.) was used for statistical processing. The mean values of the axonal outgrowth distance between groups I and II for each measurement week were compared using the Mann-Whitney U test, with a significance level of $p < 0.05$. After conducting analysis of variance (ANOVA) for wet weight, NCS, and ATF3 expression, Tukey's post-hoc tests were conducted with a significance level of $p < 0.05$.

Results

Marker expression in dADSCs

ADSCs that had been differentiated were confirmed to contain S100- and GFAP-positive cells by immunofluorescence. In western blotting, there was stronger expression of S100 and GFAP in dADSCs compared with that in ADSCs (Fig. 1).

Axonal outgrowth distance

At 2 weeks, the mean distance in group I was 3.14 mm (SD = 0.18) and the mean distance in group II was 4.12 mm (SD = 0.77) ($p = 0.029$). At 4 weeks, the mean distance in group I was 4.80 mm (SD = 1.14) and the mean distance in group II was 6.31 mm (SD = 0.13) ($p = 0.029$). Thus, axonal outgrowth was significantly promoted in group II during these times. At 8 weeks, axons reaching the distal end of the tube were confirmed in only one case in group I, but 3 cases were confirmed in group II. Assuming that axons that reached the distal end had an axonal outgrowth distance of 15 mm, then group I had a mean distance of 8.60 mm (SD = 4.84) and group II had a mean distance of 14.35 mm (SD = 1.3) ($p = 0.114$) (Fig. 2, 3).

Transplanted cell tracing

DiI-positive cells were found in the tubes at both 2 weeks and 4 weeks after transplantation. S100-positive cells were also found among the DiI-positive cells, and these were found around the ends of the regenerating axon (Fig. 4).

Wet weight ratio of tibialis anterior muscle

At 2 weeks, the mean wet weight ratio was 63.86% (SD = 6.48) in group I, 62.15% (SD = 8.17) in group II, and 59.00% (SD = 3.43) in group III. At 4 weeks, the ratio was 39.73% (SD = 3.22) in group I, 33.50% (SD = 2.72) in group II, and 36.65% (SD = 8.19) in group III. There was no significant difference among the 3 groups at these time points ($p > 0.05$). However, at 8 weeks, the mean wet weight ratio was 30.15% (SD = 7.38) in group I, 22.7% (SD = 4.12) in group II, and 53.28% (SD =

6.19) in group III, with only group III achieving significant recovery. At 12 weeks, the ratio was 14.55% (SD = 4.15) in group I and 22.72% (SD = 4.00) in group II. In group III, the mean wet weight was 50.84% (SD = 7.37), which was the same as the measurement at 8 weeks (Fig. 5).

NCS of tibialis anterior muscle

The mean distal latency was 3.23 ms (SD = 0.24) in group I, 2.80 ms (SD = 0.11) in group II, and 2.70 ms (SD = 0.17) in group III; group I had significantly extended distal latency compared to that of group II ($p = 0.014$). The mean CMAP was 0.50 mV (SD = 0.22) in group I, 0.7 mV (SD = 0.14) in group II, and 3.93 mV (SD = 0.93) in group III; there was no significant difference between groups I and II with respect to CMAP ($p = 0.836$) (Fig. 6).

Expression of ATF3 mRNA

At 2 weeks, the mean expression of ATF3 mRNA in the DRG of each treatment group relative to that on the healthy side was 54.86 (SD = 2.47) in group I, 50.46 (SD = 11.57) in group II, and 41.08 (SD = 14.85) in group III. At 4 weeks, this value was 21.72 (SD = 3.79) in group I, 55.84 (SD = 7.79) in group II, and 39.1 (SD = 8.97) in group III. Both groups II and III maintained their ATF3 expression levels from 2 to 4 weeks, while there was a significant reduction in group I (Fig. 7).

Discussion

Tubulization in the critical gap

In research on nerve regeneration in peripheral nerve defect models using rats, a 10-mm gap is often used for the sciatic nerve¹³. However, a 10-mm gap in rats is a condition that can be bridged even with a hollow silicon tube¹, which is inadequate for observing the effect of interventions such as cell transplantation. Therefore, in this study, we used a 15-mm gap model to test a critical gap that cannot be bridged with a hollow tube alone.

There are a large number of reports using critical gap models, including bridging with engineered neural tissue using Schwann cells and dADSCs^{14,15}, bridging with collagen tubes and Schwann cell transplantation¹⁶, bridging with chitosan tubes^{17,18}, and bridging with poly(L-lactide-co-ε-caprolactone) tubes^{19,20}. A common aspect of these reports is that better nerve regeneration is demonstrated through intervention, by introducing cells and devising structures for bridging, compared to that in the groups without intervention. However, in studies comparing results with autologous nerve grafting, reports have indicated that bridging with artificial material is difficult to compare with autologous nerve grafting^{14–18,20}. In our study, axonal regeneration using a PGA-c tube was further promoted by injecting dADSCs, but there was a significant difference in peripheral muscle innervation compared to that observed with autologous nerve grafting, as reported previously. There are a few reports evaluating nerve regeneration using a combination of tubulization and ADSCs in the critical gap model^{14,20}, the results of which are comparable with our findings, even though our intervention procedure was simpler and easier to use in clinical applications. Peripheral nerve regeneration was certainly promoted by dADSCs in the critical nerve gap model, but it is not sufficient for clinical applications in terms of functional recovery.

Effect and action of ADSCs and necessity of differentiation

There have been a large number of reports using tubulization with transplanted ADSCs for peripheral nerve defects, but the mechanism promoting regeneration is often attributed to trophic effects, which are caused by the secretion of humoral factors. ADSCs have been reported to express a diverse range

of neurotrophic factors, including vascular endothelial growth factor, glial growth factor, brain-derived neurotrophic factor, and nerve growth factor^{2,4,6,21,22}, which are thought to promote nerve regeneration. In contrast, there have been few reports that support repair effects, where transplanted cells are involved in myelination as Schwann cells. There was one report in which induced dADSCs reverted to an undifferentiated state within 3 days after removal of differentiation-inducing medium in *in vitro* experiments²³. Thus, it is important to determine how grafted cells function in the body. Using cell tracing, cells transplanted without differentiation induction were shown to remain in place without expressing Schwann cell markers^{2,7}, while other reports demonstrated that cells transplanted as dADSCs maintained Schwann cell marker expression⁷. According to a study by Tomita *et al.* where ADSCs were injected into the nerve after nerve compression⁹, even undifferentiated ADSCs expressed Schwann cell markers after transplantation, which suggests that differentiation may have occurred *in vivo*, although it was significantly less than that of dADSCs. However, this occurred due to the abundant contact between native Schwann cells and the grafted cells, and this differentiation *in vivo* is considered to be unlikely with tube transplantation. We consider differentiation induction to be essential if the grafted cells are expected to be involved in myelination in tubulization. However, in reports on peripheral nerve defect models, there have been no differences in nerve regeneration outcomes (wet muscle weight and sciatic functional index) despite whether differentiation was induced or not, and some reports have stated that differentiation induction is unnecessary^{7,8}. Thus, no consensus of opinion has occurred to date. In this study, the grafted cells maintained Schwann cell marker expression, consistent with previous reports, and we confirmed the persistence of expression. When the gap length is long and contact with native Schwann cells is considered to be less likely, as in this study, there will be greater merit in transplanting differentiated cells.

Change in muscle wet weight and motor function recovery

The muscle wet weight after denervation was reduced to 50–60% that of the healthy side in ~2 weeks, and then declined to 10–20% in ~12 weeks²⁴. There was a report that delayed repair of nerve injury exceeding 4 weeks post-injury resulted in the muscle wet weight of the affected side being reduced to

~20% that of the healthy side even 3 months after surgery²⁵. Specifically, determining how to induce axonal regeneration to the distal end at an early stage post-injury (up to 4 weeks), is thought to affect the prognosis of muscle recovery in nerve gap models. The results of this study showed that group III recovered wet weight from 4 to 8 weeks, which indicates that innervation of the muscle occurred through the regenerated axons, but the wet weight continued to decline up to 8 weeks in groups I and II. At 12 weeks, the wet weight in group I declined further, following the natural course of denervated muscle, but the decline in wet weight had halted in group II. We believe that this occurred because group II had better innervation than group I, but the change was not able to increase the muscle wet weight. Considering the natural course of muscle denervation and re-innervation²⁴, the delayed innervation caused degeneration of the muscle fibers and neuromuscular junctions, which resulted in insufficient recovery. There was no difference between groups I and II in the CMAP of the nerve conduction study, and this correlates with the wet weight results. Conversely, the distal latency was significantly shorter in group II, and it is thought that myelination was promoted by cell transplantation.

ATF3 expression

ATF3 has been reported to be upregulated in DRG nerve cells via axonal transport when nerve injury has occurred peripheral to the DRG. The natural course after nerve injury is a temporary elevation in expression from immediately after injury, persistence of high expression for ~2 weeks, and then a reduction in expression²⁶. ATF3 protects nerve cells from cell apoptosis by upregulating HSP27, as well as promoting axonal outgrowth^{27–30}. Saito and Dahlin reported a correlation between ATF3 expression and axonal regeneration in the delayed repair of axonal injury (i.e., maintenance of ATF3 expression is considered advantageous for axonal regeneration)³¹. In the present study, ATF3 expression declined from 2 to 4 weeks only in group I, which used the tube alone; this is considered to resemble the natural course of axonal injury. Conversely, in groups II and III, ATF3 expression was maintained at 4 weeks, at a level equal to that seen at 2 weeks. Thus, we believe that transplanting dADSCs triggers a cascade in the proximal neuron from the site of injury, and nerve cell protection equal to that seen in autologous nerve grafting can be expected.

Number of grafted cells

Many reports on ADSC research transplant approximately 10^5 – 10^6 cells. In our study, the theoretical number of grafted cells was 10^5 cells, which is similar to that reported by other investigators. We were able to confirm that the grafted cells remained in the cell tracing experiments, but it is difficult to accurately evaluate how many of the transplanted cells persisted in the tube and were involved in axonal regeneration. However, with injections of cell suspensions, leakage of the grafted cells is inevitable; hence, the number of effective cells is expected to be smaller. Based on this study and research from other investigators, even if ADSCs are incomplete, they may serve as a substitute for Schwann cells. Thus, if a larger number of cells can be reliably retained in the tube, then a stronger effect to promote regeneration could be expected.

Study limitations

In this study, we were able to use only a limited number of tubes ($n = 4$ per evaluation group), which is a smaller number than that reported by other researchers. Further, the expression of Schwann cell markers was confirmed based on only qualitative, and not quantitative, analysis. In addition, since this study was conducted in small animals, it will be necessary to perform future evaluations in larger animals to progress toward clinical applications.

Conclusions

We evaluated nerve regeneration in a 15-mm nerve defect model in rats using dADSCs. The dADSCs maintained their differentiated state even after transplantation, leading to improvement in axonal ingrowth but not functional recovery. Further innovation is needed, including improvements in the cell transplantation method.

Abbreviations

ADSCs, adipose-derived stem cells; ATF3, activating transcription factor 3; CMAP, compound muscle action potential; dADSCs, differentiated adipose-derived stem cells; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DRG, dorsal root ganglia; GFAP, glial fibrillary acidic protein; NCS, nerve conduction study; PGA-c, polyglycolic acid-collagen

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Figure legends

Fig. 1. Comparison of dADSCs and ADSCs using (A) immunofluorescence staining (scale bar is 50 μ m) and (B) western blotting. ADSCs, adipose-derived stem cells; dADSCs, differentiated adipose-derived stem cells

Fig. 2
Axonal outgrowth of group I and II was revealed using β III-tubulin staining at each week. The right side is the proximal end of the tube in all images. Scale bar is 300 μ m.
(A, B) Inverted triangles (∇) show the measurement points at the regeneration edges. (C) High-power magnification of the distal end of the tube in group II at 8 weeks. Many axons reached the distal end of the tube.

Fig. 3
Changes in axonal regeneration distance. Axonal outgrowth was significantly promoted in group II.
*: $p < 0.05$

Fig. 4
Cell tracing using DiI labeling.
(A) S100 staining of dADSCs before transplantation with DiI labeling. Scale bar is 50 μ m. (B) High-power magnification of regeneration tip at 2 weeks after transplantation of DiI-labeled dADSCs. Cells positive for both S100 and DiI can be seen at the regeneration tip. Scale bar is 50 μ m. (C) Low-power magnification at 4 weeks. Cells positive for both S100 and DiI can be seen around the growth cone. Scale bar is 300 μ m. (D) High-power magnification at 4 weeks. Scale bar is 50 μ m.
dADSCs, differentiated adipose-derived stem cells; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

Fig. 5

Changes in muscle wet weight.

There was no significant difference among the 3 groups at 2 weeks. At 8 weeks, only group III achieved significant recovery. *: $p < 0.05$.

Fig. 6.

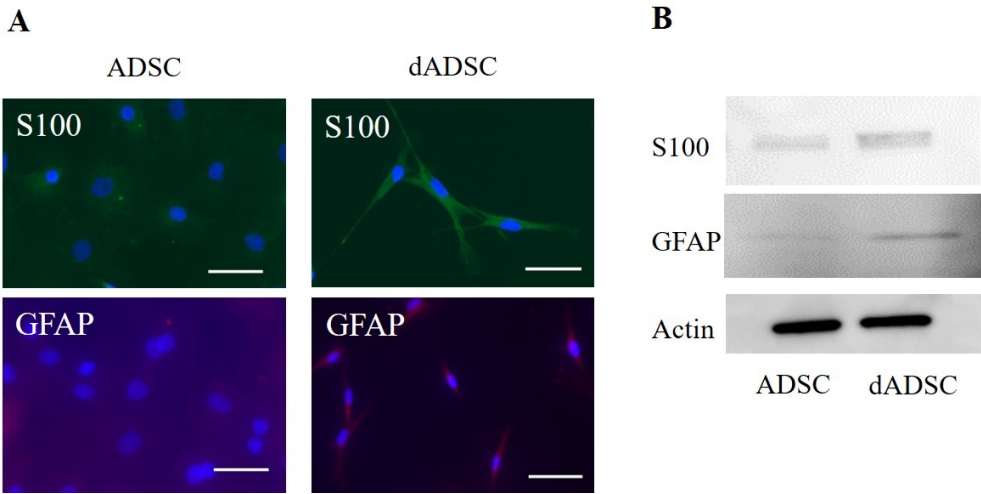
Results of NCS. (A) Distal latency of the tibialis anterior muscle. The distal latency of group I was significantly prolonged compared to that of group II. (B) CMAP of the tibialis anterior muscle. There was no significant difference between groups I and II.

*: $p < 0.05$. NCS, nerve conduction study; CMAP, compound muscle action potential

Fig. 7

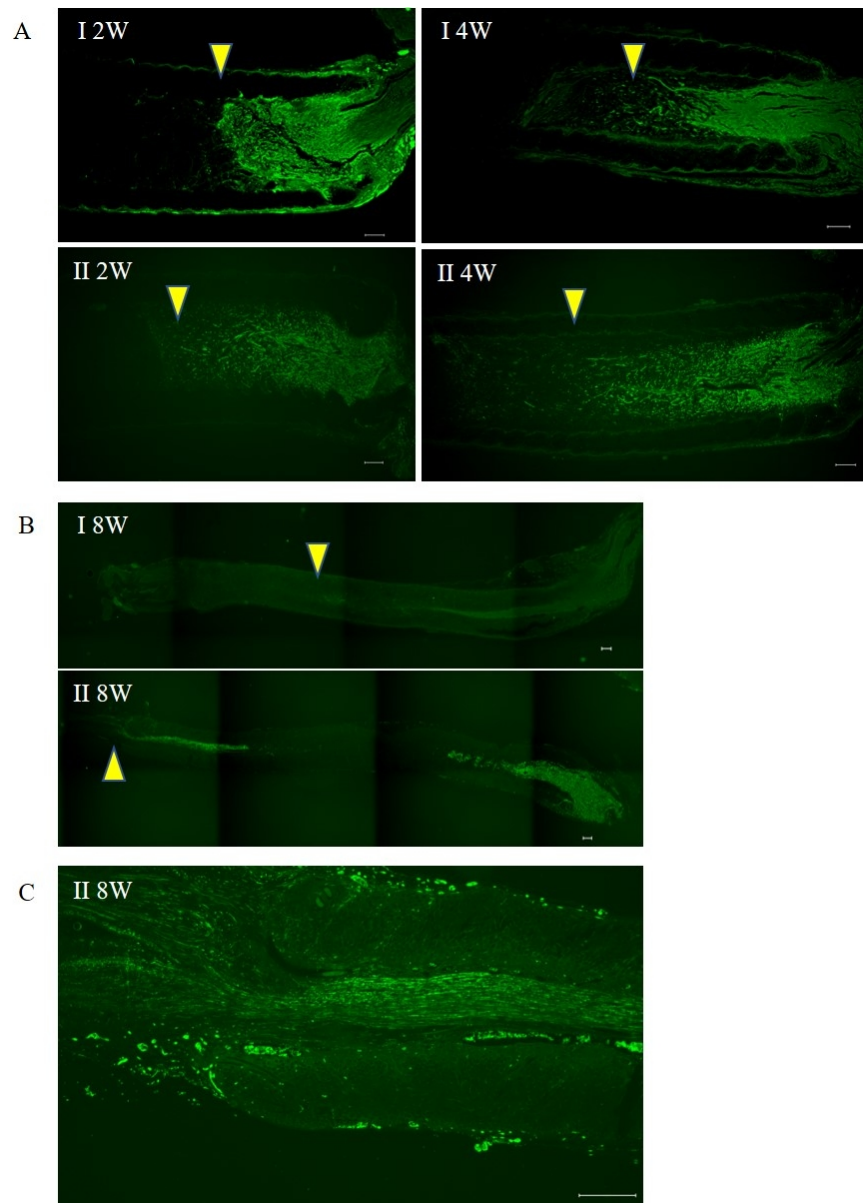
ATF3 expression relative to that observed in the non-operated side (N.O.; value = 1). Both groups II and III maintained ATF3 expression levels from 2 to 4 weeks, whereas there was a significant reduction in group I.

*: $p < 0.05$. ATF3, activating transcription factor 3



Comparison of dADSCs and ADSCs using (A) immunofluorescence staining (scale bar is 50 μ m) and (B) western blotting. ADSCs, adipose-derived stem cells; dADSCs, differentiated adipose-derived stem cells

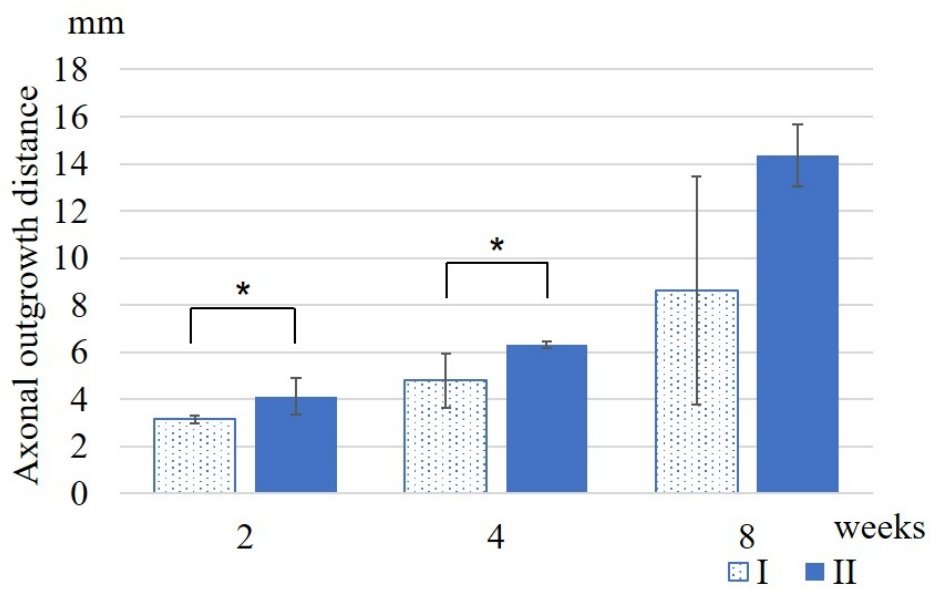
199x100mm (150 x 150 DPI)



Axonal outgrowth of group I and II was revealed using β III-tubulin staining at each week. The right side is the proximal end of the tube in all images. Scale bar is 300 μ m.

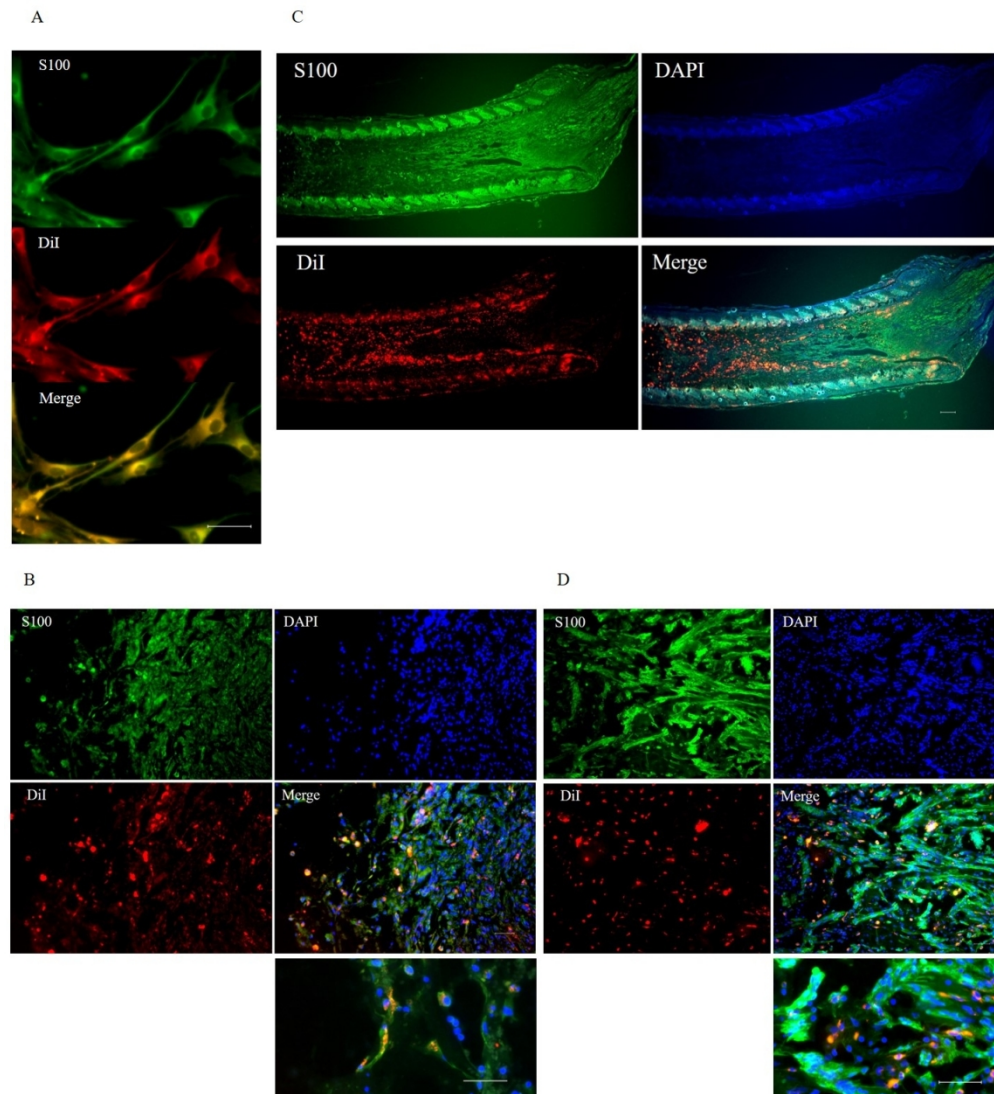
(A, B) Inverted triangles (▽) show the measurement points at the regeneration edges. (C) High-power magnification of the distal end of the tube in group II at 8 weeks. Many axons reached the distal end of the tube.

148x203mm (150 x 150 DPI)



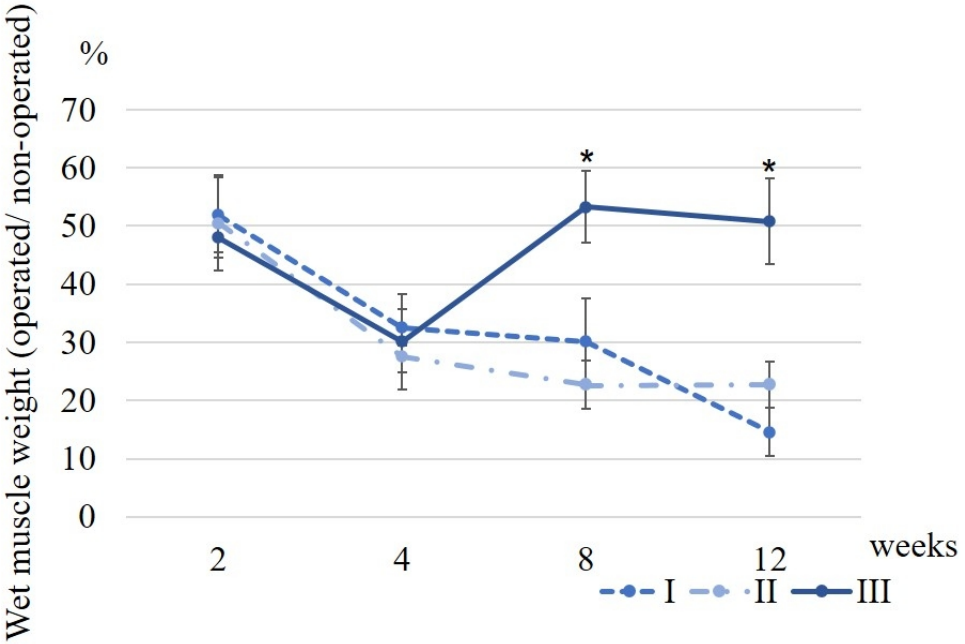
Changes in axonal regeneration distance. Axonal outgrowth was significantly promoted in group II.
*: $p < 0.05$

152x93mm (150 x 150 DPI)



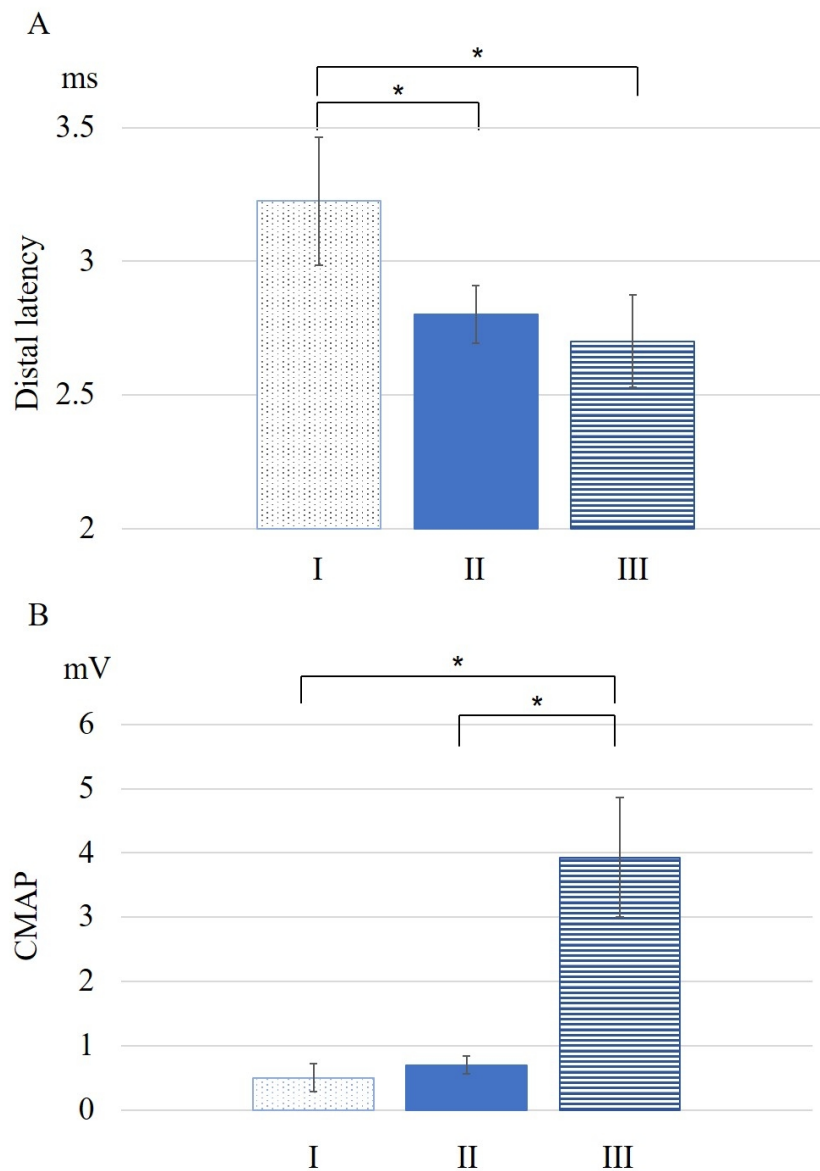
Cell tracing using DiI labeling. (A) S100 staining of dADSCs before transplantation with DiI labeling. Scale bar is 50 μ m. (B): High-power magnification of regeneration tip at 2 weeks after transplantation of DiI-labeled dADSCs. Cells positive for both S100 and DiI can be seen at the regeneration tip. Scale bar is 50 μ m. (C): Low-power magnification at 4 weeks. Cells positive for both S100 and DiI can be seen around the growth cone. Scale bar is 300 μ m. (D): High-power magnification at 4 weeks. Scale bar is 50 μ m. dADSCs, differentiated adipose-derived stem cells; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate

242x265mm (150 x 150 DPI)



Changes in muscle wet weight.
There was no significant difference among the 3 groups at 2 weeks. At 8 weeks, only group III achieved significant recovery. *: p < 0.05.

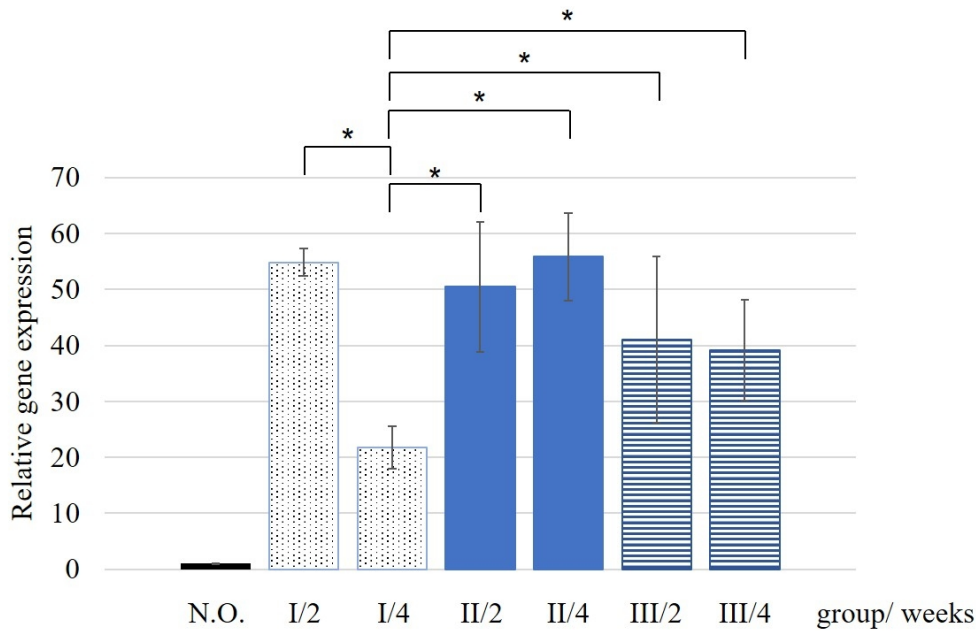
158x136mm (150 x 150 DPI)



Results of NCS. (A) Distal latency of the tibialis anterior muscle. The distal latency of group I was significantly prolonged compared to that of group II. (B) CMAP of the tibialis anterior muscle. There was no significant difference between groups I and II.

*: $p < 0.05$. NCS, nerve conduction study; CMAP, compound muscle action potential

167x239mm (150 x 150 DPI)



ATF3 expression relative to that observed in the non-operated side (N.O.; value = 1). Both groups II and III maintained ATF3 expression levels from 2 to 4 weeks, whereas there was a significant reduction in group I. *: $p < 0.05$. ATF3, activating transcription factor 3

191x130mm (150 x 150 DPI)