

Peripheral nerve storage and allotransplantation using green tea polyphenol in rats—its possibility for clinical application—Review of our works.

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Abstract

Background. We examined whether peripheral nerve segments immersed in polyphenol solution could be preserved and allotransplanted into rats without using immunosuppressants.

Methods. Sciatic nerve segments, 20 mm long, were harvested from male Lewis or DA rats and transplanted into 15-mm sciatic nerve deficits in Lewis rats immediately, or after polyphenol treatment (immersion in 1 mg/mL polyphenol solution for one week and in Dulbecco's modified Eagle's medium for three weeks more), without using immunosuppressants. In the FA group, DA rat nerve segments were transplanted into Lewis rats immediately. In the PA group, DA rat nerve segments were transplanted into Lewis rats after polyphenol treatment. In the FI group, Lewis rat nerve segments were transplanted immediately into Lewis rats. In the PI group, Lewis rat nerve segments were transplanted into Lewis rats after polyphenol treatment. Nerve regeneration was assessed electrophysiologically and histomorphologically. Nerve segments from male Lewis or DA rats were also transplanted into female Lewis rats as in the PI, PA, FI, and FA groups. Genomic DNA was

extracted from each transplanted nerve segment and subjected to polymerase chain reaction amplification specific for the sex-determining region of Y-chromosome and β -actin genes. Immunostaining for S-100 and glial fibrillary acidic protein, and Y-chromosome-specific in situ hybridization were also performed.

Results. Nerve regeneration in the PI and PA groups was similar to that in the FI group and was significantly greater than that in the FA group. The ratios of the Sry/ β -actin polymerase chain reaction products for PA, PI, FI, and FA groups were 0.144, 0.294, 0.615, and 0, respectively. Some of the S-100-positive and glial fibrillary acidic protein-positive cells displayed Sry signals in the PI and FI groups, but not in the FA group.

Conclusion. Some Schwann cells survived for one month in polyphenol-treated nerve segments that were transplanted allogeneically without immunosuppressants.

Key words: peripheral nerve storage, polyphenol solution, sex-determining region of Y-chromosome

Introduction

“Polyphenol” is a general term for a mixture of catechins. These have various biological and biochemical effects, such as in anti-oxidation, cell cycle modulation, anti-cancer,¹ and anti-arteriosclerosis² effects. Epigallocatechin gallate (EGCG) is the main component of the catechins and has the strongest effects.

Hyon et al.³ found that rat pancreatic island cells cultured in Dulbecco's modified Eagle's medium (DMEM) containing polyphenols for one week and in a polyphenol-free DMEM solution for the subsequent three weeks started to produce insulin again; insulin production peaked two weeks after the cells had been transferred into the polyphenol-free DMEM solution. They concluded that polyphenols might induce the dormancy of cells. Several studies have reported that EGCG inactivates T-cell functions and reduces immune reactions.^{4–6} We hypothesized that polyphenols might be used as tissue-storing liquids for allogeneic transplantation because of their cell-preserving and T-cell-inactivating effects.

Isogeneic nerve grafting has been the gold standard for repairing peripheral nerve injuries with an inter-stump gap; it is associated with a limited source of harvestable isogeneic nerves. However, nerve allografts have just started to be used clinically, because immune tolerance is hard to achieve in allogeneic peripheral nerve transplantation. Such allografts require the long-term administration of immunosuppressants to the recipients, which can generate life-threatening side effects, such as malignancies or severe infections. It is still controversial and ethically problematic to use immunosuppressants that have a potential to produce life-threatening side effects, when treating peripheral nerve injuries that do not affect a patient's life expectancy directly. If peripheral nerve segments could be preserved for a period in polyphenol solutions and transplanted allogeneically while reducing immune rejection, the use of such storage would be a powerful tool for allogeneic nerve transplantation.

This study was performed on rats to examine whether peripheral nerve segments could be preserved for one month with polyphenol solution and whether such segments could be transplanted allogeneically without using immunosuppressants.

Materials and Methods

Animals

Isogeneic and inbred Lewis rats (RT-1^l) and DA rats (RT-1^a) that were major histocompatibility complex (MHC) mismatched to Lewis rats were used. All rats were 10–12 weeks old, weighing 120–140 g. All experiments were performed in accordance with the guidelines of the animal Research Committee, Graduate School of Medicine, Kyoto University, Japan.

Polyphenols

The polyphenol mix used in this study was extracted from green tea and purchased from PFI Inc. (Kyoto, Japan). It consisted mainly of (–)-epigallo-catechin-3-O-gallate (28%); (–)-epigallocatechin (15.0%); (–)-epicatechin-3-O-gallate (4.6%); (–)-epigallocatechin-3-O-gallate (11.6%); (+)-gallocatechin (14.8%); (–)-epicatechin (7.0%); and (+)-catechin (9.5%), at a purity of > 90%.

Polyphenol solution

The polyphenol mix was dissolved in DMEM, adjusted to 1 mg/mL, and filtered to prevent bacterial and viral transmission.

Surgery and experimental groups

All rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal; 40 mg/kg) and ketamine chloride (Ketalar; 40 mg/kg). The polyphenol allograft (PA) group underwent allogeneic transplantation of a nerve segment that had been preserved in polyphenol solution (n=20). Sciatic nerve segments (20 mm long) harvested from male DA rats were immersed in the polyphenol solution described above for one week and then in DMEM alone for the subsequent three weeks at 4°C. Each nerve segment was then transplanted into a 15 mm sciatic nerve deficit created in a male Lewis rat. The polyphenol-treated isograft (PI) group underwent isogeneic transplantation of a nerve segment that had been preserved in polyphenol solution (n=20). Sciatic nerve segments (20 mm long) were harvested from male Lewis rats and immersed in polyphenol solution for one week and in DMEM for the subsequent three weeks at 4°C. Each nerve segment was then transplanted into a 15-mm sciatic nerve defect created in an isogeneic male Lewis rat. The fresh isograft (FI) group underwent fresh isogeneic nerve transplantation (n=20). Sciatic nerve segments (20 mm long) were harvested from male Lewis rats and immediately transplanted into 15-

mm sciatic nerve deficits created in isogeneic male Lewis rats. The fresh allograft (FA) group underwent fresh allogeneic nerve transplantation ($n=20$). Sciatic nerve segments (20 mm long) were harvested from male DA rats and immediately transplanted into 15-mm sciatic nerve deficits created in Lewis rats.

Assessment of cell viability and histology of nerve segments before transplantation ($n=4$ per group)

Nerve segments in each group were divided into individual nerve fibers using collagenase digestion. Double fluorescent staining using a calcein-acetoxymethyl ester (AM)/ethidium homodimer vital stain (Live/Dead Viability/Cytotoxicity assay, Molecular Probes, Inc., Eugene, OR, USA) was performed on the nerve fibres. They were examined using confocal fluorescence microscopy to determine the viability of the Schwann cells of each nerve fiber.⁷

Electron microscopy of nerve segments ($n=4$ per group)

The nerve segments of each group were fixed in 1% glutaraldehyde and 1.44% paraformaldehyde, followed by 1% osmic acid. A transverse thin section (1 mm thick) was cut in the middle portion of each nerve and examined under an electron microscope (Hitachi H7000, Tokyo, Japan).

Electrophysiology and histomorphometry

The motor nerve conduction velocity of the transplanted nerve segments and the amplitude of the action potential evoked in the pedal adductor muscles were examined and expressed as percentages of those measured in the contralateral healthy limbs.^{8–10} The transplanted nerve segments were then harvested and fixed as described above. Transverse sections (5 mm thick) were obtained from the distal-most portions of the transplanted nerve segments and examined by light microscopy. The images were installed on a personal computer and subjected to morphometry using the image analysis software Image-Pro Plus (Media Cybernetics, Inc., Bethesda, MD, USA). The mean myelinated axon numbers and diameters were measured for each section as described.^{8–10}

Immunological studies

Peripheral blood was taken from three rats in each group one week after transplantation, and the populations of peripheral lymphocytes with CD4 or CD8 surface antigens were measured using flow cytometry, as described previous-

ly.^{11,12} The proportions of CD4-positive cells and CD8-positive cells and the CD4⁺/CD8⁺ ratios were calculated.

Identification and quantification of surviving donor-originating cells in the transplanted nerve segments

Sciatic nerve segments were harvested from male Lewis or DA rats, treated with the methods described for the PA, PI, FI, and FA groups ($n=8$ per group), and transplanted into sciatic nerve deficits created in female Lewis rats. In six of the eight rats in each group, a 15-mm nerve segment was harvested from the middle portion of the transplanted nerve segment. Genomic DNA was extracted from each of these nerve segments and subjected to polymerase chain reaction (PCR) amplification with primers specific for the genes for the sex-determining region of the Y chromosome (Sry) or b-actin 12 weeks after transplantation, using PCR conditions as described.^{8–10,13} The optical densities of the amplified Sry and b-actin fragments were measured on 2% agarose gels after electrophoresis and analyzed using National Institutes of Health (NIH) Image Software (<http://rsbweb.nih.gov/nih-image/about.html>). The ratio of Sry to b-actin was calculated to estimate the population of donor-originating cells in each transplanted nerve segment.^{8–10}

In situ hybridization specific for the Y chromosome and immunohistochemical staining specific for the S-100 and glial fibrillary acidic protein (GFAP) were performed on serial transverse sections taken from the midpoints of the transplanted nerve segments that had been harvested from the two remaining female rats 12 weeks after transplantation. For immunohistochemistry, these sections were incubated with primary antibodies for 1 h in TBST (50 mM Tris-HCl, pH7.6, 0.15 M NaCl+0.05% Tween). Primary antibodies used in this study were anti-rabbit anti-S-100 antibody (1:200; DAKO, Glostrup, Denmark) and anti-rabbit anti-GFAP antibody (1:200; DAKO). Following rinses in TBST, ENVISION+/HRP (DAB; DAKO) was applied for 30 min. Following a rinse in phosphate-buffered saline (PBS), hematoxylin was used for nuclear staining. Adjacent sections were reacted with a fluorescent probe for the Y-chromosome to observe transplanted male Schwann cells using a hybridization method.^{14–17} The paint probe used in this study was the rat Y-Cy3/12-fluorescein isothiocyanate (FITC) paint probe mix (Cat. No. CA 1630; Cambio, Cam-

bridge, UK). After the mix had been applied, the sealed slides were denatured at 75°C for 10 min and hybridized overnight at 37°C in a humid chamber. The coverslip was then removed and washed with PBS, and the sections were covered with 4',6-Diamidino-2-phenylindole (DAPI)-containing a mounting agent and examined using a fluorescence microscope.

Statistical analysis

Data are expressed as the mean and standard deviation. One-way ANOVA was used to compare the data of the four different groups. If a significant difference was identified between any groups, a post hoc Bonferroni-Dunn test was applied; $P < 0.05$ was deemed to be significant.

Results

Assessment of the viability of Schwann cells in nerve segments

The calcein-AM/ethidium homodimer vital stain is hydrolyzed in the cytoplasm of viable cells and appears green under confocal fluorescence microscopy (Fig. 1). In dead cells, the ethidium homodimer passes through the broken nuclear membrane and binds to nucleic acids, appearing red. Just before transplantation, most of the nerve fibres in each segment of the four groups were green, with no or only a partial red colour. This indicates that the cell viability in the nerve segments in all four groups (PI, PA, FI, and FA) was almost completely preserved after treatment with or without polyphenols. As a control, we stained the nerve fibres of Lewis rat sciatic nerve segments (20 mm) that had been immersed in only DMEM for 28 days (M group) with the calcein-AM/ethidium homodimers.

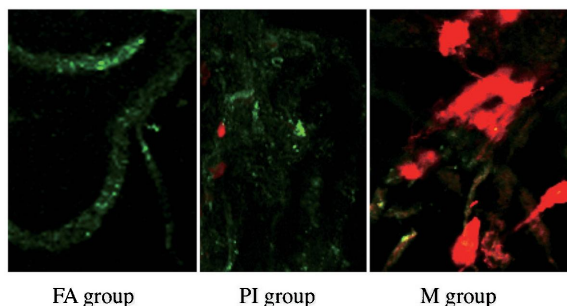


Fig. 1 Confocal examination of nerve fibers stained with calcein-AM/ethidium homodimer before transplantation. (FA) Fresh allograft group. (PI) Polyphenol-treated isograft group. (M) Only in DMEM group. The red color indicates that all the cells in the fibers were dead.

These nerve fibres stained uniformly red, indicating that all the Schwann cells were dead.

Electron microscopy of nerve segments

The PI and PA groups showed marked demyelination of each nerve fibre (Fig. 2). However, the structure of the Schwann cells remained intact. No Schwann cells remained in the nerve segments that had been immersed in DMEM for 28 days (M group).

Electrophysiology

There were no significant differences among the four groups in the mean values of the motor conduction velocity, at either 12 or 24 weeks (Fig. 3A). At both these times, the mean pedal adductor muscle amplitudes of the PA, FI, and PI groups were significantly greater than that of the FA group, but no significant difference was observed among the PA, FI, and PI groups (Fig. 3B).

Histomorphometry

The mean numbers of myelinated axons of the PA and PI groups were significantly greater than that of the FA group, and no significant difference was observed between the PA and PI groups at 12 weeks. No significant difference was observed among the PA, FI, and PI groups at 24 weeks, but the mean numbers of myelinated axons in the PA, FI, and PI groups were significantly greater than in the FA group (Fig. 4A).

The mean myelinated axon densities of the PA, FI, and PI groups were significantly greater than that of the FA group, but no significant differences were observed among the PA, FI, and PI groups at either 12 or 24 weeks (Fig. 4B).

Immunological study

The proportion of CD4⁺ cells in the population of all lymphocytes in the peripheral blood

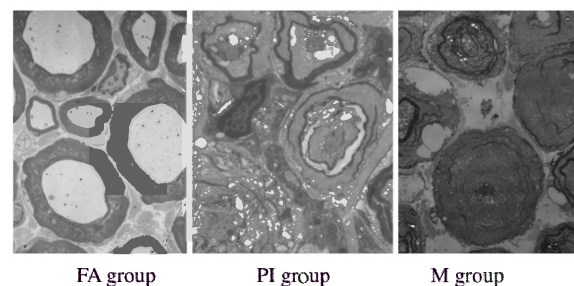


Fig. 2 Electron microscopic examination before transplantation. (FA) Fresh allograft group. (PI) Polyphenol-treated isograft group. (M) Only in DMEM group. The Schwann cells remain in the PI group, whereas the Schwann cells of the M group have disappeared.

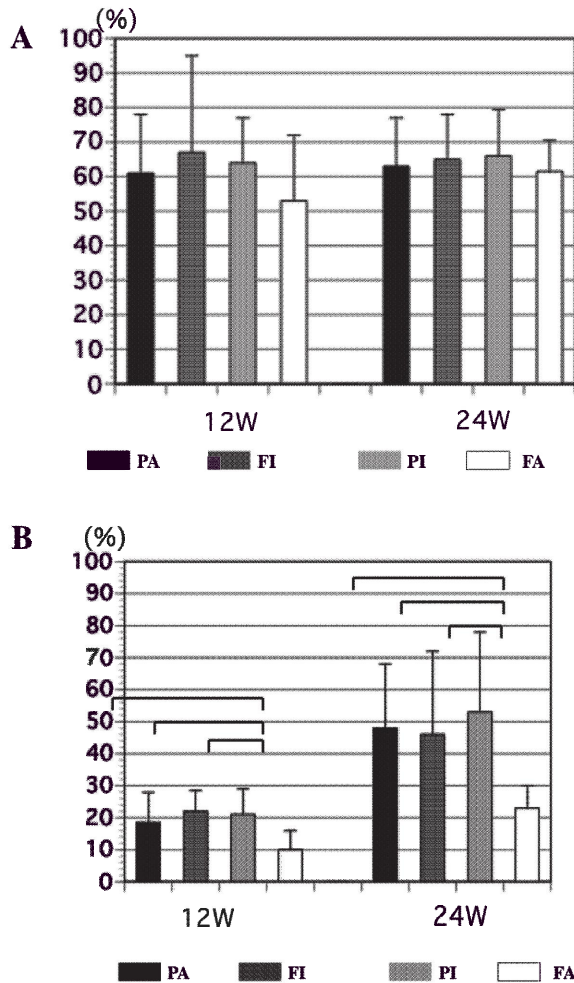


Fig. 3 Electrophysiological studies. (A) Mean motor nerve conduction velocities for each group 12 and 24 weeks after transplantation. (B) Mean amplitudes of the action potentials evoked in the pedal adductor muscle 12 and 24 weeks after transplantation. Brackets indicate significance, $P < 0.05$.

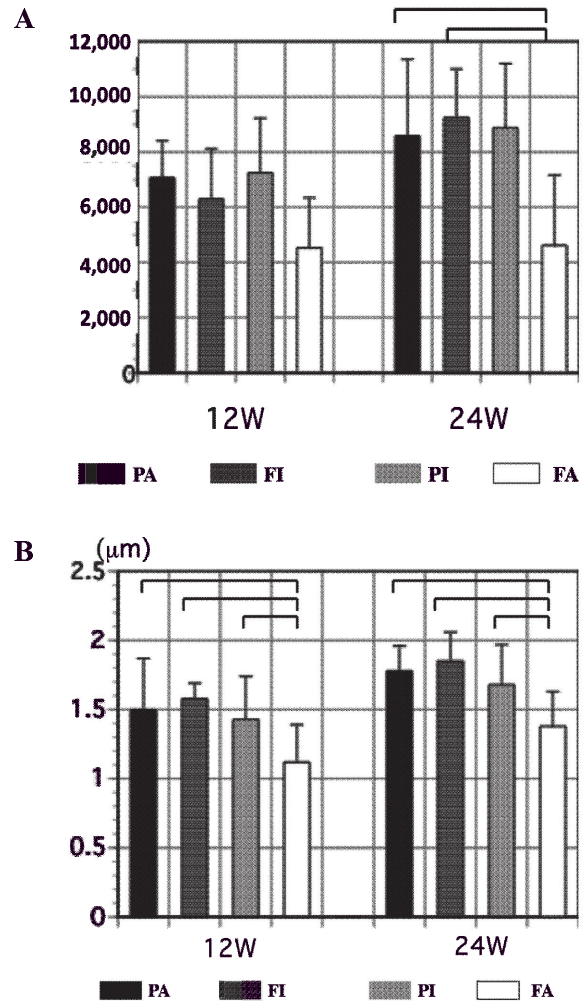


Fig. 4 Histomorphometric studies. Mean total myelinated axon numbers (A) and mean myelinated axon diameters (B) in transverse sections from the distal-most region of the transplanted nerves of each group. Brackets indicate significance, $P < 0.05$.

one week after transplantation in the FA group was significantly smaller than those of the PA, PI, and FI groups, but no significant difference was observed among the latter three groups (Fig. 5A). There was no significant difference in the proportion of CD8⁺ cells in the population of all lymphocytes among the four groups (Fig. 5B). The ratio of CD4⁺ cells to CD8⁺ cells (CD4⁺/CD8⁺) in the FA group was significantly smaller than the ratios of the PA, PI, and FI groups, but no significant difference was observed among the latter three groups (Fig. 5C).

Identification and quantification of surviving donor-originating cells in the transplanted nerve segments by PCR

Amplified Sry and b-actin bands were obser-

ved in the DNA extracted from the nerve segments of the PA, FI, and PI groups at 12 weeks after transplantation but not in the FA group. The ratios of Sry to b-actin products for the PA, PI, FI, and FA groups were 0.144, 0.294, 0.615, and 0, respectively (Fig. 6).

In situ hybridization and immunohistochemistry

Some nuclei of cells that were S-100- and GFAP-positive by immunohistochemistry also demonstrated Y-Cy3/12FITC signals by situ fluorescent hybridization, indicating that some of the Schwann cells remaining in the nerve segments 12 weeks after transplantation had originated from the transplanted nerve segments (Fig. 7). No cells of donor origin were found in

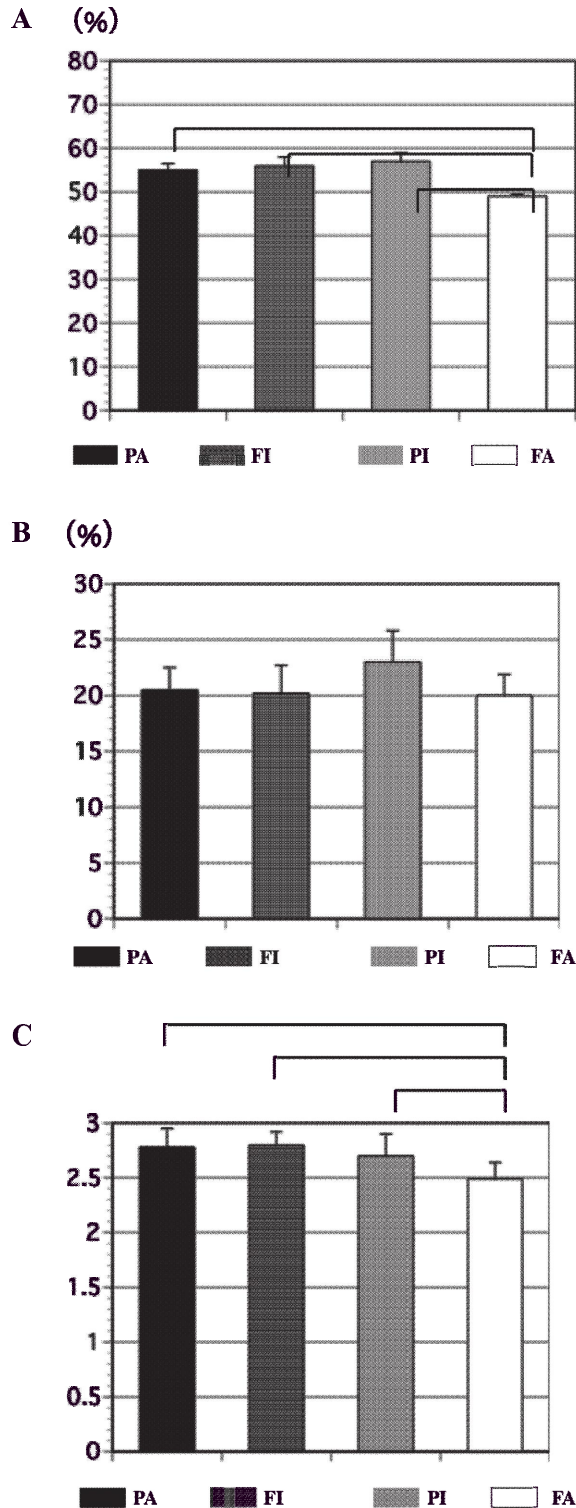


Fig. 5 (A) The proportion of CD4⁺ cells in the population 12 weeks after transplantation. (B) The proportion of CD8⁺ cells in the population 12 weeks after transplantation. (C) The ratio of CD4⁺/CD8⁺ cells. The brackets indicate a significant difference.

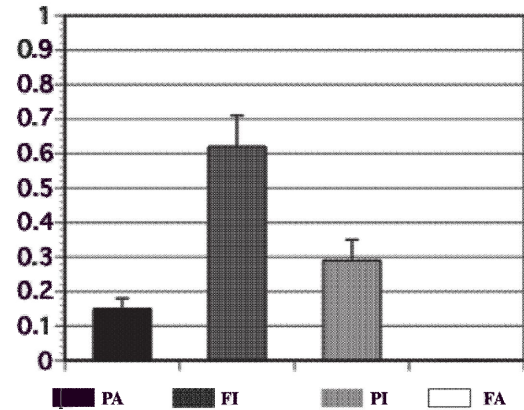


Fig. 6 Mean ratios of the Sry gene product to the b-actin gene product in each transplantation group.

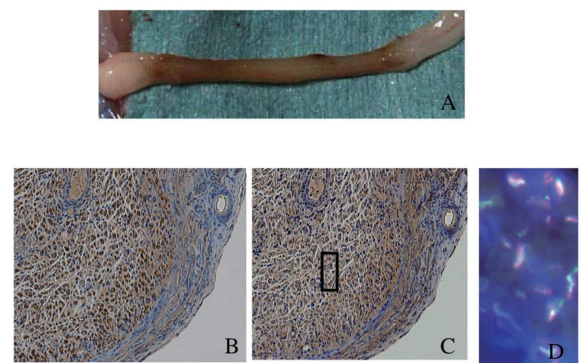


Fig. 7 A transplanted nerve segment treated with polyphenol solution (PA group) (A). Immunohistochemical staining for S-100 (B) and GFAP (C) in transverse sections harvested from the nerve segment. (D) shows an in situ hybridization study specific for the Y chromosome.

the nerve segments of the FA group in either the in situ hybridization or PCR studies.

Discussion

Double staining with calcein-AM and an ethidium homodimer demonstrated that viable cells remained in the isogeneically transplanted nerve segments after preservation for one month in polyphenol solution (PI group; Fig. 1). Electrophysiology and histomorphometry revealed that nerve regeneration in this group was almost equivalent to that in the fresh isogenic nerve transplantation (FI) group at 12 and 24 weeks after transplantation (Figs. 3 and 4). The PCR studies demonstrated that at 12 weeks after transplantation, 30% of cells in the PI group originated from the donor nerve segments and that the population of cells originating from

the donor nerve in the nerve segments of this group was almost half that in the fresh isogeneic nerve segments (FI group; Fig. 6). The *in situ* hybridization studies revealed that Schwann cells originating from the donor nerve segments that had been treated with polyphenol remained viable in the nerve segments 12 weeks after allogeneic transplantation (PA group) (Fig. 7).

Nerve regeneration in the nerve segments of the PA group was almost equal to that in the fresh isogeneic nerve transplantation group at both 12 and 24 weeks after transplantation. The population of donor-originating cells remaining in the PA group was 23% of that in the FI group at 12 weeks. Wramner et al.¹¹ reported that the numbers of lymphocytes in the CD4⁺ and CD8⁺ subsets in the peripheral blood decrease after the renal allogeneic transplantation, followed by a rapid recovery to preoperative levels within one week in recipients not exhibiting immune rejection. Davenport et al.¹² demonstrated that the ratio of CD4⁺ cells to CD8⁺ cells decreased significantly between days 2-3 and day 7 after the liver allotransplantation in the peripheral blood of recipients with rejection episodes. In the present study, there was no significant difference in the numbers of CD4⁺ cells or in the CD4⁺/CD8⁺ ratios of the FI and PA groups one week after transplantation. This indicates that polyphenol treatment reduced the donor-host immune response that occurs during allogeneic nerve transplantation.¹⁵ In contrast, the proportion of CD4⁺ cells and the CD4⁺/CD8⁺ ratio in the FA group were significantly lower than those of the other three groups (Fig. 5), indicating the occurrence of immune rejection in this group.¹⁰ Several researchers tried to preserve peripheral nerve segments using a cryopreservation technique¹⁸ or the University of Wisconsin cold storage solution.¹⁹ However, no living cells were found in nerve segments preserved for more than three weeks in those studies.

The present study has revealed that the preservation of peripheral nerves for one month is possible using a polyphenol solution. However, the question arises of whether whole organs can be preserved in this way. In a preliminary study, a vascularized groin flap harvested from a Lewis rat that had been systemically irrigated with polyphenol solution was preserved in the same solution for 24 h and transplanted into another inbred isogeneic Lewis rat using a vascular anastomosis technique. The flap looked viable 24

hours after transplantation but showed complete necrosis at 72 hours (data not shown). Matsumoto et al.^{20,21} examined the histology of canine sciatic nerves that had been preserved in polyphenol solution at 4°C for one month, and found that Schwann cells remained within 0.5 to 0.7 mm of the perineurium in the superficial fascicles but that no Schwann cells remained in the deeper fascicles. These studies indicate that there might be a limit to the permeability of polyphenols for cell preservation. Polyphenols might not reach the peripheral tissues during systemic irrigation because of their high molecular weight. They are effective in preserving nerve segments of 1-1.4 mm in diameter, which are similar to rat sciatic nerves, but thicker tissues or organs might not be stored successfully by simple immersion in such solutions. From a clinical perspective, nerve segments with a greater diameter should be preserved after they have been divided into fascicles of less than 1-1.4 mm in diameter.

In the present study using rat sciatic nerves, nerve regeneration after polyphenol-treated allogeneic or isogeneic nerve grafting was almost equivalent to that seen with fresh isogeneic nerve grafts. However, the proportions of donor cells surviving in the polyphenol-treated isogeneic and allogeneic nerve grafts were 48 and 23% of that seen in the fresh isogeneic nerve grafts, respectively. This reduced population of donor-originating cells in the transplanted nerve segments might be followed by a delay or deterioration of nerve regeneration in long nerve grafts if this technique were to be applied in human therapy. We must develop a technique or device to increase the population of donor-originating cells in nerve segments treated with polyphenols.

In conclusion, because polyphenols reduce not only ischemic stress but also transplantation-induced immune responses to peripheral nerves, such solutions could be used for allogeneic nerve storage.

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