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# *In Vivo* Study on the Survival of Neural Stem Cells Transplanted into the Rat Brain with a Collagen Hydrogel That Incorporates Laminin-Derived Polypeptides

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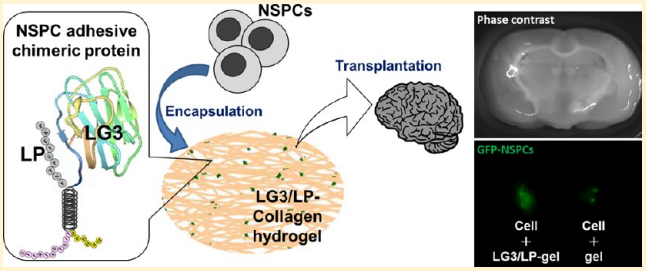
**ABSTRACT:** Poor viability of cells transplanted into the brain has been the critical problem associated with stem cell-based therapy for Parkinson's disease. To overcome this problem, a collagen hydrogel incorporating an integrin-binding protein complex was prepared and used as a carrier for neural stem cells. The protein complex consisted of two polypeptides containing the G3 domain of a laminin $\alpha$1 chain and the C-terminal oligopeptide of a laminin $\gamma$1 chain. These polypeptides were fused with $\alpha$-helical segments which spontaneously formed a coiled-coil heterodimer and with the collagen-binding peptide that facilitated the binding of the heterodimer to collagen networks. In this study, neural stem cells stably expressing the enhanced green fluorescent protein (EGFP) were suspended in the hydrogel and transplanted into the striatum of healthy rats. The viability of transplanted cells was evaluated by histological analysis and quantitative reverse-transcriptase polymerase chain reaction for EGFP mRNA present in the tissue explants. Our results showed that the collagen hydrogel incorporating the integrin-binding protein complex serves to improve the viability of neural stem cells (NSCs) in the early stage after transplantation into the striatum.




## INTRODUCTION

Parkinson's disease is caused by the deficiency of the neurotransmitter dopamine due to degeneration of the basal ganglia. To develop an effective method for the treatment of Parkison's disease, many attempts have been made to optimize the dopamine level in the brain by transplanting neural precursor cells.[1−3] However, the therapeutic effect is still limited primarily due to the poor viability of transplanted cells.[4] It appears that this involves two major factors: cell apoptosis caused by the disruption of cell−cell or cell−substrate interactions[5,6] and acute inflammatory responses against transplanted cells through the infiltration of activated microglia.[7,8]

To improve the viability of transplanted cells, several hydrogels such as cross-linked hyaluronic acid[9] and Matrigel[10] have been tested for their feasibility as cell carriers. However, most of the hydrogels studied to date were not effective enough to improve the viability of transplanted cells. Although Matrigel was shown to be effective as a carrier, this hydrogel cannot be clinically used because Matrigel is derived from the sarcoma of mice. We have also made attempts to develop cell carriers from collagen hydrogels that incorporate cell adhesive polypeptide[11,12] or epidermal growth factor.[13] Our *in vitro* studies demonstrated that rat neural stem cells (NSCs) survived longer periods in these collagen hydrogels than in the hydrogel made of collagen alone on account of the incorporated polypeptides or growth factor. Among these hydrogels, those incorporating a cell-adhesive protein complex consisting of two types of laminin-derived polypeptides[11] gave the best results in our *in vitro* study, demonstrating that apoptotic cell death was suppressed by integrin ligation.

The protein complex employed in the previous study consisted of two types of engineered polypeptides: one of the polypeptides contained the G3 domain of a laminin $\alpha$1 chain, and the other contained the C-terminal oligopeptide of a laminin $\gamma$1 chain. These polypeptides and oligopeptides were fused with 35-mer $\alpha$-helical segments, which spontaneously form a coiled-coil heterodimer. In addition, the chimeric polypeptide consisting of the G3 domain and the $\alpha$-helical segment was further linked with the collagen-binding peptide that facilitates the binding of the heterodimer to collagen networks. In our previous study,[11] the effect of the cell-adhesive protein complex was investigated using an *in vitro* cell culture system in which the effect of integrin ligation could be studied without interference from inflammatory microglia.

In the present study, we carried out a transplantation study in which rat NSCs were suspended in the collagen hydrogel precursor solution and infused into the striatum of healthy rats.

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Special attention was paid to the suppression of microglial infiltration into the region where cells were transplanted. To discriminate transplanted cells from host brain cells, NSCs obtained from transgenic rats stably expressing enhanced green fluorescent protein (EGFP) were used. The infiltration of microglia into the transplanted sites was analyzed by immunofluorescent staining, and the viability of transplanted cells was determined from the total level of EGFP mRNA expressed in the transplanted sites.

## EXPERIMENTAL PROCEDURES

**Collagen-Binding LG3 and Histidine-Tagged LP.** The preparation of collagen-binding LG3 (CLG3) and histidine-tagged LP (HLP) was reported elsewhere.[11] The domain structure of CLG3 and HLP is shown in Figure 1A. As reported previously,[11] these proteins were designed to spontaneously bind to collagen networks and form a CGL3-HLP heterodimer on account of the coiled-coil association of 35-mer $\alpha$-helical polypeptides K5 and E5 (Figure 1B). These proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and their purities were assessed from the band intensities determined using Scion Image software (Scion Corporation, MD).

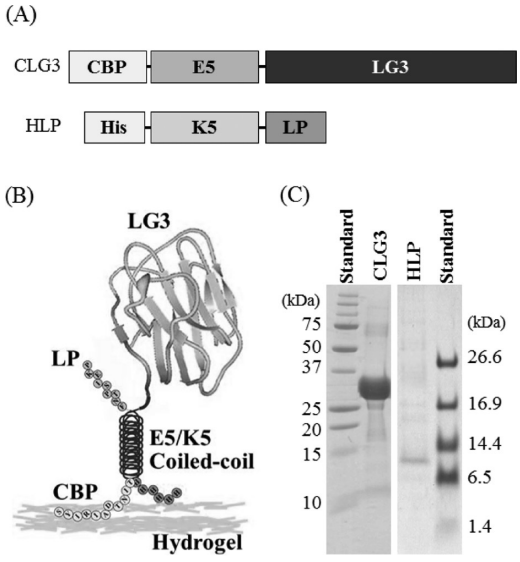


**Figure 1.** (A) Domain structure of the laminin-derived polypeptides, CLG3 and HLP. LP, FNTPSIEKP; E5, $[KELASVE]_5$; K5, $[EKLASVK]_5$; CBP, SYIRIADTNIT. (B) Illustration of a CLG3/HLP heterodimer bound to collagen. (C) The results of the SDS–PAGE analysis for CLG3 and HLP.

**Cell Isolation and Culture.** The striatum was isolated from fetuses (E16) of EGFP-transgenic Sprague–Dawley rats [SD-Tg(CAG-EGFP) rats, Japan SCL, Inc., Hamamatsu, Japan] according to the guidelines of the Animal Welfare Committee of the institute and dissociated into single cells by treating with 0.05% trypsin solution containing 0.53 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA). The single cells obtained were suspended in DMEM/F12 (1:1) (Invitrogen, Carlsbad, CA) containing 3 mM glutaMAX (Invitrogen), 5 $\mu$g/mL heparin, 100 unit/mL penicillin, 100 $\mu$g/mL streptomycin, 2% B27, 20 ng/mL basic fibroblast growth factor (Wako Pure Chemical industries, Osaka, Japan), and 20 ng/mL epidermal growth factor (Wako Pure Chemical industries). The cells were cultured for 4–5 d at 37 °C under 5% $CO_2$ to form neurospheres to enrich NSCs. Then, the neurospheres were dissociated into single cells by treating with 0.05% trypsin solution containing 0.53 mM EDTA and seeded onto the epidermal growth factor-anchored substrates[14] at a density of $2.9 \times 10^4$ cells/cm$^2$. The cells were cultured for 7 d in DMEM/F12 (1:1) containing 3 mM glutaMAX, 5 $\mu$g/mL heparin, 100 unit/mL penicillin, 100 $\mu$g/mL streptomycin, and 2% B27 at 37 °C under 5% $CO_2$. According to the result of immunostaining, more than 95% of the cells expressed nestin, a marker for neural stem cells. Although Sox2 has also been analyzed as one of the markers for NSCs by many research groups,[21,22] we used in this study cell populations rich in nestin-expressing cells. This population is referred to as EGFP-NSCs in this article.

**Preparation of Collagen Hydrogel.** Forty-five microliters of 10 mg/mL collagen solution (Nippon Meat Packers, Osaka, Japan) was mixed with 10 $\mu$L of buffer solution (50 mM NaOH, 260 mM $NaHCO_3$, and 200 mM HEPES) and 20 $\mu$L of 5-fold concentrated DMEM/F12 (1:1) containing 15 mM glutaMAX, 25 $\mu$g/mL heparin, 500 unit/mL penicillin, 500 $\mu$g/mL streptomycin, and 10% B27. To this collagen solution, a mixed solution of CLG3 and HLP was added to the final concentration of 3 $\mu$M for each protein. EGFP-NSCs were harvested by trypsinization from the epidermal growth factor-anchored substrate and suspended in the collagen/CLG3/HLP solution (50 $\mu$L) at $3 \times 10^5$ cells/$\mu$L. To delay gelation, the suspension was kept at 4 °C until transplantation.

**Transplantation.** Adult (9 weeks old, 290–310 g) male Sprague–Dawley (SD) rats were housed under a 12-h light and 12-h dark cycle with ad libitum access to food and water. Cell transplantation experiments were carried out under anesthesia with Nembutal (50 mg/kg weight) according to the guidelines of the Animal Welfare Committee of our institute. A suspension of EGFP-NSCs ($1.5 \times 10^6$ cells, 5 $\mu$L) in a collagen hydrogel precursor solution with or without CLG3/HLP or in a culture medium [DMEM/F12 (1:1), 3 mM glutaMAX, 5 $\mu$g/mL heparin, 100 unit/mL penicillin, 100 $\mu$g/mL streptomycin, and 2% B27] was stereotactically infused into the striatum (coordinates: 0.5 mm anterior and 2.0 mm lateral to bregma, and 5.0 mm ventral)[15] using a 10-$\mu$L Hamilton microsyringe. The microsyringe was first plunged 5.0 mm below the dural surface and immediately withdrawn 1.5 mm just before injection to avoid back-flow. Injections were performed at a rate of 1 $\mu$L/min.

**Histochemistry.** Rats were sacrificed 3, 7, or 15 d after transplantation by the intraperitoneal injection of overdose Nembutal and transcardially infused with 350 mL of phosphate buffered saline (PBS) followed by 5% paraformaldehyde (350 mL) for fixation. The brain was enucleated from the rat and postfixed with 5% paraformaldehyde in PBS overnight. Then the fixed brain was kept in 20% sucrose solution for 3 d at 4 °C, which served to preserve tissue integrity during paraffin embedding. The brain tissues were trimmed and embedded in paraffin. Ten-micrometer-thick sections were cut in the coronal section using microtome. After deparaffinization, these sections were permeabilized with 0.5% Triton-X100 solution for 10 min and incubated in 50% Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 2 h. The sections were immunofluorescently stained using primary antibodies to EGFP (1:1000, chicken polyclonal, Chemicon, Temecula, CA) and microglial cells (1:250, rabbit monoclonal Iba1, Wako Pure Chemical Industries). Antichicken IgG Alexa 488 (1:500, Invitrogen) and antirabbit IgG Alexa 594 (1:500, Invitrogen)


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were used as secondary antibodies. The localization of secondary antibodies was analyzed with an epifluorescent microscope (IX71, Olympus). Moreover, the localization of collagen hydrogel was analyzed by Masson-trichrome staining.

The apoptosis of transplanted cells was evaluated using DeadEnd Fluorometric TUNEL System (Promega). To discriminate the transplanted living cells from host cells, the brain sections were immunofluorescently stained using antibodies to EGFP (antichicken IgG Alexa 594, 1:500, Invitrogen) followed by nucleus staining with Hoechst.

The histochemical analyses and the TUNEL assay were performed for thin sections obtained from 6 rats for EGFP and lba1 staining and 6 rats for EGFP and TUNEL staining.

**Viability of Transplanted Cells.** Rats were intracerebrally infused with EGFP-NSCs suspensions in collagen hydrogel precursor solution with ($n$ = 24) or without ($n$ = 22) CLG3/HLP or in the culture medium ($n$ = 6) and sacrificed 3 h or 3, 7, or 15 d after transplantation by an intraperitoneal injection of an overdose of Nembutal and transcardially infused with 350 mL of phosphate buffered saline (PBS). The brain was enucleated from the rat, and a region containing transplanted cells was isolated while observing with a fluorescent stereomicroscope (Leica MZ FLIII, Solms, Germany). To avoid EGFP-expressing cells being left behind, a slightly larger volume of tissue samples (typical size: 10 × 5 × 3 $mm^3$) was carefully prepared. The explanted tissues (150 ± 0.5 mg) were lysed with 10 mM Tris-HCl buffer (pH 7.5) containing 4 M guanidine thiocyanate and 0.97% $\beta$-mercaptoethanol. The volume of the lysate was adjusted to 1.5 mL by adding the buffer solution. Total RNA was isolated from 175 $\mu$L of the lysate using SV Total RNA Isolation Kit (Promega, Madison, WI). The amount of total RNA obtained ($R_{total}$) was determined from absorbance at 260 nm. Then, 5 $\mu$g of total RNA was reverse-transcribed in a 20 $\mu$L reaction mixture to obtain complementary DNA (cDNA) using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). One-tenth of the resultant solution (2 $\mu$L) was used as a template for quantitative real-time polymerase chain reaction (qPCR) using the following specific primers: EGFP, (forward) 5′-CTACGGCGTGCAGTGCTTCA-3′; (reverse) 5′-AAGTTCACCTTGATGCCGTT-3′; and 3-phosphate dehydrogenase (GAPDH; internal standard), (forward) 5′-TGAACGGGAAGCTCACTGG-3′; (reverse) 5′-TCCACCACCCTGTTGCTGTA-3′. The reaction mixtures (20 $\mu$L) containing the cDNA template, Power SYBR reaction mix (Applied Biosystems), 0.5 $\mu$M forward primer, and 0.5 $\mu$M reverse primer were subjected to qPCR using StepOne Real-Time PCR System (Applied Biosystems) under thermal cycling conditions of denaturation at 98 °C for 10 s and annealing/extension at 60 °C for 1 min. After 40 cycles, the homogeneity of amplified DNA was confirmed by analyzing the melting temperature. The amount of EGFP ($D_{EGFP}$) and GAPDH ($D_{GAPDH}$) cDNAs was determined from the cycle numbers at which the fluorescent intensity of SYBR reached the predetermined threshold in the logarithmic phase of amplification.[16] To normalize the amount of cDNA templates added to each reaction mixture, $D_{EGFP}/D_{GAPDH}$ was calculated to determine the equivalent number of EGFP-expressing cells ($N$) using the standard curve (EGFP-expressing cell numbers vs $D_{EGFP}/D_{GAPDH}$) obtained from qPCR analysis for the known number of cognate cells. Accordingly, the total number of EGFP-expressing cells present in the excised tissue sample ($N_{total}$) could be expressed as follows:

$$N_{total} = \left[\left(R_{total} \times \frac{1500}{175}\right)/5\right] \times \frac{20}{2} \times N$$

## RESULTS

**Preparation of CLG3 and HLP.** As shown in Figure 1C, CLG3 and HLP were separated as single bands by SDS−PAGE analyses. The molecular weights estimated from band mobility were in good agreement with those predicted from their amino acid sequences (CLG3, 28.0 kDa; HLP, 6.6 kDa). The purities of CLG3 and HLP determined from their band intensities were approximately 94% and 78%, respectively.

**Transplantation.** EGFP-NSCs were suspended in a mixture of collagen/CLG3/HLP or pure collagen, and these suspensions were infused into the striatum of healthy SD rats. After 3 d, a brain slice was prepared, and the EGFP-emitting living cells were imaged with a fluorescent stereoscope. The result is shown in Figure 2. EGFP fluorescence is observed in the region where cells were transplanted with collagen/CLG3/HLP indicated by an arrow. In contrast, fluorescence is hardly seen in the control site indicated by an arrowhead. These results suggest that EGFP-emitting living cells are more clearly seen when infused with collagen/CLG3/HLP than with collage alone.

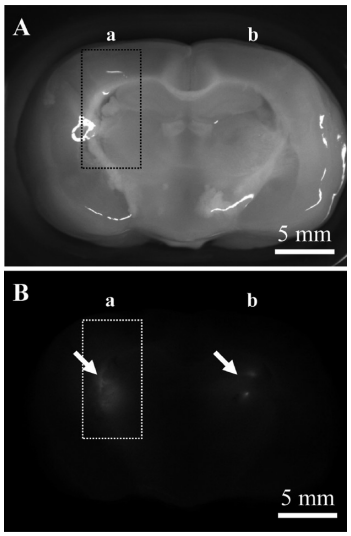


**Figure 2.** (A) Stereoscopic and (B) fluorescent micrographs of EGFP-NSCs 3 d after transplantation into the rat brain. Cells were suspended in collagen hydrogels (a) with or (b) without the CLG3/HLP complex. The bright, ring-shaped region seen on the left of stereoscopic micrograph A is an artifact caused by the reflection of an illuminated light source. Arrows in photograph B represent the fluorescence from transplanted cells. The dotted square represents the typical size of tissue (10 × 5 mm;thickness, 3 mm) harvested for evaluating the viability of transplanted cells. Scale bar: 5 mm.

**Histological Analysis.** Figure 3 shows the result of histological analyses for EGFP-expressing cells and host microglia. EGFP-expressing cells transplanted with collagen/CLG3/HLP are prominently seen in Figure 3A (3 d post-transplantation). On the 15th day (Figure 3C), cells had the extended shapes. However, when cells were suspended in a collagen gel without CLG3/HLP, EGFP-expressing cells are unremarkable after 3 d post-transplantation (Figure 3D). Although EGFP expression appears to be more prominent at 7 d post-transplantation than at 5 d (Figure 3E), it is hard to


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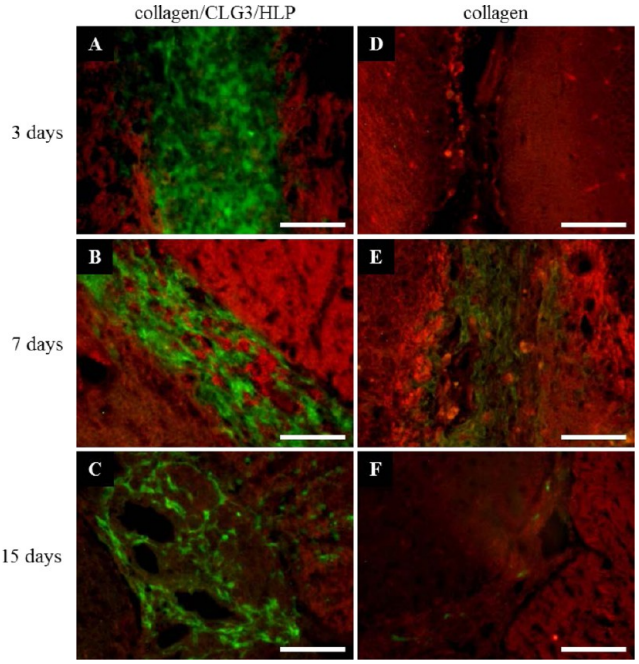


**Figure 3.** Localization of transplanted EGFP-NSCs and host microglia. Tissue sections (3, 7, and 15 d post-transplantation) were immunologically stained using antibodies against EGFP (green) and Iba 1, a microglial marker (red). Scale bar: 50 $\mu$m. Voids in C may be artifacts caused during sectioning. A small number of EGFP-expressing cells are seen in D. This is probably due in part to the fact that the gels after 3 d post-transplantation were easily left out from the tissues during immunostaining procedures.

see EGFP staining after 15 d (Figure 3F). These qualitative observations will be confirmed by qPCR assays as described below.

It should be noted that the regions with microglial cells of the recipient tissues are clearly separated from the regions with the hydrogel with transplanted cells (Figure 3A). This result suggests that the infiltration of microglia is effectively blocked by the hydrogel present in the identical regions with the cells (Figure 4A). After 7 d (Figure 3B), it is seen that microglial cells are present within the regions with EGFP-expressing cells. This is probably because of the partial degradation and resorption of a hydrogel (Figure 4B), reducing the blockade effect against infiltrating microglia. However, microglial cells within the transplanted region disappeared after 15 d (Figure 3C), even though most of the hydrogel was resorbed (Figure 4C). It is likely that the acute inflammatory reactions ceased after 15 d.

**Apoptosis of Transplanted Cells.** After 3 and 7 d of transplantation, brain tissue sections were prepared and subjected to TUNEL assays followed by immunofluorescent staining using the anti-EGFP antibody. The results are shown in Figure 5. As can be seen in Figure 5 A and C, cells positive

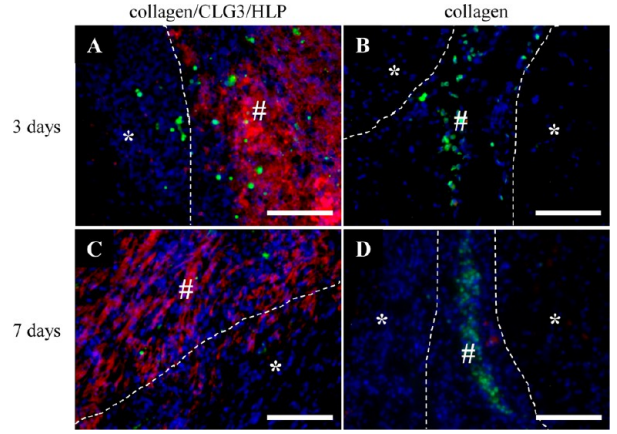


**Figure 5.** Results of TUNEL assays for apoptotic cells. Cells were suspended in collagen hydrogels (A,C) with or (B,D) without the CLG3/HLP complex and transplanted in the rat brain. Assays were performed at (A,B) 3 and (C,D) 7 d post-transplantation. Red, EGFP (living cells); green, TUNEL (apoptotic cells); and blue, cell nuclei. Dotted lines represent the border between the hydrogel and the host tissue, assigned from transplanted cells and microglia visualized by immunostaining and collagen visualized by Masson-trichrome staining (data not shown) on the serial tissue sections. #, cell transplanted region; *, host tissue. Scale bar: 100 $\mu$m.

for TUNEL staining are notable 3 and 7 d post-transplantation in the regions where EGFP-NSCs were transplanted with collagen/CLG3/HLP. In contrast, EGFP-NSCs infused with collagen alone are hardly seen both 3 and 7 d post-transplantation, while TUNEL-positive cells are obvious in the transplanted regions compared to those in the collagen/CLG3/HLP hydrogel. Although these results are still qualitative, it is suggested that apoptosis of transplanted EGFP-NSCs is suppressed by incorporating CLG3/HLP into a collagen hydrogel.

**Viability of Transplanted Cells.** To study the viability of transplanted cells, EGFP mRNA was determined by qPCR for excised brain tissues. In addition to the suspensions of EGFP-NSCs in collagen/CLG3/HLP and pure collagen, EGFP-NSCs suspended in a medium were also infused to examine the effect of the collagen hydrogel itself. As shown in Figure 6, only 40% of transplanted cells were detected after 3 h post-transplantation when the medium was used as a vehicle. This reduction in cell number is largely due to the back-flow of the cell suspension during the retraction of the needle, as we

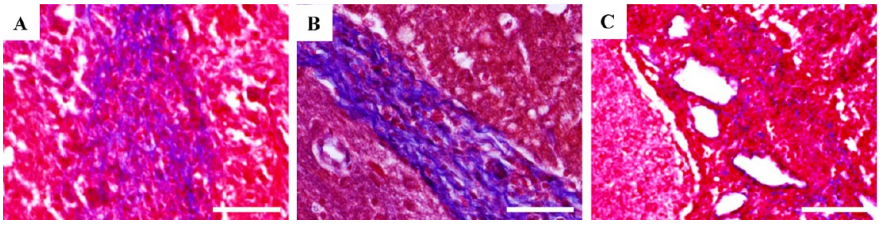


**Figure 4.** Localization of collagen hydrogels (A) 3, (B) 7, and (C) 15 d after transplantation of EGFP-NSCs. Collagen was visualized by Masson-trichrome staining. Collagen exhibits a blue-purple color with Masson-trichrome staining. Scale bar: 50 $\mu$m. Voids in C may be artifacts caused during sectioning.


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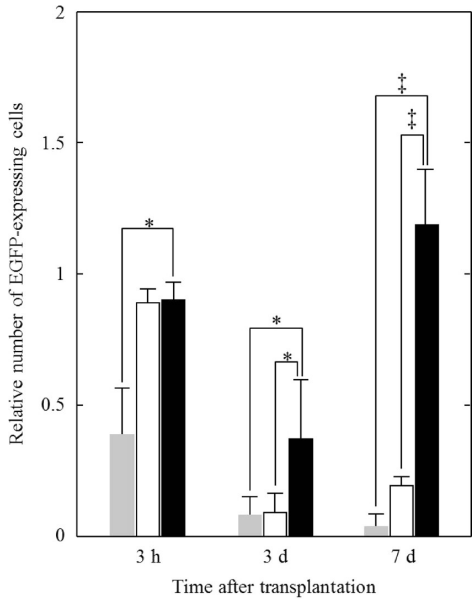


**Figure 6.** Number of living cells determined 3 h, 3 d, and 7 d after transplantation into the rat brain. Cells were suspended in collagen hydrogels (closed bar) with or (open bar) without the CLG3/HLP complex, or (hatched bar) in medium. The number of EGFP-expressing cells relative to that of transplanted cells ($1.5 \times 10^6$ cells) was determined, and the data are expressed as the mean ± standard deviation (day 0, $n = 3$; day 3, $n = 10$; day 7, $n = 9$). The symbols * and ‡ indicate statistical significance (Tukey's HSD test, * $p < 0.05$ and ‡ $p < 0.01$).

frequently observed. However, the retention of cells infused with pure collagen was ca. 90% when evaluated 3 h post-transplantation. This is significantly higher than the relative number of cells infused with a medium. These results indicate that the form of hydrogel serves to prevent cell loss during the infusion procedure. However, the number of living cells considerably decreased during 3 d. In contrast to this, the majority of cells infused with collagen/CLG3/HLP (>90%) remained after 3 h, and the cell number was still high (ca. 40%) after 3 d. When examined after 7 d, the number of living cells was found to increase 3-fold during 4 d (from the third to seventh day). This result demonstrated that cells in the collagen/CLG3/HLP hydrogels rather increased their numbers after 7 d by proliferation, in marked contrast to the cells in a pure collagen hydrogel and medium.

## ■ DISCUSSION

The poor survival of grafted cells is one of the major issues in stem cell-based therapy for Parkinson's disease.[4,17−19] It is considered that the following two aspects are involved in this problem: the acute inflammation and the destruction of cell−cell and cell−matrix interactions associated with the transplantation procedure. The present *in vivo* study demonstrated that infiltration of microglial cells was physically blocked by hydrogels (Figure 3) and that apoptotic cell death was minimized by ligating integrins with the CLG3/HLP complex incorporated in a collagen hydrogel (Figure 5). Accordingly, cell viability was significantly improved at the early stage after transplantation (Figure 6).

Several research groups reported that less than 5% of neural stem/progenitor cells survived several days after transplantation into the brain of Parkinson's model animals.[4,17,18] To improve the survival of transplanted cells, attempts were made to overexpress an antiapoptotic Bcl-xL gene in neural stem or progenitor cells.[23,24] Although such cells are particularly useful for investigating the behavior of transplanted cells,[25] the genetically modified cells are not favorable for their clinical applications. In contrast, our strategy is to use the hydrogel system for improving the survival of transplanted cells. Our results showed that approximately 40% of cells transplanted with the hydrogel survived 3 d in the rat brain and rather increased their numbers after 7 d. These results demonstrate the feasibility of our strategy.

It is considered that the inflammatory response in the brain is caused by microglia activated by foreign substances such as graft cells. The activated microglia infiltrates into the graft regions to release various cytotoxic cytokines. It was previously reported that the use of materials such as hydrogels would be beneficial to the protection of transplanted cells from inflammatory responses in the brain.[10] Our results shown in Figure 3A demonstrate that the infiltration of microglia is inhibited at the early stage after transplantation, most likely due to the effect that the collagen hydrogel acts as a physical barrier against microglial infiltration. Although a collagen hydrogel itself may also initiate inflammation,[20] we speculate that this is overwhelmed by the effect of isolating graft cells from microglia.

Cell−cell and cell−extracellular matrix interactions are destroyed upon the preparation of cell suspensions. This also initiates apoptotic cell death,[7,8] leading to the poor survival of transplanted cells. As demonstrated in our previous *in vitro* study,[11] NSCs suspended in a collagen hydrogel that incorporates CLG3/HLP survive longer periods than in a pure collagen hydrogel owing to the ligation of $\alpha_6\beta_1$ integrin by the CLG3/HLP complex. Therefore, the improved survival of NSCs demonstrated in the present *in vivo* study might be the consequence of two antiapoptotic effects: integrin ligation and the suppression of microglial infiltration. The observed increase in cell numbers 7 d after transplantation with collagen CLG3/HLP (Figure 6) is probably due to the effect that integrin ligation promotes the proliferation of surviving cells.

We conducted here the histochemical analyses that provided reproducible results, and representative micrographs are shown in Figures 3 and 5 to qualitatively discuss the prevention of microglial infiltration and cell apoptosis. On the one hand, more replicates for histochemical analyses would provide quantitative insights with regard to cell survival after transplantation. On the other hand, we suspect that accurate quantification of activated microglia and apoptotic cells is difficult using consecutive sections because it is hard to identify individual cells dispersed in a complicated manner in three dimension. For more quantitative analyses of infiltrated microglia and apoptotic cells, we need other experimental approaches totally different from those in this study. In addition, 5-brom-2-deoxyuridine (BrdU) uptake would also provide quantitative information on dividing cells. However, our principal interest in this study was to analyze the total number of living cells which might contain nondividing cells. Moreover, BrdU incorporation possibly directly affects the growth of transplanted cells. Therefore, we did not adopt the BrdU assay for our purpose.

As demonstrated above, the CLG3/HLP-loaded collagen hydrogel serves to improve the viability of NSCs in the early stage after transplantation. As shown in Figure 4, most of the hydrogel is resorbed after 15 d. This is an advantage for the

integration of transplanted cells to the host tissue. Accordingly, the CLG3/HLP/collagen hydrogel system provides an important step for developing therapeutic methodology for the treatment of Parkinson's disease. The present study was focused on cell survival in the early stage after transplantation. At this moment, cells surviving and proliferating in our hydrogel have not been thoroughly characterized in terms of differentiation by, for example, analyzing the expression of tyrosine hydroxylase, a marker for dopaminergic neurons. For developing efficient treatments for Parkinson's disease, we probably need to have additional modifications of the hydrogel system to direct the differentiation of transplanted cells *in situ* into dopaminergic neurons.

## ■ CONCLUSIONS

The CLG3/HLP-loaded collagen hydrogel serves to improve the viability of NSCs in the early stage after transplantation into the striatum. This effect is caused by two antiapoptotic effects: integrin ligation and the suppression of microglial infiltration. These effects as well as biodegradability of the hydrogel will be useful for developing therapeutic methodology for the treatment of Parkinson's disease.

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### Notes

The authors declare no competing financial interest.

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