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ARTICLE

Interleukin-10 chimeric protein to protect transplanted neural progenitor cells from immune responses

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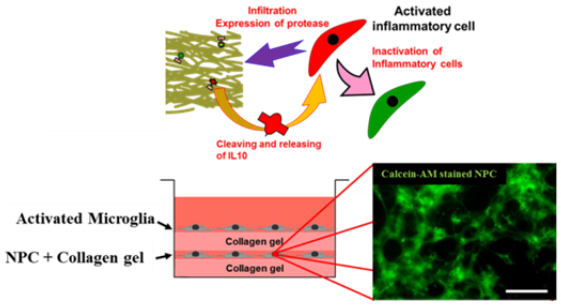
Provision of adhesive scaffolding and protection from inflammatory responses are important in enhancing the graft survival. We previously developed a functional hydrogel that strongly enhances the survival of cells transplanted into the midbrain striatum. Although graft survival reached approximately 40% using this hydrogel, the survival of transplanted cells required further enhancements because it ultimately produced a decrease in the number of transplanted cells. Therefore, we developed a hydrogel system that can locally prevent the inflammatory response. This hydrogel was modified by the addition of the interleukin 10 chimeric protein (IL10CP), which is selectively released from the hydrogel when triggered by an inflammatory response. This design protects transplanted cells from the inflammatory response, while other host cells remain unaffected. The IL10 domains are selectively released from the hydrogel and act locally on immune cells to prevent the inflammatory response without the administration of an immune suppressor. The selective release of IL10 domains from the hydrogel and their activity to prevent immune responses were evaluated using various approaches. Moreover, the ability of the IL10CP-modified hydrogel to protect cells was investigated using in vitro co-culture with activated microglia. The IL10 incorporated into the hydrogel was selectively released by the activity of matrix-metalloproteinase 9 (MMP9), and neural progenitor cells encapsulated in the IL10CP-immobilized hydrogel were protected from activated microglia by the release of IL10s from the hydrogel by the MMP9 produced by the activated microglia. These results show that the IL10CP-modified hydrogel will be useful as a biomaterial for improving survival of transplanted cells.



Novel system for protecting transplanted cells from inflammatory responses.

Introduction

Drug administration[1, 2] and cell transplantation[3, 4] have been considered as potential methods for treating intractable nerve disorders. For the treatment of Parkinson's disease, especially, transplantation of neural stem/progenitor or neuronal cells into the damaged tissue in the brain has been proposed, to recruit the dopaminergic neurons that are destroyed through degeneration and dropout during the course of the disease.[5,6] However, this type of cell-based therapy is not currently feasible because the graft survival of cells is very low, and no method has been proposed to protect and regulate the transplanted cells.

Transplanted cells require a scaffold to allow

and for protection from inflammatory responses evoked immediately following transplantation. When cells are transplanted into the striatum of the brain, graft survival has been shown to be less than 5%.[7-9] Although long-term engraftment using drugs such as immune suppressors has previously been reported, [10] long-term drug administration is regarded as posing a potential risk to the patient. Therefore, a novel system for engrafting transplanted cells needs to be developed.

Our research group has previously developed an atelocollagen-based hydrogel to provide a graft cell scaffold and to physically insulate grafted cells from inflammatory cells.[11, 12] This hydrogel was infused with neural cell adhesive chimeric protein (LGCP) derived from the laminin G domain, which is known to promote integrin-dependent adhesion. Using the LGCP-loaded collagen hydrogel, graft survival increased to approximately 40% because of the suppression of anoikis-induced cell death[8] and the prevention of microglia infiltration.

However, greater improvement of graft survival would enhance the therapeutic effect and ultimately produce a decrease in the number of transplanted cells. Although our collagen hydrogel has the ability to suppress microglia infiltration, encapsulated cells cannot be protected from inflammatory responses such as attack by inflammatory cytokines. Therefore, we designed a novel hydrogel that incorporates the ability to suppress inflammatory responses immediately surrounding the graft area. Interleukin 10 (IL10), an anti-inflammatory cytokine, is known to inhibit the activation of microglia, which are the inflammatory cells in the brain.[13] Here, we developed a novel hydrogel system from which IL10 is selectively released immediately following the induction of the inflammatory response; the released IL10 intercepts activated microglia (Fig. 1).

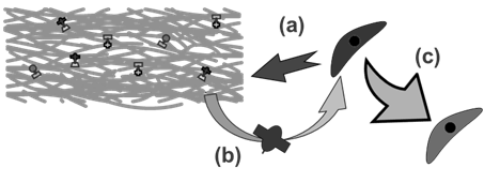


Fig. 1. Schematic illustration of a novel system for preventing attack by inflammatory cells. To prevent the inflammatory response evoked by cell transplantation, an anti-inflammatory cytokine selectively released by the infiltration of microglia was designed. This protein was named "interleukin-10 chimeric protein (IL10CP)." (a) Matrix metalloproteinases (MMPs) are released from activated microglia. (b) cleavage site of Matrix metalloproteinase-9 (M9CP) in IL10CPs stably anchored in the hydrogel is cleaved by MMP9, and cleaved IL10s are released from the hydrogel. (c) Released IL10s react to activated microglia, and prevent the inflammatory reaction.

Based on this idea, we developed a collagen hydrogel modified with IL10 chimeric protein (IL10CP). In this study, it was demonstrated that IL10CP was selectively released by the presence of activated microglia, and that cells were protected by the IL10CP-immobilized collagen hydrogel.

Results and Discussion

Synthesis and characterization of IL10 chimeric protein

Microglia and other immune cells perform important functions throughout the body for remodeling of damaged areas, and the inflammatory response was expected to be locally and selectively reduced immediately around the graft area only. Here, we developed a chimeric protein that is cleaved between the IL10 domain and the substrate-anchoring domain by reaction with matrix metalloproteinase 9 (MMP9), which is produced by immune cells, with the expectation that it would act around the graft area only when the inflammatory response was raised by transplantation. This protein was named IL10 chimeric protein (IL10CP). As shown in Fig. 1, the IL10CP incorporated into the hydrogel, which acts as a scaffold for graft cells, is selectively cleaved by the MMP9 produced by activated microglia, and the cleaved IL10 domains are released from the gel. The IL10 domains inactivate the microglia, and the graft cells encapsulated in the hydrogel are thus protected from inflammatory responses, especially from the inflammatory cytokines produced by activated microglia.

IL10CP is a fusion protein of IL10, a MMP9 cleavage sequence (M9CS), and a collagen binding peptide (CBP). It was synthesized using the *Escherichia coli* (*E. coli*) expression system (Fig. 2a). The measured molecular weight of IL10CP was concordant with the theoretical molecular weight (22.9 kDa) calculated from the amino acid sequence (Fig. 2b). Moreover, this protein produced a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, indicating that highly pure IL10CP was obtained.

The secondary structure of IL10CP was evaluated using circular dichroism (CD) spectroscopy. The CD spectrum of IL10CP was in accord with that of recombinant human IL10 (rhIL10) not including the region under 200 nm. This suggests that the IL10 domain in the chimeric protein has the same secondary structure as rhIL10. In contrast, the Cotton effect produced by random and turn structures (170–200 nm) showed slight differences between IL10CP and rhIL10. This result can be

explained by variability in the structure of the flexible linker of M9CS and CBP in the chimeric protein.

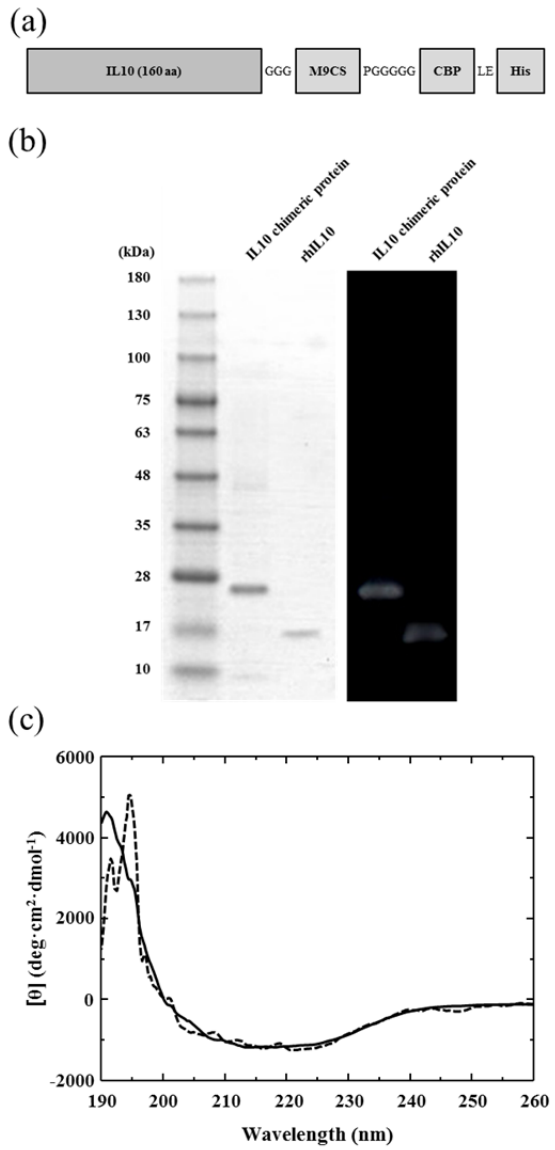


Fig. 2. (a) Schematic illustration of interleukin-10 (IL10) fused with a matrix metalloproteinase-9 cleavage site (M9CS), a collagen binding peptide (CBP), and an oligohistidine (His) (IL10 chimeric protein, IL10CP: 22.9 kDa). (b) SDS-PAGE (silver staining) and western blotting analyses of IL10CP. (c) CD spectra of IL10CP (solid line) and rhIL10 (dotted line). Sequence of M9CS and CBP peptides: GPPGVVGEQGEQGPP and SYIRIADTNIT. M9CS peptides were cleaved between G_4 and V_5.

Bioactivity of IL10CP

The bioactivity of IL10CP was evaluated by assaying its ability to prevent inflammatory cytokine production in rat microglia. Activated microglia is known to produce IL1β, IL6, and TNFα, which are inflammatory cytokines, similar to macrophages.[13] In this evaluation, rhIL10 was used as a control. As shown in Fig. 3, microglia activated with lipopolysaccharide (LPS) and interferon γ (IFNγ) was observed to have a flattened form. In contrast, when rhIL10 and IL10CP were administered to the activated microglia, the number of flattened cells significantly decreased and the microglia showed an inactivated morphology (Fig. 3a).

Inflammatory cytokines produced by inactivated microglia, activated microglia, and activated microglia incubated in medium containing IL10CP or rhIL10 were quantitatively investigated using enzyme-linked immunosorbent assay (ELISA). The amount of cytokines produced decreased with the addition of IL10CP. Moreover, cytokine production decreased as the concentration of IL10CP increased. Additionally, IL10CP and rhIL10 inhibited cytokine production equally. These results indicated that IL10CP has similar bioactivity to rhIL10.

Release of immobilized IL10 from a substrate

The selective release of IL10 anchored to a substrate by reaction with MMP9 (cleavage of M9CS in IL10CP) was quantitatively investigated. To evaluate the relationship between the amount of IL10 released and the concentration of or incubation time with MMP9, IL10CPs immobilized on a 2-dimensional glass substrate were reacted with various concentrations of MMP9. The amounts of IL10CP remaining on the surface and of the IL10 domain released from the substrate to the supernatant were determined using micro bicinchoninic acid (BCA) assay and western blotting, respectively. Fig. 4a shows the amount of protein remaining on the surface after reaction with MMP9 at concentrations of 0.5, 1.0, and 2.5 μg/mL. The surface density of protein on the surface decreased with the reaction time with MMP9, and rapidly decreased as the MMP9 concentration increased. Moreover, IL10 domains were detected in the solution by western blotting after reaction with MMP9. It appeared that the amount of protein on the surface was decreased by the release of the IL10 domain by cleavage of the M9CS sequence, and the IL10 domain was selectively released from the substrate by the reaction with MMP9. Additionally, as shown in Fig. 4b, the amount of IL10 domains released plateaued with the reaction with 1.0 μg/mL MMP9, indicating that nearly all IL10 domains had been released from the substrate.

The amount of surface protein (surface density) after reaction with MMP9 was approximately 110 ng/cm^2 (Fig. 4a and b). This was considered to represent the linker peptides remaining

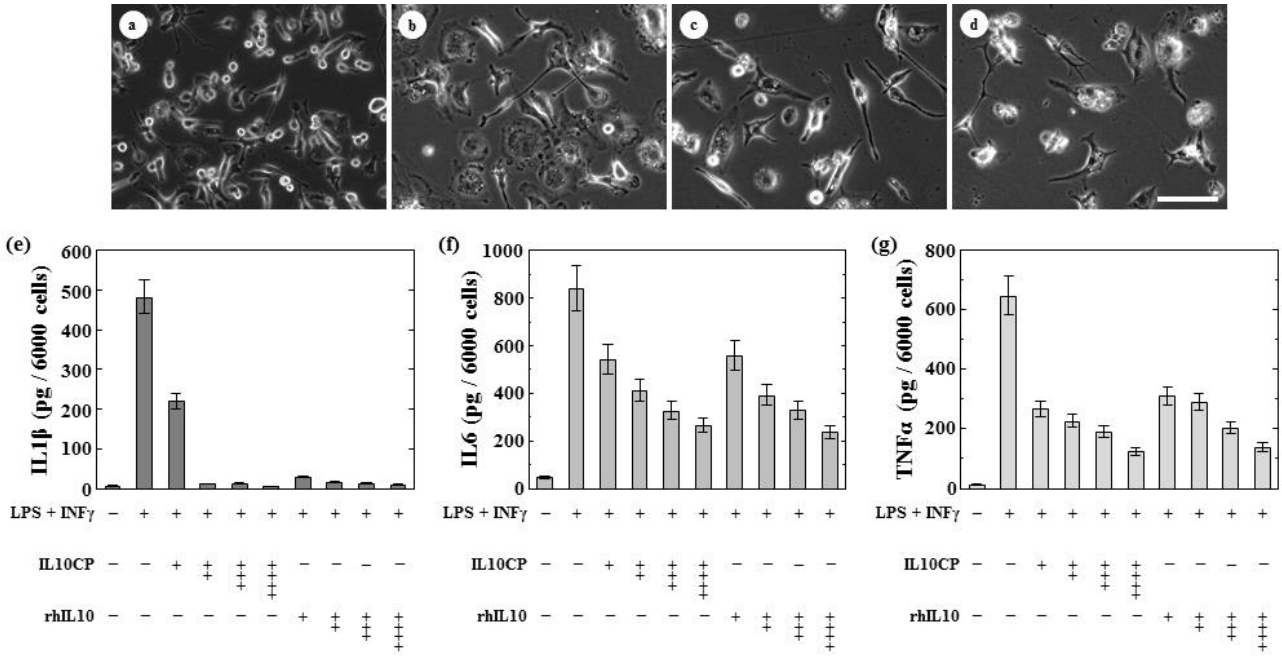


Fig. 3. The bioactivity of IL10CP was evaluated using rat microglia. Phase-contrast images of microglia (a), microglia activated with 1 μg/mL LPS and 100 ng/mL IFNγ (b), and activated microglia incubated in medium containing 200 ng/mL IL10CP (c) and 200 ng/mL rhIL10 (d). Scale bar: 100 μm. The amounts of IL1β (e), IL6 (f), and TNFα (g) expressed by inactivated or activated microglia were determined by ELISA. Activated microglia was incubated for 24 h in medium containing IL10CP or rhIL10 at each concentration (+: 2 ng/mL, ++: 20 ng/mL, +++: 200 ng/mL, ++++: 2 μg/mL).

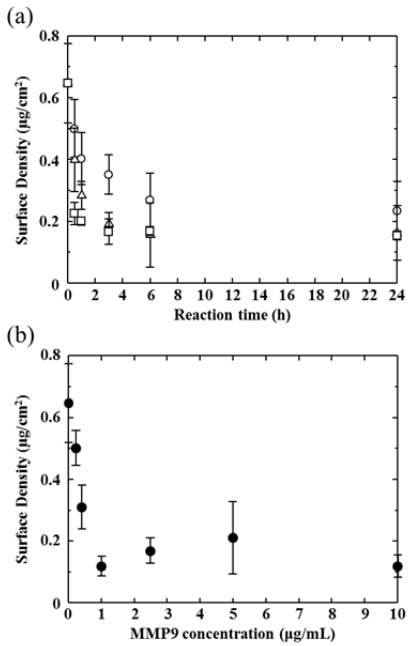


Fig. 4. Controlled release of IL10s immobilized on a substrate. IL10CPs immobilized on a glass substrate were incubated in medium containing matrix metalloproteinase-9 (MMP9). The amount of IL10CP remaining on the substrate was determined using a microBCA assay. (a) The IL10CP-anchored substrate was incubated for 0.5, 1, 3, 6, and 24 h in medium containing (○) 0.5, (△) 1.0, or (□) 2.5 μg/mL MMP9. (b) The IL10CP-anchored substrate was incubated for 3 h in medium containing MMP9 at various concentrations (0.2, 0.5, 1, 2.5, 5, and 10 μg/mL).

after cleavage of the IL10 domain. Reaction with MMP9 cleaves IL10CP into an IL10 domain (167 amino acids) and a linker oligopeptide (38 amino acids). In other words, the percentage of the weight of IL10 domain against total weight of IL10CP is 82% theoretically. This weight ratio of IL10 was concordant with the reduction ratio of surface density of IL10CP following MMP9 reaction. This result also indicates that the decreased surface density was caused by the release of the IL10 domain.

Moreover, the selective release of the IL10 domain from the hydrogel was evaluated using ELISA. A hydrogel constructed from 1 mL of hydrogel precursor solution was immersed in medium containing MMP9 (0.5 and 1 μg/mL). After incubation, the amount of IL10 domains dissolved in the supernatant was determined by ELISA. As shown in Table 1, release of the IL10 domains from the hydrogel increased with the concentration of and the incubation time with MMP9, whereas release of the IL10s was not observed from the hydrogel incubated in medium without MMP9. This result demonstrated that IL10 incorporated into the hydrogel can be selectively released by MMP9, similar to IL10 immobilized on a 2-dimensional substrate.

Additionally, IL10 domain release was investigated when the hydrogel was re-incubated for 2 h in medium without MMP9, following incubation for 10 min in medium containing 1

Table 1. Amount of IL10 domains released from the hydrogel by reaction with MMP9.

Incubation conditions	Released IL10 domains (ng/g gel) [b, c]
In medium (10 min, 37°C)	$2.0 \pm 1.7 \times 10^{-3}$
In medium (3 h, 37°C)	$1.1 \pm 1.3 \times 10^{-3}$
In 0.5 μg/mL MMP9 (10 min, 37°C)	124 ± 21
In 0.5 μg/mL MMP9 (3 h, 37°C)	513 ± 69
In 0.5 μg/mL MMP9 (24 h, 37°C) [a]	1299 ± 163
In 1 μg/mL MMP9 (10 min, 37°C)	349 ± 39
In 1 μg/mL MMP9 (3 h, 37°C)	1085 ± 74
In 1 μg/mL MMP9 (24 h, 37°C) [a]	2884 ± 394

a) The supernatant was exchanged after 12 h because MMP9 may become inactivated.
b) The data are shown as mean ± standard deviation for three independent samples.
c) The total amount of IL10CP immobilized on the hydrogel was 12.9 ± 0.74 μg/g gel.

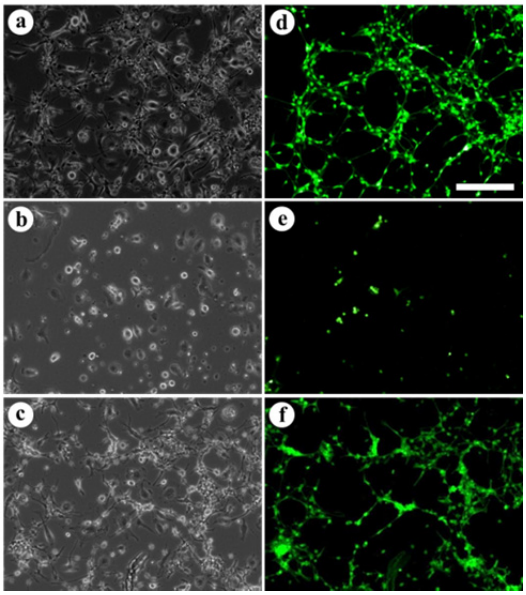


Fig. 5. Phase contrast (a–c) and fluorescent (d–f) images of NPCs co-cultured with microglia in medium without LPS/IFNγ (a, d), with LPS/IFNγ (b, e), and with LPS/IFNγ and 200 ng/mL IL10CP (c, f). To facilitate observation of the surviving cells, NPC stably expressing EGFP was used in the co-culture assay. Scale bar: 200 μm.

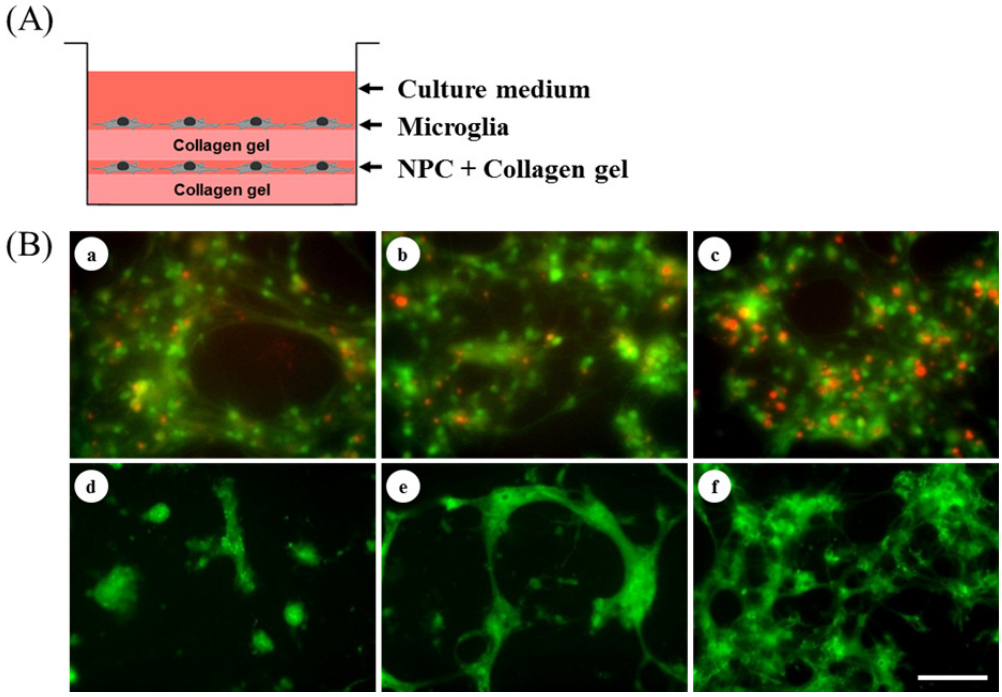


Fig. 6. NPCs were co-cultured with microglia in a collagen hydrogel using the sandwich culture method. (A) Schematic illustration of co-culture method for NPCs and microglia. As shown in the illustration, a collagen gel (upper layer) was stacked on NPCs adhered on a collagen gel (bottom layer). Microglia were seeded on the collagen gel of the upper layer. For activating microglia, LPS (1 μg/mL) and IFNγ (100 ng/mL) were added into the culture medium. (B) Fluorescent images of activated microglia (a, b, c) and NPCs (d, e, f) cultured on and in the hydrogel. In this evaluation, microglia stained red using Cell-Tracker CMRA were seeded on the hydrogel. After co-culture for 2 days following activation of microglia, cultured cells (NPCs and microglia) were stained with calcein-AM to visualize living cells. (a, d) pure collagen hydrogel, (b, e) LGCP-immobilized collagen hydrogel, (c, f) LGCP- and IL10CP- immobilized collagen hydrogel. Scale bar: 100 μm.

μg/mL MMP9. The amount of IL10s in the supernatant after incubation for 10, 30, 60, and 120 min was 342 ± 81 ng/g gel, 424 ± 24 ng/g gel, 468 ± 34 ng/g gel, and 479 ± 11 ng/g gel, respectively. The release of IL10s released from the hydrogel plateaued with the incubation time in pure medium, indicating that release of IL10s from the hydrogel ceased when MMP9 was removed. This shows that the release of the IL10 domains depends only on the production of MMP9 by activated microglia, i.e., the inflammatory response.

Effect of IL10 chimeric protein

Neuronal progenitor cells (NPCs) co-incubated with activated microglia were found to be protected by IL10CP. First, NPCs and microglia (each at 3.0×10^4 cells/cm^2) were co-cultured in medium containing LPS and IFNγ, which are microglia activation factors (Fig. 5). In this experiment, NPCs stably expressing EGFP were used to observe the survival of NPCs. When NPCs were cultured for 2 days with inactivated microglia, almost all cells survived and adhered to the substrate (2.6×10^4 cells/cm^2, recovery: 86%). Meanwhile, NPCs cultured with activated microglia showed a drastic decrease in survival after incubation for 1 day (0.24×10^4 cells/cm^2, recovery: 8%). In contrast, when NPCs and microglia were cultured in medium containing LPS, IFNγ and IL10CP, NPC survival scarcely decreased compared with that of NPCs co-cultured with activated microglia (2.1×10^4 cells/cm^2,: 70%). These results indicated that NPC survival is increased by the presence of IL10CP, because the microglia were inactivated by IL10.

The direct influence of LPS, IFNγ and IL10CP on NPCs was also investigated, and it was observed that the growth and differentiation of NPCs was the same when these cells were cultured in a general medium. Therefore, it was concluded that the death of NPCs cultured with activated microglia was caused by direct/indirect attack by microglia. The effect of microglia was also evaluated to determine the cause of death of NPCs co-cultured with microglia. NPCs were cultured in conditioned medium derived from culture of activated microglia for 1.5 days. Survival of NPCs cultured in this medium was significantly decreased (recovery: 37 ± 1.4%). Consequently, the death of NPCs was considered to be mainly caused by the inflammatory cytokines produced by activated microglia.

Protection of NSPCs in collagen gel containing IL10CP

A sandwich gel culture method (Fig. 6A) was used to evaluate the protection of NPCs from activated microglia *in vitro*. As a control, a pure collagen hydrogel and a collagen hydrogel incorporating LGCP (but without IL10CP) were also evaluated. Fluorescent images of microglia and NPCs cultured for 1 day after activation of microglia are shown in Fig. 6B. Moreover, Fig. 7A shows the number of living NPCs cultured in each hydrogel before and after activation of microglia.

Almost all NPCs in the pure hydrogel died, in spite of the inactivated state of the microglia (Fig. 7A). This result was consistent with a previous report[11, 12] and was considered to be

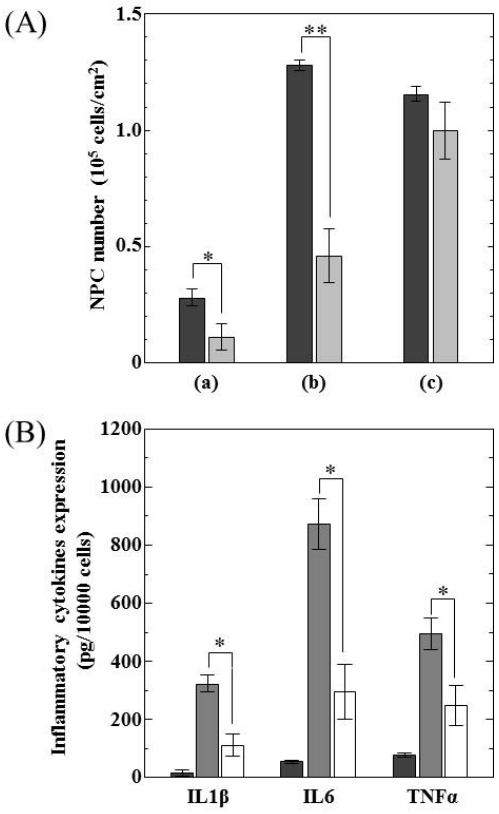


Fig. 7. (A) The number of NPCs following 2 days of co-culture with inactivated (dark gray bar) or activated (light gray bar) microglia in (a) pure, (b) LGCP-immobilized, and (c) LGCP- and IL10CP- immobilized collagen hydrogel. Data are expressed as the mean ± standard deviation for n = 4. * and ** indicate statistically significant differences (* $p < 0.05$ and ** $p < 0.01$, Tukey's HSD test). In this evaluation, EGFP-NPCs were used to count only NPCs recovered from the hydrogel. (B) The amounts of inflammatory cytokines (IL1β, IL6 and TNFα) released from inactivated microglia (dark gray bar), activated microglia (light gray bar), or activated microglia co-cultured with NPCs in an LGCP- and IL10CP- immobilized collagen hydrogel system (white bar). Data are expressed as the mean ± standard deviation for n = 4. * indicates a statistically significant difference ($p < 0.05$, Tukey's HSD test).

caused by the absence of adhesive molecules (LGCPs). In contrast, the cells cultured in LGCP-immobilized hydrogel showed high viability when the microglia was inactivated. However, the survival of NPCs decreased by 65% with the activation of the microglia (Fig. 6B(e) and Fig. 7A), indicating that NPCs were attacked by the inflammatory cytokines produced by the microglia, rather than by the microglia directly, because the NPCs were separated from the microglia by a hydrogel layer (see Fig. 6A). However, in the hydrogel modified with IL10CP, the viability of NPCs was maintained after the activation of the microglia (Fig. 7A), and the NPCs formed a network (Fig. 6B(f)). Incidentally, it was indicated in preliminary experiment (data not shown) that NPCs were not received the damages and the other influences absolutely by incubation of 1% collagenase solution for 1 h. These results strongly suggested that NPCs can be protected by the provision of an adhesive scaffold and the suppression of microglia activation using an LGCP- and IL10CP-immobilized collagen hydrogel.

The produced amount of MMP9 from the co-cultured microglia was also evaluated by ELISA. In this model system (co-culture system of NPCs and microglia), MMP9 was produced 128 ± 49 ng/mL (6.4 ± 2.5 ng/6000 cells) in culture medium. Although the produced amount cannot be directly compared in vivo environment, it was indicated that the selective release of IL10 domains was stimulated by relatively-low concentration of MMP9, and NPCs encapsulated in hydrogel were protected from inflammatory response with activated microglia. From these results, the IL10CP-anchored hydrogel system can be strongly expected as an effective tool for the protection of transplanted NPCs.

To demonstrate the protection of NPCs by the incorporation of IL10CP into the hydrogel, the amount of inflammatory cytokines in the culture supernatant was investigated by ELISA (Fig. 7B). The production of IL1β, IL6, and TNFα by activated microglia increased 16-, 15-, and 5.6-fold, respectively, compared with that of inactivated microglia. In contrast, the production of cytokines was decreased to approximately 1/3 by the immobilization of IL10CP into the hydrogel. These results were concordant with the results of the assessment of NPC viability in the sandwich gel culture (Fig. 7A). Consequently, it was demonstrated that the survival of NPCs was enhanced by prevention of production of inflammatory cytokines; the activation of microglia was inhibited by IL10 domains

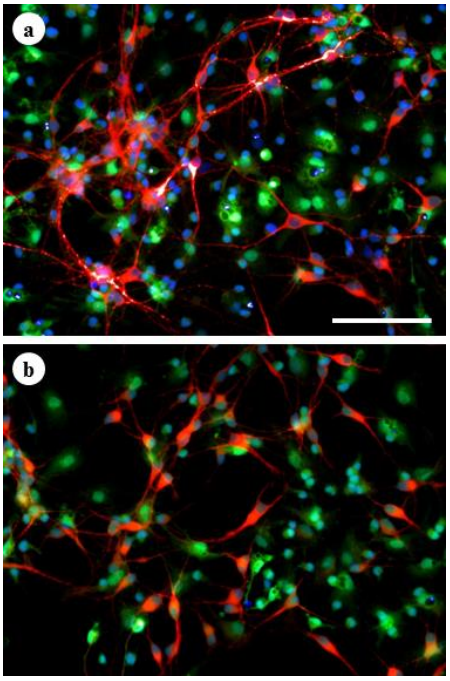


Fig. 8. Immunofluorescent images of EGFP-NPCs (a) cultured for 10 days with (a) and without (b) microglia in medium containing 1 μg/mL LPS, 100 ng/mL IFNγ, and 200 ng/mL IL10CP. (red) MAP2, (green) EGFP. Scale bar: 100 μm.

released from the hydrogel by cleavage with MMP9 produced by activated microglia.

In the present study, an IL10CP-immobilized collagen hydrogel was designed to provide a tool for improvement of graft survival of NPCs. Previously, we were able to increase graft survival to 40% using a LGCP-loaded collagen hydrogel.[12] Here, the incorporation of IL10CP into the hydrogel was shown to enhance the viability of encapsulated cells. Although various strategies are known to be effective in preventing the inflammatory response, we aimed to facilitate graft survival via local suppression of the inflammatory response with selective release of IL10. Microglia operates throughout the brain to repair damaged neuronal cells and maintain neural functions; administration of systemic immune suppressors could therefore impair microglial activity throughout the brain tissue, not just in the region of the transplant. This effect must be avoided to maintain the health of host tissue. Moreover, inactivated microglia is known to enhance the growth and differentiation of neuronal cells.[14] When NPCs were co-cultured for 10 days with microglia inactivated by IL10 domains, differentiation of NPCs into mature neurons was enhanced (Fig. 8); the inactivated microglia may also produce neurotrophic factors, such as brain-derived and glial cell line-derived neurotrophic factors. Therefore, by using a hydrogel

incorporating IL10CP, it is expected that microglia infiltrating into the transplanted region would be inactivated by the released IL10 domains and that these inactivated microglia might maintain and enhance the function of the transplanted cells. Thus, the IL10CP-loaded collagen hydrogel developed here shows the potential to act as a high-performance biomaterial not only for the protection of transplanted NPCs but also for the regulation of surviving NPCs. As the next step of our research, we plan to investigate the effectiveness of IL10CP-immobilized collagen hydrogels *in vivo*.

Experimental

Synthesis of Interleukin 10 Chimeric Protein (IL10CP)

IL10CP is a functional protein produced by fusion of a flexible linker peptide (FLP, amino acid sequence: PGGGGGGG), a matrix metalloproteinase-9 (MMP9) cleavage peptide (M9CS, amino acid sequence: GPPGVVGEQGEQGPP), [15, 16] a collagen-binding peptide (CBP, amino acid sequence: SYIRIADTNIT), [11] and an oligohistidine to the C-terminus of IL10. This protein was synthesized using the *E. coli* expression system.

The gene encoding the IL10CP was synthesized using the overlap extension PCR method[17] with the primers shown in Table 2. First, the IL10 gene was amplified with primers 1 and 2 from a human IL10 cDNA clone inserted in plasmid DNA (pCMV6-XL5) (SC300099, OriGene Technologies, Rockville, MD, USA). The oligopeptide-corded gene fused to IL10 was obtained by ligation of primers 3 and 4 using overlap extension PCR and amplification of the gene using primers 5 and 6. Finally, IL10- and oligopeptide-corded genes were ligated with overlap extension PCR. The obtained gene was amplified with primers 1 and 7. Using this procedure, the IL10-M9CS-FLP-CBP (IL10CP) gene was obtained.

The IL10CP gene was digested with the *Nde*I and *Xho*I restriction enzymes and inserted into a pET-22 plasmid (Merck Novagen, Darmstadt, Germany) that had been digested with the same restriction enzymes using a DNA Ligase Kit (Mighty Mix, TaKaRa Bio Inc., Sigma, Japan) (pET22-IL10CP). The plasmids were transfected into DH5α *E. coli* and amplified. The sequence of the IL10CP gene was verified using a Genetic sequencer (3130xl Genetic Analyzer, Applied Biosystems, Foster City, CA, USA).

To obtain the refolded protein, IL10CP was synthesized using the *E. coli* expression system to co-express the molecular chaperone.[18] pET22-IL10CP plasmids were transfected into BL21(DE3)*pGro7*[19] using the heat shock method. The transfectants were suspended in TB medium containing 50 μg/mL ampicillin, 20 μg/mL chloramphenicol, and 500 μg/mL L-arabinose, and cultured at 30°C with shaking at 175 rpm until the absorbance at OD_{600} reached 0.45. One hundred milli-molarity IPTG solution was added to the *E. coli* suspension at a final concentration of 0.75 mM, and this suspension was cultured for 24 h at 20°C with shaking at 175 rpm.

E. coli expressing IL10CP was recovered by centrifugation (8000 × *g*, 5 min), and IL10CP was extracted using Bugbuster Protein Extraction Reagent (Merck Novagen) containing 200 μg/mL lysozyme and 10 units/mL benzonase. The supernatant containing IL10CP was purified with affinity chromatography using His-trap FF (GE Healthcare, Buckinghamshire, UK) and fractionated with a molecular cutoff filter (Amicon Ultra 15, MWCO 50 kDa and 30 kDa, Merck Millipore, Darmstadt, Germany). The purified protein solution was sterilized with a 0.22-μm syringe filter and stored at −80°C

Table 2. Primers for synthesis of IL10CP-corded gene.

Primer No.	Sequence	Remarks
1	5′-catgcatatgagcccaggccagggcacccag-3′	Fw primer for IL10 and IL10CP genes
2	5′-gtttcgtatcttcattgtcatgtagg-3′	Rv primer for IL10 gene
3	5′-ctacatgacaatgaagatacgaaacggtggcgggggaccacctggtgtagtgggagaacaag gggagcagggaccaccgcc-3′	Fw primer for overlap extension PCR
4	5′-gctcgagggtgatattggtatcagcaatgcggatgtaggaaccgcccccacccccgccacctg gcggtggtccctgctcccct-3′	Rv primer for overlap extension PCR
5	5′-ctacatgacaatgaagatac-3′	Fw primer to amplify the gene obtained from primers 3 and 4
6	5′-gctcgagggtgatattggta-3′	Rv primer to amplify the gene obtained from primers 3 and 4
7	5′-gctcgagggtgatattggtatcagc-3′	Rv primer for IL10CP genes

until further use.

Synthesis of laminin-derived neuronal cell-adhesive chimeric protein (LGCP)

Cell-adhesive chimeric protein had previously been developed in our group, and was constructed by the complex of the G3 domain of the laminin α-chain and 9 residues (FNTPSIEKP) at the C-terminus of the laminin γ-chain. As reported previously,[11, 12] LGCP was expressed in *E. coli*, purified by affinity column chromatography using a His-trap column and ultrafiltration using an Amicon Ultra 15 filter unit, and refolded by the step-wise dialysis method.

Characterization of IL10CP

The molecular weight and purity of IL10CP were evaluated by SDS-PAGE and western blotting analyses. The protein solution was electrophoresed in Tris/Tricine/SDS buffer on a 16% polyacrylamide gel (Bio-Rad, Hercules, CA, USA). The protein was stained with an Ultra Fast Protein Silver Staining Kit (Nag Research Laboratories, Fremont, CA, USA). Following electrophoresis, proteins from the gel were transferred to poly vinylidene difluoride (PVDF) membrane using a semi-dry blotting machine (Bio-Rad). This membrane was washed for 3 min with phosphate buffer saline containing 0.05% Tween-20 (PBS-T) and immersed for 1.5 h in blocking reagent. Proteins on the membrane were immunochemically stained with an antibody against IL10 (anti-IL10 rabbit IgG, polyclonal, Santa Cruz Biotechnology, Dallas, TX, USA). After reaction with a secondary antibody against rabbit IgG (anti-rabbit IgG HRP conjugate, GE Healthcare), the membrane was treated with ECL Prime Plus (GE Healthcare), and the IL10CP band was detected with a chemical luminescence imager (AE-9200, ATTO Corporation, Tokyo, Japan).

The secondary structure of IL10CP was investigated by CD spectroscopy (J-820, JASCO Corporation, Tokyo, Japan). rhIL10 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a control protein. Measurements were performed under the following conditions: concentration of amino acid residue = 0.9 mM, pass length = 0.5 mm, response time = 0.5 sec, scan speed = 100 nm/min, scan time = 8, temperature = 25°C.

The bioactivity of IL10CP was evaluated using microglia derived from newborn Wistar rats (MB-X0501, DS Pharma Biomedical Co., Ltd., Osaka, Japan). The maintenance culture conditions of microglia are described below (in the Cell Culture section). Microglia was activated by culture for 6 h at 37°C and 5% CO_2 in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) containing 1 μg/mL lipopolysaccharide (LPS) and 100 ng/mL interferon γ (IFNγ). After changing to fresh medium, IL10CP or rhIL10 at a concentration of 2, 20, 200 ng/mL and 2 μg/mL, respectively, were added to the medium, and activated microglia was incubated for 2 h. Finally, the medium was collected and analyzed with ELISA Kits against IL1β, IL6, and TNFα (Affymetrix eBioscience, San Diego, CA, USA).

To evaluate the selective release of the IL10 domains from the substrate, a glass surface on which IL10CP had been immobilized was prepared, using a previously reported method.[20-22] IL10CP was anchored to the surface through the interaction between oligohistidine and Ni-NTA chelation. The substrate was immersed for 0–24 h in DMEM/F12 containing matrix metalloproteinase 9 (MMP9, R&D Systems, Minneapolis, MN, USA) at a concentration of 0–10 μg/mL. The amount of protein remaining on the surface was measured by microBCA assay, and the amount of IL10 released into the medium was measured using an ELISA Kit against IL10 (Affymetrix eBioscience).

The release of the IL10 domain from the collagen hydrogel was evaluated. A collagen hydrogel loaded with IL10CP was immersed in medium containing 0.5 or 1.0 μg/mL MMP9, and the amount of IL10 domain released was determined using an ELISA Kit against IL10.

Hydrogel preparation

The collagen hydrogel loaded with IL10CP and LG was prepared using the method reported previously.[11,12] Five hundred microliters of Types I and III atelocollagen solutions (10 mg/mL, Nippon Meat Packers, Inc., Osaka, Japan, referred to as simply "collagen" in this paper) in HCl solution (pH 2−3) was mixed with 5× concentrated DMEM/F12 (200 μL), B27 supplement (20 μL), 10× concentrated reconstruction buffer (50 μL, Nitta Gelatin Inc., Osaka, Japan), LGCP (at a final concentration of 3 μM), and IL10CP (at a final concentration of 3 μM) at 4°C. PBS was added to this solution to raise the volume to 1 mL (hydrogel precursor solution).

Incubation for 15–30 min at 37°C transforms the precursor solution into a hydrogel to allow the regeneration of collagen fibrils. Therefore, cells were mixed with the hydrogel precursor solution at 4°C and incubated at 37°C to allow the encapsulation of cells in the hydrogel.

Cell culture

NPCs were isolated from the striatum of Fischer344 rat or GFP-stably expressing SD rat embryos (E15), according to the guidelines of the Animal Welfare Committee at our institution. The striatum was dissociated into single cells by reaction for 15 min at 37°C with 0.05% trypsin solution containing 0.53 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA) (trypsin/EDTA solution, Nacalai Tesque, Inc., Kyoto, Japan). Single cells were suspended in a neuronal cell base medium [DMEM/F12 (1:1) (Invitrogen, Carlsbad, CA) containing 3 μM glutamax (Life Technologies–Invitrogen, Carlsbad, CA), 5 μg/mL heparin (Nacalai Tesque, Inc.), 100 unit/mL penicillin and 100 μg/mL streptomycin]. These cells were cultured for 4 days in the neuronal cell base medium supplemented with 2% B27, 20 ng/mL basic fibroblast growth factor (bFGF, Wako Pure Chemical Industries, Ltd.), and 20 ng/mL epidermal growth factor (EGF, Wako Pure Chemical Industries, Ltd.) to form neurospheres. Neurosphere-forming cells were dissociated into single cells with trypsin/EDTA solution. Single cells were re-suspended in the neuronal cell base medium.

Microglia derived from newborn Wistar rats was purchased from DS Pharma Biomedical Co. Ltd. The microglia cells were suspended in the microglia growth medium [DMEM/F12 containing 10% FBS, 5 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF), 100 unit/mL penicillin and 100 μg/mL streptomycin] and cultured for 7 days in T-25 flasks; fresh medium was provided every 2–3 days. These cells were treated with 0.25% trypsin/2.5 mM EDTA solution for 1–2 min and harvested using a cell scraper. The cells were re-suspended in fresh medium and seeded on tissue culture polystyrene (TCPS) dishes or onto collagen hydrogel at a density of 3×10^4 cells/cm^2.

Co-culture of NPCs and microglia

For co-culture, NPCs derived from the striatum of a GFP rat fetus (EGFP-NPCs) were used to identify NPCs and microglia. The isolation and pre-culture (for proliferation) of EGFP-NPCs were carried out by the same methods of NPCs (see *Cell culture* section). EGFP-NPCs and microglia cells were suspended in neuronal cell base medium and seeded onto laminin-coated TCPS dishes at a density of 3×10^4 cells/cm^2. After incubating for 1 day for complete adherence, these cells were incubated in the neuronal cell base medium containing 1 μg/mL LPS and 100 ng/mL IFNγ, with or without 200 ng/mL IL10CP. After 1 day, the survival of NPCs was determined using an epifluorescent microscope (IX71, Olympus Optical Co. Ltd., Tokyo, Japan)

To evaluate the survival of NPCs in collagen hydrogel, NPCs (not expressing EGFP) were cultured using the sandwich method[11] for ease of observation. Microglia was stained with CellTracker CMRA (Life Technologies–Invitrogen) to facilitate observation of their infiltration into the hydrogel. First, 300 μL of the hydrogel precursor solution described above was added to 12-well non-treated TCPS plates (4 cm^2/well) and incubated for 30 min at 37°C. NPCs suspended in hydrogel precursor solution (1.2×10^5 cells and 3×10^4 cells/cm^2) were seeded on the bottom gel and incubated for 30 min to allow gelation. Next, 300 μL of hydrogel precursor solution was added to the hydrogel of the cell layer and re-incubated for 30 min. Neuronal cell base medium supplemented with 2% B27, 20 ng/mL bFGF, and 20 ng/mL EGF was added to the hydrogel, and the cells were cultured for 2 days to adhere to and grow in the hydrogel. Microglia (1.2×10^5 cells and 3×10^4 cells/cm^2) was seeded on the hydrogel and incubated for 6 h to allow adhesion. These cells were activated by adding LPS (final concentration: 1 μg/mL) and IFNγ (final concentration: 100 ng/mL) to the medium. After 1 day, these cells were observed with an epifluorescent microscope (IX71)

Cell staining assay

EGFP-NPCs co-cultured with activated microglia on TCPS dishes (2-dimensional surface) were immunocytochemically stained. These cells were washed with PBS solution warmed to 37°C, and fixed with 4% *p*-formaldehyde/PBS solution. Fixed cells were permeabilized with 0.2% TritonX-100/PBS solution, and treated for 2 h with 20% Blocking One (Nacalai Tesque)/PBS-T solution. Cells were stained for 2 h with primary antibodies for anti-microtube associated protein-2 (MAP2, 1:200, mouse monoclonal IgG, MAB3418, Merck Millipore). After washing with PBS-T solution, these cells were stained with the fluorescence secondary antibody, Alexa Fluor 594 anti-mouse IgG (1:500, Life Technologies–Molecular Probes, Carlsbad, CA) and counter-stained with Hoechst33258 (Dojindo Laboratories, Kumamoto, Japan). Immunostained cells were observed with an epifluorescent microscope (IX71).

To evaluate the survival of NPCs co-cultured with activated microglia in the hydrogel, cells were stained with calcein-AM (Dojindo Laboratories). Co-cultured cells were washed with DMEM/F12 medium without other reagents and incubated for 30 min in medium containing calcein-AM (2 μg/mL). Cells were washed and incubated for 10 min in DMEM/F12 medium. The washing and incubation were repeated 3 times. The stained cells were observed with an epifluorescent microscope (IX71).

Quantitative evaluation of NPCs co-cultured with activated microglia in the hydrogel

After co-culture for 2 days with inactivated and activated microglia, the collagen hydrogels were incubated for 60 min in 1% collagenase/PBS and degraded completely. These solutions were collected and centrifuged to recover the cells. The cells were washed with PBS and re-suspended in medium. Cells stained green with calcein-AM and red with Cell-Tracker (for microglia) were applied onto hemocytometer and observed using an epifluorescent microscope. Cells stained only with green, which are NPCs, were counted because cells double-stained with red and green were microglia. Each sample was counted three times, and the data were averaged. The data are shown as mean ± standard deviation for four independent samples.

Assay of inflammatory cytokines

The inflammatory cytokines produced by activated microglia co-cultured with NPCs in hydrogel were quantitatively evaluated with ELISA. After 2 days of culture, the medium was collected and reacted to surface-bound anti-IL1β, anti-IL6, and anti-INFγ antibodies on ELISA plate for 2 h. ELISA analysis kits were purchased from Affymetrix eBioscience. The data are shown as mean ± standard deviation for four independent samples.

Conclusion

We designed a novel hydrogel system to locally and selectively prevent the inflammatory response and to facilitate graft survival of NPCs. Cells encapsulated in IL10CP-immobilized hydrogel were protected from activated microglia by selective release of IL10 domains cleaved with MMP9. The advantage of this hydrogel is that the inflammatory response is locally suppressed only in the transplanted area and IL10 is selectively released immediately following the inflammatory response. The results of co-culture assays of NPCs and activated microglia established the high functionality of the IL10CP-immobilized hydrogel. Although the effectiveness of the hydrogel remains to be demonstrated *in vivo*, this hydrogel system shows excellent potential to contribute to cell transplantation therapy for treatment of Parkinson's disease.

Notes and references

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(1) F. Zhang, S. Wang, L. Gan, P. S. Vosler, Y. Gao, M. J. Zigmond and J. Chen, *Prog. Neurobiol.*, 2011, **95**, 373–395.
(2) F. Wang, M. Kameda, T. Yasuhara, N. Tajiri, Y. Kikuchi, H. B. Liang, J. T. Tayra, A. Shinko and T. Wakamori, *Neurosci. Res.*, 2011, **71**, 92–98.
(3) T. Agari, I. Date and Okano, H. *Neurosci. Res.*, 2002, **69**, 698–707.
(4) S. Goldman, *Nat Biotechnol.*, 2005, **23**, 862–871.
(5) Y. Takagi, J. Takahashi, H. Saiki, A. Morizane, T. Hayashi, Y. Kishi, H. Fukuda, Y. Okamoto, M. Koyanagi, M. Ideguchi, H. Hayashi, T. Imazato, H. Kawasaki, H. Suemori, S. Omachi, H. Iida, N. Itoh, N. Nakatsuji, Y. Sasai and N. Hashimoto, *J. Clin. Invest.*, 2005, **115**, 102–109.
(6) P. Piccini, N. Pavese, P. Hagell, J. Reimer, A. Björklund, W. H. Oertel, N. P. Quinn, D. J. Brooks and O. Lindvall, *Brain*, 2005, **128**, 2977–2986.
(7) R. J. Armstrong and C. N. Svendsen, *Cell Transplant.*, 2000, **9**, 139–152.
(8) D. M. Marchionini, T. J. Collier, M. Camargo, S. McGuire, M. Pitzer and C. E. Sortwell, *J. Comp. Neurol.*, 2003, **464**, 172–179.
(9) D. E. Kim, K. Tsuji, Y. R. Kim, F. J. Mueller, H. S. Eom, E. Y. Snyder, E. H. Lo, R. Weissleder and D. Schellingerhout, *Radiology*, 2006, **241**, 822–830.
(10) A. C. Lepore, B. Neuhuber, T. M. Connors, S. S. W. Han, Y. Liu, M. P. Daniels, M. S. Rao and I. Fischer, *Neuroscience*, 2006, **142**, 287–304.
(11) T. Nakaji-Hirabayashi, K. Kato and H. Iwata, *Bioconjugate Chem.*, 2012, **23**, 212–221.
(12) T. Nakaji-Hirabayashi, K. Kato and H. Iwata, *Bioconjugate Chem.*, 2013, **24**, 1798–1804.
(13) T. Arimoto, D. –Y. Choi, X. Lua, M. Liu, X. V. Nguyena, N. Zheng, C. A. Stewart, H. -C. Kimd and G. Bing, *Neurobiol. Aging*, 2007, **28**, 894–906.
(14) G. M. Halliday and C. H. Stevens, *Movement Disorders*, 2011, **26**, 6–17.
(15) J. L. Lauer-Fields, T. Sritharan, M. S. Stack, H. Nagase and G. B. Fields, *J. Biol. Chem.*, 2003, **278**, 18140–18145.
(16) H. F. Bigg, A. D. Rowan, M. D. Barker and T. E. Cawston, *FEBS J.*, 2007, **274**, 1246–1255.
(17) T. Nakaji-Hirabayashi, K. Kato, Y. Arima and H. Iwata, *Bioconjugate Chem.*, 2008, **19**, 516–524.
(18) J. G. Thomas, A. Ayling and F. Baneyx, *Appl. Biochem. Biotech.*, 1997, **66**, 197–238.
(19) T. Nakaji-Hirabayashi, K. Kato and H. Iwata, *Biomaterials*, submitted.
(20) T. Nakaji-Hirabayashi, K. Kato, Y. Arima and H. Iwata, *Biomaterials*, 2007, **28**, 3517–3529.
(21) T. Nakaji-Hirabayashi, K. Kato and H. Iwata, *Biomaterials*, 2008, **29**, 4403–4408.
(22) T. Nakaji-Hirabayashi, K. Kato and H. Iwata, *Bioconjugate Chem.*, 2009, **20**, 102–110.