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The Pro-Inflammatory Cytokines Expression of Porcine Embryonic Stem Cells Xenotransplanted into the Brain and Spinal Cord in Rats

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Abstract

In this study, we transplanted green fluorescent protein-expressing porcine embryonic stem (pES/GFP$^+$) cells and their derived cells, D12 neuronal progenitors (D12 NP) and D18 neuronal progenitors (D18 NP), into the brain and spinal cord of Sprague-Dawley (SD) rats to investigate the differences of inflammation after xenotransplantation. On day 3, 7, and 14 after transplantation, we collected the brain and spinal cord tissues to investigate the gene expression of interleukin 1-α and -β (*IL-1α* and *IL-1β*), interleukin-6 (*IL-6*), and tumor necrosis factor α (*TNF-α*). One month after transplantation, histopathological changes of the brain and spinal cord were examined by H&E staining. The expression patterns of inflammatory-related factors in the spinal cord were more dramatic than those in the brain. On day 14 after transplantation, only D18 NP significantly elevated the expressions of *IL-1α* and *IL-1β* in the brain, but almost all grafted cells evoked the expression of *IL-1α*, *IL-1β*, *IL-6*, and *TNF-α* in the spinal cord. Following H&E staining, no dramatic histopathological abnormality was revealed, indicating that no acute damage can be observed following the experimental period albeit inflammatory gene expressions were temporarily triggered. Although the gene expression patterns varied during the experimental period, no lethality appeared in the rats after xenotransplantation of pES/GFP$^+$ cells. These indicate that pES/GFP+ cells may be a safe cell resource for future application on regenerative medicine.

Keywords:

Inflammation; Xenotransplantation; Porcine; Embryonic stem cells; Brain; Spinal cord

Introduction

Xenotransplantation offers a potentially unlimited source for the transplantation of tissues and organs, but the induction of strong inflammation and immune responses pose a major roadblock to its application in the clinic. The grafted cells present various antigens that is recognized by the host immune system to evoke inflammation and immune rejection via proinflammatory cytokines. Hence, the reduction of inflammation and immune rejection after transplantation is critical for the success of xenotransplantation. The characteristics of grafted materials, donors and recipients, and the transplantation method and location determine the level of immune repsonses [1]. The three major groups of antigens involved in the immune responses of xenotransplantation are major histocompatibility complex (MHC) antigens, minor histocompatibility antigens, and ABO blood antigens [2,3]. Among these antigens, MHC-I is the strongest trigger for immune responses [4]. The mediators known as pro-inflammatory cytokines, including interleukin 1-α and -β (IL-1α and IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α), are released and coordinate the local and systemic inflammation following transplantations [5].

The immunologically privileged sites are specialized location in the human body exempt from immune responses [6]. The sites are thought to include central nervous system (CNS), eye, pregnant uterus, testis, ovary, etc [7]. Among these sites, the eye, the brain and the pregnant uterus are regarded as well-developed immune privilege sites in the body [8]. Due to the absence of antigen-presenting cells (APC), sparse lymphatic drainage, and blood-brain barrier (BBB), the brain was one of the first organs to be recognized as an immunologically privileged site. Therefore, it was hypothesized that the brain could potentially accept grafted materials [6]. However, this theory was proved to be incorrect. This immune status is far from absolute and varies with age as well as different regions in the brain [5]. In fact, the perivascular space of the human brain is rich in antigens [9]. Donor APCs are the major regulator that determines whether the recipients accept grafted materials. Moreover, active lymphocytes could pass through BBB, and there is evidence that microglia in the brain might have an APC capability [4]. These results imply that immune response might be evoked in the brain.

The cell surface of embryonic stem cells (ES cells) and their derived

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cells may express antigens, and therefore the immune system could recognize those antigens to trigger immune responses. The expression of MHC-I/II in grafted cells was depended on the differentiated state [10]. The undifferentiated mouse [11] or human [12] ES cells expressed low MHC-I/II. However, the expression increased 2-4 folds when ES cells differentiated into embryoid body, and up to 8-10 folds when they were induced to teratoma [12]. Compared with somatic cells, the expression of MHC-I/II in ES cells was minor although the expression was increased after differentiation. Hence, ES cells transplantation might evoke less immune rejection, but the efficacy for application in the clinic requires further investigation.

In our previous studies, pES/GFP$^+$ cells-derived neuronal progenitors were successfully transplanted, which ameliorated Parkinson's disease [13] and spinal cord injury [14] in rat models. In addition, regeneration of periodontal furcation defects in a porcine model was improved by transplantation with pES/GFP$^+$ cells [15]. Fortunately, we did not observe any teratoma formation or obvious immune rejection in the animals of these studies. In order to further investigate the biological response between host and pES/GFP$^+$ cells, we transplanted pES/GFP$^+$ cells and their derived cells into the brain and spinal cord of SD rats to investigate inflammatory responses.

Materials and methods

In vitro culture of GFP-expressing pES cells

The pES cells expressing green fluorescent protein (pES/GFP$^+$) used in this study were obtained by GFP electroporation of pES cell line M215-3 that was derived from the pre-implantation blastocysts of the Livestock Research Institute Black Pig No. 1 (a top crossing breed established from Taoyuan and Duroc pigs). They were maintained in ES-cell culture medium (ESM) consisted of Dulbecco's modified eagle medium (DMEM, high glucose and no pyruvate, Invitrogen, Grand Island, NY, USA) supplemented with 1 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM β-2-mercaptoethanol (Sigma-Aldrich), 10 mM MEM non-essential amino acids (Sigma-Aldrich), 0.03 mM adenosine (Sigma-Aldrich), 0.03 mM guanosine (Sigma-Aldrich), 0.03 mM cytidine (Sigma-Aldrich), 0.03 mM uridine (Sigma-Aldrich), 0.01 mM thymidine (Sigma-Aldrich), antibiotics (50 units/mL penicillin G and 50 g/mL streptomycin sulfate, Invitrogen) and 16% fetal bovine serum (FBS, Invitrogen). The pluripotent pES/GFP$^+$ cells were propagated on a feeder layer of mitomycin C (Sigma-Aldrich)-inactivated STO cells (mouse embryonic fibroblasts, CRL-1503, USA) in gelatin-coated Multidish 4 Wells® (Nunc 176740, Roskilde, Denmark) and cultured at 37°C in an atmosphere of 5% CO_2 in air [13-16].

In vitro induction of neuronal differentiation

In vitro neuronal differentiation of pES/GFP$^+$ cells was induced in a suspension culture with induction medium containing different combinations of neurogenic stimulators and then re-plated onto gelatin-coated 4-well dishes in expansion medium. The neurogenic stimulators used for neuronal induction were retinoic acid (RA, 1 μM, Sigma-Aldrich), sonic hedgehog (Shh, 200 ng/ml, R&D systems, Minneapolis, MN, USA), and fibroblast growth factor (FGF, 100 ng/ml, Sigma-Aldrich). Confluent pES/GFP$^+$ colonies were trypsinized by 0.25% trypsin-EDTA and subjected to a two-step *in vitro* induction protocol for neural differentiation. They were firstly taken into a single-cell suspension culture at a concentration of 1×10^5 cells/mL in an Easy Flask® (Nunc 169900) with serum-free ESM containing RA+Shh+FGF for neuronal induction. After 12 days of induction, the pES/GFP$^+$ cell-derived cells were re-plated onto gelatin-coated 4-well dishes in 5×10^3 cells/mL. These cells were cultured in expanding medium containing DMEM/F12 medium (Sigma-Aldrich), 1:50 B27 supplementation (Invitrogen), 2 mM L-glutamine, 50 units/mL penicillin G and 50 g/mL streptomycin sulfate (Invitrogen), and supplemented with 20 ng/mL human recombinant epidermal growth factor (hrEGF, Invitrogen), 20 ng/mL human recombinant basic fibroblast growth factor (hrbFGF, Invitrogen), and 1:100 N2 (Invitrogen) [13,14]. The undifferentiated pES/GFP$^+$ cells were regarded as D0 pES/GFP$^+$ cells (D0 pES). The pES/GFP$^+$ cells-derived cells collected at the end of the 12 day neuronal induction were regarded as the D12 neuronal progenitors (D12 NP), and the pES/GFP$^+$ cells-derived cells collected on day 6 after the 12 day neuronal induction were regarded as the D18 neuronal progenitors (D18 NP) [13,14].

Transplantation of pES/GFP$^+$cells and derived neuronal progenitors

All animal experiments in this study were carried out in accordance with ethical guidelines and following the approval of the Livestock Research Institutional Animal Care and Use Committee. Sixty male Sprague-Dawley (SD) rats at 12 weeks of age weighted 250-300 g were bought from Bio-LASCO Taiwan Co., Ltd. Forty-five of them were used for real-time PCR analysis and fifteen of them were used for histopathological study. They were anesthetized with 4% chloral hydrate by intraperitoneal injection, and the cells were transplanted into the brain and spinal cord. For the brain transplantations, 1×10^6 D0 pES, D12 NP, or D18 NP were transplanted into striatum with stereotaxic instrument at the site of anterior-posterior (AP)=0.0 mm, medial-lateral (ML)=-3.0 mm, and dorsal-ventral (DV)=-5.0 mm. For the spinal cord transplantations, thoracic vertebral level 9 to 10 were exposed and transplanted with 1×10^6 D0 pES, D12 NP, or D18 NP. The SD rats injected with cell-free PBS were regarded as the control groups, while those in the sham group were sham-operated to expose the injection site only. After transplantation, all SD rats were rendered basic attention according to NIH guidelines.

Investigation of inflammatory cytokine expression by real-time PCR

To investigate the subacute inflammation, real-time PCR assay was performed. A total of 45 SD rats were used in five groups, control, sham, D0 pES, D12 NP, and D18 NP, where 3 SD rats were sacrificed per group on day 3, 7, and 14 after transplantation to collect brain and spinal cord tissues for analysis. The SD rats were sacrificed with CO_2 and the sample of the brain striatum at transplantation site (0.0, -3.0, -5.0) was dissected with 5×5 mm in size. The brain striatum at opposite site (0.0, +3.0, -5.0) was also sampled in each group and regarded as internal control. The spinal cord was sampled by dissecting 5 mm in length at transplantation site, and the same length of spinal cord 1 cm in front and rear of the transplantation site were also dissected and regarded as internal controls. The total RNA was extracted and reverse-transcribed to cDNA. Real time-PCR was performed by Applied Biosystems StepOne™ and StepOnePlus™ with SYBR green (Invitrogen) to quantify the gene expression of *IL-1α*, *IL-1β*, *IL-6*, and *TNF-α*. The gene expression level of each sample from the brain and spinal cord was presented by transplantation site/internal control. The primer sequences are shown in table 1.

Histopathological study of rat brain and spinal cord by H&E staining

One month after transplantation, a total of fifteen SD rats were

Gene	Primer	Accession Number
IL-1α	Forward 5'-AAGACAAGCCTGTGTTGCTGAAGG-3' Reverse 5'-TCCCAGAAGAAAATGAGGTCGGTC-3'	D00403
IL-1β	Forward 5'-CACCTCTCAAGCAGAGCACAG-3' Reverse 5'-GGGTTCCATGGTGAAGTCAAC-3'	NM_031512
IL-6	Forward 5'-TCCTACCCCAACTTCCAATGCTC -3' Reverse 5'-TTGGATGGTCTTGGTCCTTAGCC-3'	NM_012589
TNF-α	Forward 5'-ATCATCTTCTCAAAACTCGAGTGACAA-3' Reverse 5'-CTGCTCCTCTGCTTGGT-3'	NM_012675
RATP31 (Cyclophilin-α)	Forward 5'-TATCTGCACTGCCAAGACTGAGTG-3' Reverse 5'-CTTCTTGCTGGTCTTGCCATTCC-3'	NM_017101

Table 1: The primer list of immune response associated factors

sacrificed to exam chronic inflammation. The brain and spinal cord were retracted and prepared for histopathological study. Briefly, samples were fixed in 4% paraformaldehyde overnight and embedded in paraffin after serial dehydration. Sections of 3 μm in thickness were stained with hematoxylin and eosin. For semiquantitative grading, lesion severity was graded using the criteria developed by Shackelford et al. Lesion severity was graded as follows: 1=minimal (<10%), 2=slight (11-25%), 3=moderate (26-50%), 4=moderate/severe (51-75%), and 5=severe/high (76-100%) [17].

Statistical Analysis

Analysis of variance was performed with the General Linear Model procedure and Duncan's multiple range test by SAS (SAS Enterprise Guide 4.1. SAS Institute Inc., Cary, North Carolina, USA). $P<0.05$ was considered to be statistically significant.

Results

The expression of *IL-1α* was increased on day 7 after transplanted with D12 NP and D18 NP

On day 3 after transplantation, only D0 pES triggered significant expression level of *IL-1α* in the brain compared with other groups in the brain ($P<0.05$). No significant responses were observed after the brain or spinal cord grafted with D12 NP and D18 NP. However, the expression levels were significantly increased on day 7 after grafting with D12 NP and D18 NP in the brain and the spinal cord ($P<0.05$). On day 14 after transplantation, *IL-1α* expressions in all treatment groups of the brain, except the D18 NP treatment group, were decreased. For the spinal cord, D0 pES and D18 NP treatment groups continued to exhibit higher expression compared with other groups in the spinal cord ($P<0.05$) (Figure 1A).

The expression of *IL-1β* was increased on day 3 and 7 after transplantation

The expression of *IL-1β* was significantly increased on day 3 after the brain was grafted with D0 pES ($P<0.05$), but in the spinal cord the significant response delayed until day 7. However, after the D12 NP and D18 NP were grafted, the expressions in both the brain and the spinal cord were significantly increased on day 3 and day 7, respectively ($P<0.05$). Again, the *IL-1β* expressions in all treatment groups of the brain returned to the basal level similar to the control group on day 14, except the D18 NP treatment group. However, the expression levels in all treatment groups of the spinal cord were still significantly higher than those in the control group on day 14 ($P<0.05$) (Figure 1B).

The expression of *IL-6* was much less in the brain

After transplantation, the expressions of *IL-6* in the brain grafted with D0 pES and D18 NP were significantly increased on day 3 and 7, respectively ($P<0.05$). The expression showed no significant difference in the brain grafted with D12 NP. On the other hand, the spinal cord grafted with D12 NP exhibited the highest *IL-6* expression on day 3, and the expression in the D0 pES and D12 NP treatment groups were also significantly higher on day 7 ($P<0.05$). On day 14 after transplantation, the expression in all treatment groups of the brain was as low as the control group, but in the spinal cord, higher expression of *IL-6* was observed in the D12 NP and D18 NP treatment groups ($P<0.05$). All in all, the elevated expression of *IL-6* triggered by pES/GFP$^+$ cell transplantation was mild in the brain (Figure 1C).

The global expression of *TNF-α* was smooth

On day 3 after transplantation, only the D0 pES treatment group significantly triggered the expression of *TNF-α* in the brain ($P<0.05$), similar to the expression pattern of *IL-1α* on day 3. No significant expression was observed after the brain was grafted with D12 NP and D18 NP. However, expression in all treatment groups of the spinal cord was significantly increased on day 7 ($P<0.05$). On day 14 after transplantation, D0 pES and D18 NP continued to induce *TNF-α* production significantly in the spinal cord ($P<0.05$). Interestingly, the expression in the control group of the brain was significantly higher than all treatment groups on day 14 ($P<0.05$) (Figure 1D).

Granulation emerged in the brain and spinal cord after transplantation

We suspected that the rats might show brain and spinal cord histopathological abnormalities due to the xenotransplantation of pES/GFP$^+$ cells/derived cells. Therefore, we collected the brain and the spinal cord, and investigated the tissues by H&E staining. One month after transplantation, the lesions by injection in the brain and the spinal cord were clearly distinguishable but recovered (Figures 2 and 3). In the brain, we found black spot lesions caused by traumatic liquefactive necrosis and chronic granulation with gliosis in all treatment groups (Figure 2). Moreover, numerous mononuclear cells and hemosiderosis were presented in the D12 NP and D18 NP treatment groups, respectively (Figures 2I,J; 2Q,R). In the spinal cord, hemosiderosis was also discovered in the injection site after being grafted with D18 NP for 1 month (Figure 3A). Granulation (Figure 3B) and giant cells (Figure 3C) were also found near the injection site in the spinal cord.

Discussion

Successful xenotransplantation indicates an unlimited potential source for tissue and organ replacements. Following our previous study where porcine embryonic cells and thus-derived neuronal progenitors were found to ameliorate Parkinson's Disease and spinal cord injury [13,14], we wanted to examine the inflammatory effects of the xenotransplanted pES/GFP$^+$ and derived cells in rat models. Interestingly, we have found quite different expression patterns of *IL-*

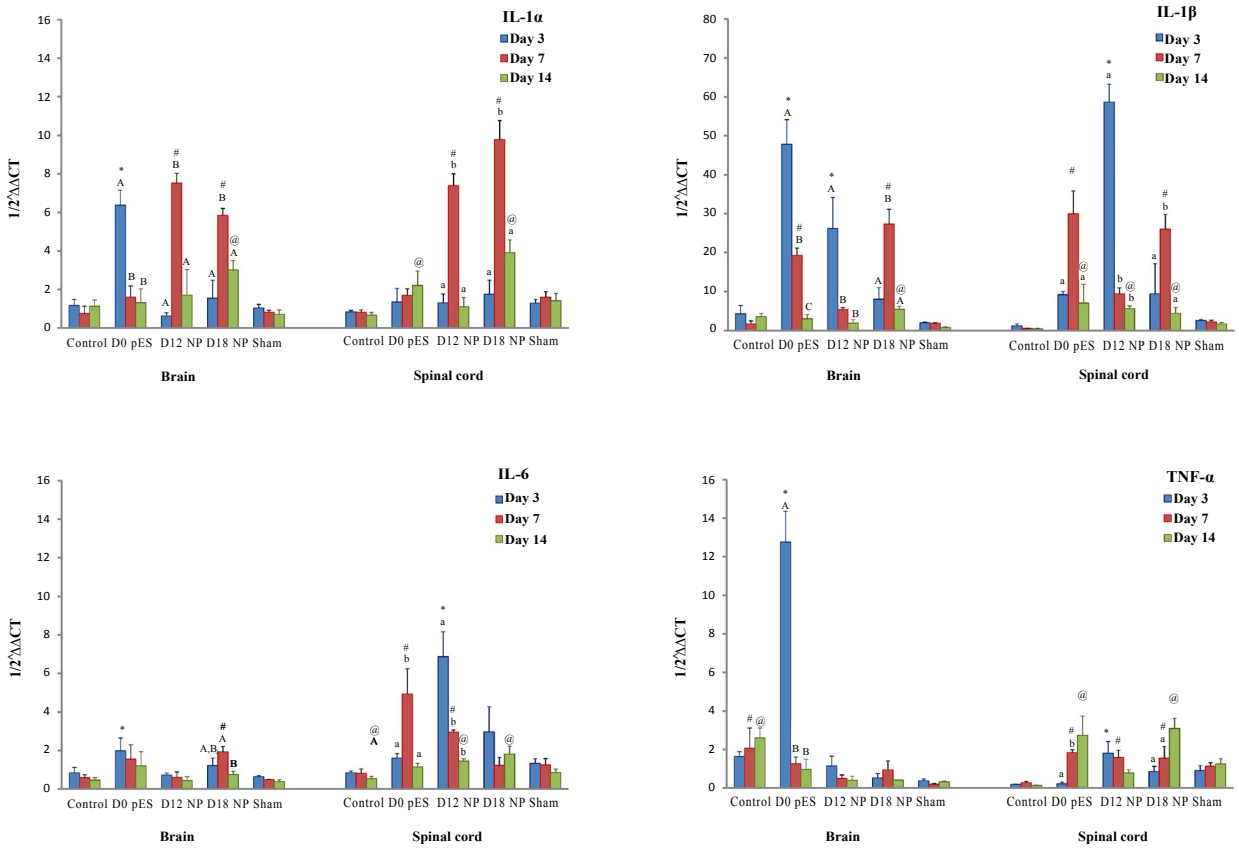

Figure 1: The expression of *IL-1α*, *IL-1β*, *IL-6*, and *TNF-α* in the brain and the spinal cord on day 3, 7, and 14 after the transplantation of D0 pES, D12 NP, and D18 NP quantified by real-time PCR. ABC in the brain and abc in the spinal cord indicate significant differences within treatments compared with day 3; *#@ in the brain and the spinal cord indicate significant differences on day 3, 7, and 14 compared with control, respectively

1α, *IL-1β*, *IL-6*, and *TNF-α* in the brain when compared with the spinal cord following transplantation, where most of the inflammatory factors examined continued to have significant expression in the spinal cord on day 14, but returned to basal levels in the brain for the D0 pES and D12 NP treatment groups. We propose that these different inflammatory factor expressions might be due to the relatively differential immunological privilege between the brain and the spinal cord, where the spinal cord is not as immunologically privileged as the brain [7,8]. In a previous study where pig umbilical cord matrix (pUCM) cells were transplanted into the brains of neurotoxin 6-hydroxydopamine-treated Lewis rats, no evidence of any significant host immune responses were observed [18]. The relatively low inflammatory reaction in the rat brain following transplantation indicates that the pES/GFP$^+$ cells and the derived NPs may also harbor some immunosuppressive capability which is similar to pUCM [18]. The continued expression of inflammatory factors (*IL-1α* and *IL-β*) on day 14 in the rat brains transplanted with the D18 NPs may be due to their high maturity when compared with the D0 pES and D12 NPs (Figure 1A and 1B). In our study, the immune responses in the brain triggered by the D0 pES were earlier than the D12 NP and D18 NP. This might be because the D0 pES tended to aggregate much more easily than the D12 NPs and D18 NPs. Hence, the colonies of D0 pES formed after transplantation would be more readily detected by the immune system and evoke higher initial immune responses.

In our study, *IL-1α* expression in the spinal cord was found to be significantly higher than the other groups on day 14 after transplantation with D0 pES and D18 NP (Figure 1A), and the expressions of *IL-1β*, *IL-6*, and *TNF-α* exhibited similar tendencies in the spinal cord (Figure 1B,C,D). These increased responses might be caused by the elevated levels of *IL-1α*, since *IL-1α* has been found to stimulate human cardiac myofibroblasts to express *IL-1β*, *IL-6*, and *TNF-α* [19]. In contrast, similar tendency was not found in the brain as that in the spinal cord (Figure 1). *IL-1β* is secreted by macrophage, natural killer cell, monocyte, and neutrophil. Increased *IL-1β* production has been reported in patients related with various infections, inflammation, surgical trauma, ischemic diseases, tumors, intravascular coagulation, autoimmune disorders, UV radiation, graft-versus-host disease, transplant rejection, and in healthy subjects after strenuous exercise [20,21]. The expression level of *IL-1β* in our study was much greater than that of *IL-1α*, *IL-6*, or *TNF-α*. The transplantation of D0 pES and D12 NP in the brain significantly evoked *IL-1β* expression on day 3 (Figure 1B). However, on day 14 after transplantation, the immune response was relieved in the brain due to the reduction of *LI-1α*, *IL-6*, and *TNF-α* in all treatment groups. However, the expression level of *IL-1β* in the brain was still significantly higher in the D18 NP treatment group. In contrast, all treatment groups of the spinal cord significantly triggered *IL-1β* expression on day 14 (Figure 1B).

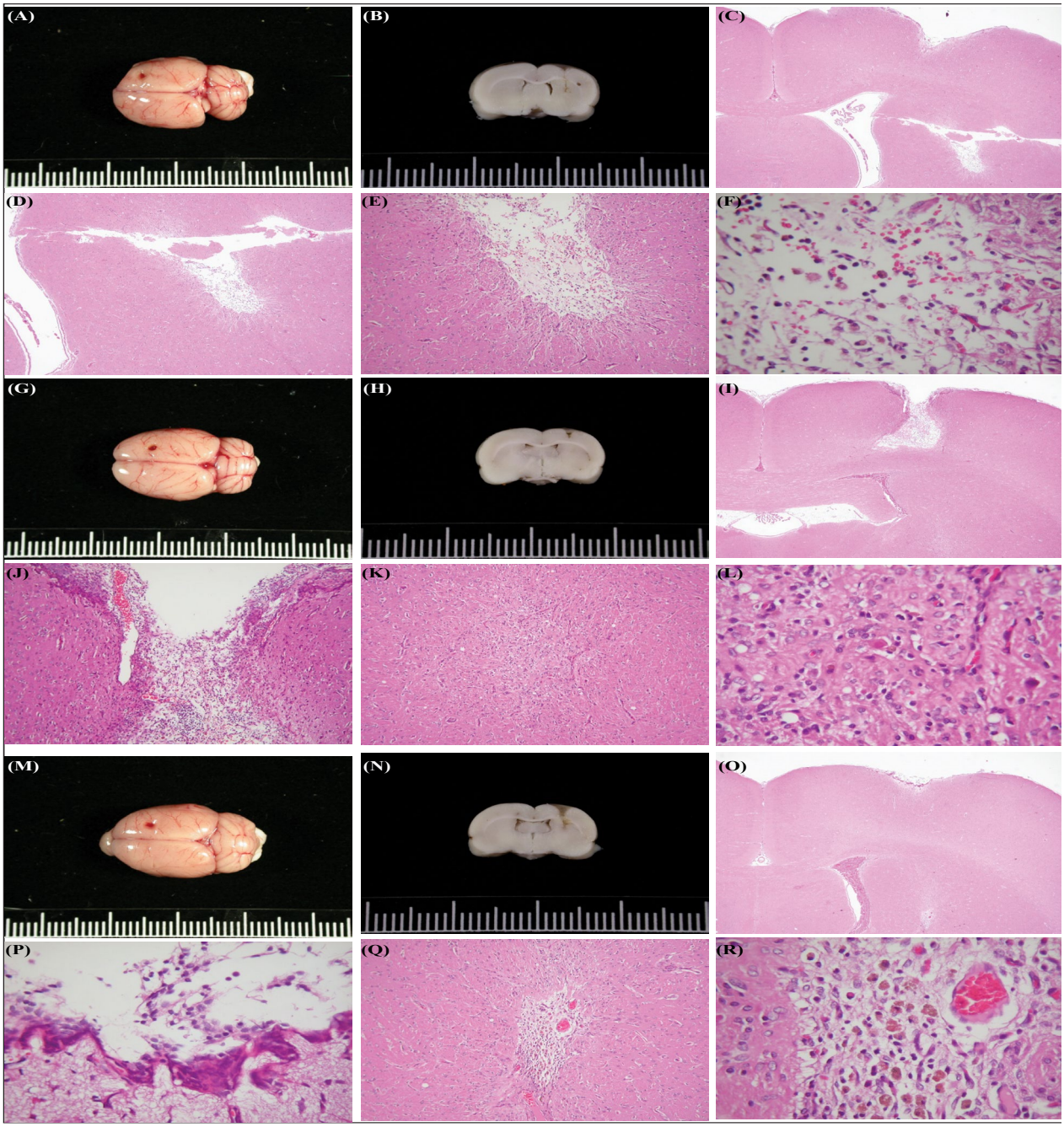

Figure 2: Gross and histopathological changes in the brain after the rats were grafted with D0 pES (A-F), D12 NP (G-L), and D18 NP (M-R) for 1 month. The sections were stained with hematoxylin and eosin. Black spot lesions were found in the transplantation site of all treatments. White arrows indicate the transplantation sites (A, B, G, H, M, and N). Microscopically, in the D0 pES treatment group, moderate traumatic liquefactive necrosis was noted in the cortex of the brain (C, Magnification×20; D, Magnification×100). Slight granulation with gliosis was found in the lateral ventricle of the brain (E, Magnification×200; F, Magnification ×400) (Black arrows). In the D12 NP group, moderate traumatic liquefactive necrosis with numerous mononuclear cells infiltration was noted in the cortex (I, Magnification×20; J, Magnification×100). Moderate granulation with gliosis was found in the parenchyma of the brain (K, Magnification×100; L, Magnification×400) (Black arrows). In the D18 NP group, moderate traumatic liquefactive necrosis (O, Magnification×20) with menigiogranulation was noted in the cortex of the brain (P, Magnification×400). Slight granulation with gliosis and hemosiderosis were found in the lateral ventricle of the brain (Q, Magnification×100; R, Magnification×400) (Black arrows)

In our study, the expression pattern of *TNF-α* was very stable in the brain in the experimental period (Figure 1D). The stable expression pattern of *TNF-α* and its short half-life lead to the expected less *IL-6* expression in the brain (Figure 1C). This may be due to the linked production of *IL-6* following the expression of *TNF-α*, where *TNF-α* has been found to increase the mRNA level of *IL-6* in osteoblast-like ROS17/2.8 cells [22] and have cooperative functions to impair co-stimulatory blockade in allograft effects. However, on day 14 after transplantation, the highest expression of *TNF-α* in the brain was found in the control group, and the reason for this high expression remains to be determined (Figure 1D). Contrary to the brain, the expression of *IL-6* and *TNF-α* in the spinal cord was still high on day 14 (Figure

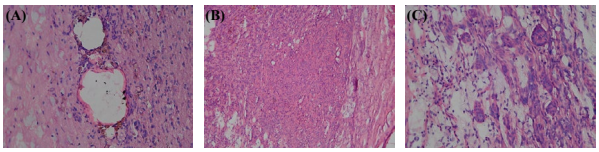

Figure 3: Gross and histopathological changes in the spinal cord after grafted with D18 NP for 1 month. Injection cavities were gently recovered and hemosiderosis was noted around the transplantation site (A, Magnification×200). indicates injection cavity and black arrow indicates hemosiderosis. Moderate granulation (B, black arrow) and giant cells (C, black arrows) were found beside the injection sites (B, Magnification×100; C, Magnification×200)

1C, D). Since increased levels of *IL-6* and *TNF-α* are associated with acute allograft rejection and T cell alloimmunity [23,24], these different expressions of *TNF-α* and *IL-6* in the brain and spinal cord in response to the grafted cells might reflect their dissimilarity in immunological privileges.

In histopathological studies, granulation was observed in the brain and the spinal cord, which may have resulted from the recovery of the injections (Figures 2 and 3). It has been previously demonstrated that microglia, macrophages, and lymphocytes derived from the host circle around the transplantation sites and trigger immune responses after the rat brain has been transplanted with neuronal precursor cells derived from mouse ES cells for 2-8 weeks. These cells secret proinflammatory cytokine, such as *IL-1β*, *IL-6*, and *TNF-α*, and possess antigen-presenting cell capacities [25,26]. In this study, we observed numerous mononuclear cells in the brain grafted with D12 NP (Figure 2I, J), and giant cells in the spinal cord grafted with D18 NP (Figure 3C). These results indicate traces of immune responses similar to those found with transplanted mouse ES cells in rat brains. Furthermore, we also found hemosiderosis in the brain and the spinal cord that might have resulted from hemorrhage after transplantation. According to our previous studies, the pES/GFP$^+$ cells and their derived cells could be detected within the brain and the spinal cord by the *in vivo* imaging system for three months after transplantation [13,14]. At the same time, the real-time images of grafted cells in recipients has been performed by *ex vivo* fibered confocal Cellvizio Imaging System (Cellvizio, Mauna Kea Technologies, Cambridge, MA, USA) for invasive tracking during the 3 months of experimental period [27]. The cells resided at the transplantation sites to ameliorate or to repair the Parkinson's disease [13] or spinal cord injury [14], respectively. In order to fully understand the safety of pES/GFP+ cells, further studies should focus on whether the protein secretion, phenotypic characteristics of host cells, and CNS were affected by grafted cells.

In conclusion, the tolerance to immune responses triggered by pES/GFP$^+$ cells/derived cells transplanted in the brain is better than that in the spinal cord. Although the expression patterns of inflammatory cytokines varied during the experimental period, there appeared to be no lethality for any rats in this study. The threshold of lethality caused by immune responses in the rats grafted with D0 pES, D12 NP and D18 NP is higher than we expected. Hence, the improvement of Parkinson's disease [13], spinal cord injury [14], and periodontal furcation defects [15] following the transplantation of pES/GFP$^+$ cells/derived cells together with well-tolerated immune responses suggest the potential for further xenotransplantation applications of pES cells in the field of regenerative medicine.

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