Intrathecal injection of a therapeutic gene-containing polyplex to treat spinal cord injury

Kentaro Hayakawa^{1,2}, Satoshi Uchida³, Toru Ogata^{1,2}, Sakae Tanaka², Kazunori Kataoka^{3,4},
Keiji Itaka^{2,3}

1. Department of Rehabilitation for the Movement Functions, Research Institute, National Rehabilitation Center for the Persons with Disabilities, Saitama, Japan
2. Sensory & Motor System Medicine, Faculty of Medicine, The University of Tokyo
3. Laboratory of Clinical Biotechnology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
4. Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, Tokyo, Japan

Correspondence should be addressed to K.I. (itaka-ort@umin.net) and K.K. (kataoka@bmw.t.u-tokyo.ac.jp)

Associate Professor Dr. Keiji Itaka, Division of Clinical Biotechnology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

Tel: (+81)3-5841-1418; Fax: (+81)3-5841-1419; E-mail: itaka-ort@umin.net

Professor Dr. Kazunori Kataoka, Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

Tel: (+81)3-5841-7138; Fax: (+81)3-5841-7139; E-mail: kataoka@bmw.t.u-tokyo.ac.jp

Abstract

Spinal cord injury (SCI) is a serious clinical problem that suddenly deprives patients of neurologic function and drastically diminishes their quality of life. Gene introduction has the potential to be effective for various pathological states of SCI because various proteins can be produced just by modifying nucleic acid sequences. In addition, the sustainable protein expression allows to maintain its concentration at an effective level at the target site in the spinal cord. Here we propose an approach using a polyplex system composed of plasmid DNA (pDNA) and a cationic polymer, poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)], that has high capacity to promote endosome escape and the long-term safety by self-catalytically degrading within a few days. We applied Brain-derived neurotrophic factor (BDNF)-expressing pDNA for SCI treatment by intrathecal injection of PAsp(DET)/pDNA polyplex. A single administration of polyplex for experimental SCI provided sufficient therapeutic effects including prevention of neural cell death and enhancement of motor function recovery. This lasted for a few weeks after SCI, demonstrating the capability of this system to express BDNF in a safe and responsible manner for treatment of various pathological states in SCI.

Keywords

spinal cord injury, Brain-derived neurotrophic factor (BDNF), gene delivery, polyplex, intrathecal injection

1. Introduction

Spinal cord injury (SCI) is a serious clinical problem that suddenly deprives patients of neurologic function and drastically diminishes their quality of life [1, 2]. Initial mechanical trauma to the spinal cord is followed by secondary injury, which is the progression of tissue damage for several days due to delayed neural cell death around the original site of injury [3-5]. Because initial injury has already occurred before hospital arrival, the therapeutic target has been focused on attenuation of secondary injury. However, there are few pharmacological strategies showing solid evidence of therapeutic effectiveness in clinical settings. One reason for this is the complex pathophysiology of secondary injury such as inflammatory responses, a variety of molecules inhibiting regeneration, and a lack of trophic support [6-10]. In addition, the pathophysiology is constant or increased for several days. Thus, therapeutic molecules need to be effective and sustainable for various pathological states in secondary injury. However, it is not only difficult to choose adequate therapeutic molecules, but it is also difficult to maintain the concentration of the molecules at an effective level at the target site in the spinal cord [11].

Gene introduction has the potential to be effective for the treatment of SCI because various proteins can be produced just by modifying nucleic acid sequences. In addition, sustainable protein expression resulting from an introduced gene is apparently beneficial for regulating the complex pathophysiology of SCI. For this purpose, viral vectors such as herpes simplex virus vectors have been evaluated [12]. However, viral vectors still have problems surrounding their genotoxicity and immunogenicity, hampering their administration for acute and serious states of SCI [13-15].

A non-viral system that can introduce genes to express therapeutic molecules has high potential for the treatment of SCI. Since neural tissue is mostly composed of highly differentiated, non-dividing cells, the system needs to have a high level of safety so as not to impede long-term neural function after gene introduction.

In this context, we propose an approach using a polyplex system composed of plasmid DNA (pDNA) and cationic polymers [16, 17]. The polyplex system can protect

pDNA from nuclease attack and increase cellular uptake through electrostatic interactions between a cationic polyplex and the anionic cell membrane. One of the critical barriers in achieving good gene expression is the inefficient translocation from endosomes to the cytoplasm. The capacity of polymers to buffer acidic environments in the endosomes, typically represented by polyethylenimine (PEI) [18], has been intensely investigated to increase the efficiency of translocation, although the toxicity of the polymers has hampered their application for therapeutic purposes [19]. Our original cationic polymer, poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)] [20, 21], effectively solves the safety issues by self-catalytically degrading within a few days, minimizing the cumulative toxicity caused by the polymers remaining in the cells [22]. Besides its high capacity to promote endosome escape, it was revealed by a pharmacogenomic analysis that PAsp(DET) did not alter the expression profiles of endogenous genes after introduction into cells, confirming the long-term safety of PAsp(DET) in not affecting innate cell function [23, 24].

The purpose of this study is to investigate the availability of the polyplex system for the treatment of SCI. As a proof-of-concept study, we used Brain-derived neurotrophic factor (BDNF) as a therapeutic agent. BDNF is a member of neurotrophins, which have gained much attention for exerting diverse effects as a trophic support in treating SCI [25, 26]. In animal studies, recombinant BDNF protein showed therapeutic effects by enhancing neural cell survival and axonal regeneration in SCI by intrathecal infusion [27, 28]. However, multiple or continuous administrations using a catheter are usually required to maintain effective concentrations of BDNF protein at the injured site, and this would cause complications such as scar formation at the catheter tip, which can lead to infusion failure and damage to the spinal cord caused by the catheter itself [29, 30].

In this study, we applied BDNF-expressing pDNA for SCI treatment by intrathecal injection of PAsp(DET)/pDNA polyplex. As shown later, a single administration of polyplex provided sufficient therapeutic effects including prevention of neural cell death and enhancement of motor function recovery. This lasted for a few weeks after SCI, demonstrating the capability of this system to express BDNF in a safe and responsible manner for treatment of various pathological states in SCI.

2. Materials and methods

2.1. Materials

Plasmid DNA (pDNA) encoding luciferase (pGL4.13: Promega, Madison, WI, USA) and brain-derived neurotrophic factor (BDNF) (pUNO1-hBDNFa: InvivoGen, San Diego, CA, USA) were amplified in competent DH5 α Escherichia coli and purified using NucleoBond Xtra EF (Nippon Genetics, Tokyo, Japan). The pDNA concentration was determined by reading the absorbance at 260 nm. Linear polyethyleneimine (LPEI) (Exgen 500, *in vivo*; MW=22,000) was obtained from MBI Fermentas (Burlington, ON, Canada). Lipofectamine 2000 Transfection Reagent was from Thermo Fisher Scientific (Waltham, MA, USA). PAsp(DET) polymer was synthesized as described previously [20]. By ^1H -NMR analysis, the polymerization degree of PAsp(DET) was determined to be 52.

2.2. Animals

C57BL6/J mice (female, eight-weeks-old) were purchased from Charles River Laboratories (Yokohama, Japan). The mice were allowed to feed and drink *ad libitum* on a 12-hour light/dark cycle prior to experimentation. All animal protocols were conducted with the approval of the Animal Care and Use Committee of the University of Tokyo.

2.3. Preparation of polyplex or lipoplex containing pDNA

To form PAsp(DET)/pDNA polyplex, PAsp(DET) polymer and pDNA were separately dissolved in 10 mM HEPES buffer at pH = 7.4. The polyplex was obtained by simply mixing both solutions at N/P ([total amines in polymer]/[DNA phosphates]) ratio of 8. LPEI-based pDNA carrier was prepared by mixing LPEI and pDNA solution at N/P ratio of 6, which is recommended as an optimal transfection condition by the manufacturer. Lipoplex was formed following the manufacturer's protocol by mixing

Lipofectamine 2000 and pDNA. The final pDNA concentration was adjusted to 200 µg/ml for all samples.

2.4. Intrathecal injection of pDNA solution

Mice were anesthetized with isoflurane (Escain: Mylan, Canonsburg, PA, USA). The skin was incised so that the superficial fat could be pried apart to dissect the muscle tissue and expose the lumbar laminae. An injection needle was inserted into the dural space from the interlaminar space between L4 and L5, and 10 µl of solution containing 2 µg pDNA was injected in 60 seconds with Hamilton syringe (Sigma-Aldrich, St. Louis, MO, USA). The tip of the needle was kept at the injection site for 5 minutes to prevent leakage of cerebrospinal fluid (CSF).

2.5. Experimental spinal cord injury

Twenty-four hours after the injection of carrier solutions, mice were anesthetized with sodium pentobarbital (50 mg/kg) (Somno-pentyl: Kyoritsu Seiyaku, Tokyo, Japan). The thoracic laminae were exposed and laminectomy was performed at the level of the 9th thoracic vertebra. A contusion SCI was induced using Infinite Horizons Impactor (Precision Systems and Instrumentation LLC, Fairfax, VA, USA) with an impact force of 60 kilodynes. Mice underwent daily check for general health, mobility within the cage, wounds, infections, and autophagy of the toes throughout the experiment. Bladders were manually pressed twice daily for the first week after operation and once daily until no longer needed.

2.6. Evaluation of luciferase expression

In vivo luciferase expression was evaluated using the IVIS™ Imaging System (Xenogen, Alameda, CA, USA) after intraperitoneal injection of D-luciferin (150 mg/kg, Sumisho Pharmaceuticals International, Tokyo, Japan), following the manufacturer's protocol.

2.7. Measurement of proinflammatory cytokines

Total RNA was isolated from extracted neural tissues using the RNeasy Mini Preparation Kit (Qiagen, Hilden, Germany). The gene expression of proinflammatory cytokines was analyzed by real-time quantitative PCR using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA), and TaqMan Gene Expression Assays (Mm00443258_m1 for tumor necrosis factor (TNF)- α , Mm00446190_m1 for interleukin (IL)-6, Mm00434228_m1 for IL-1 β , and Mm00607939 for β -actin).

2.8. Behavioral assessments

Hind-limb motor function was evaluated using two separate procedures, the Basso Mouse Scale (BMS) open field locomotor test and a computerized gait analysis using CatWalk gait analysis system (Noldus, Wageningen, Netherlands). In the former, two blinded persons assessed parameters of stepping frequency, coordination, paw position, trunk stability, and tail position, by scoring from 0 points (no ankle movement) to 9 points (complete functional recovery) [31]. In addition, since mice may not show formulaic recovery of motor function by correlation with the BMS scores, we also calculated BMS subscore by adding each score of the parameters described above. The scores of right and left hind limbs were averaged.

In the latter, we analyzed the walking pattern in a quantitative manner by assessing the parameters shown in Table 1, including those reflecting regularity and relative paw placement [32]. Each mouse was allowed to cross runway with a glass floor (L 50 cm $\times$ W 8 cm) in a darkened room. Footprints, illuminated with green LED light, were observed with a charge-coupled device (CCD) for following analyses with CatWalk XT 10.0 software (Noldus).

2.9. Evaluation of the cell apoptosis

We used the terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay. Mice were perfused transcardially under deep ether anesthesia with buffered 4% paraformaldehyde. The spinal cords were removed and postfixed in 4% paraformaldehyde at 4 °C for 4 hours, cryoprotected in buffered 20% sucrose at 4 °C for 24 hours, 30% sucrose for 24 hours, and were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA). Frozen tissues were cut into 14 µm longitudinal sections with a cryostat and mounted on glass slides. The midline section was chosen and the apoptotic cells were labeled with TMR red using *In Situ* Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol, then mounted with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). For counting of apoptotic cells, three areas were chosen in rostral, caudal, and ventral regions surrounding the spinal cord lesion (see Fig. 5A) for fluorescent microscopic observation using AxioVision (Carl Zeiss, Oberkochen, Germany) under ×200 magnification. The numbers of apoptotic cells and whole cells were counted using an image analyzer software (WinROOF: Mitani Co., Tokyo, Japan).

2.10. Evaluation of the tissue sparing

Frozen tissue sections were prepared as described in the previous section. Myelin and myelinated axons were stained with Luxol fast blue (LFB) solution [33]. Briefly, the sections were hydrated with 95% ethanol and de-fatted with 1:1 ethanol/chloroform, hydrated with 95% ethanol, and left in 0.1% LFB solution (Solvent blue: MP Biomedicals, Santa Ana, CA, USA) at 56 °C overnight. Then, the sections were rinsed in 95% ethanol and distilled water, differentiated with the lithium carbonate solution and 95% ethanol, rinsed with distilled water, differentiated in 95% and 100% ethanol, cleared with xylene, and mounted. The section was viewed with an optical microscope (BX51: Olympus, Tokyo, Japan) and the images of the serial sections with an equal distance (200 µm) spanning ± 400 µm from the epicenter were obtained with an image processing software (InStudio: Pixera Co., San Jose, CA, USA). For the quantification of tissue sparing in the images, the edges of the stained area were drawn with a pen

tablet input device (Bamboo Pen: Wacom Co., Tokyo, Japan) and the surrounding area was automatically measured using WinROOF.

2.11. Statistical analysis

Statistical analyses were performed with an unpaired two-tailed Student's *t* test for single comparisons and one-way ANOVA for multiple comparisons. In all statistical analyses, values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Evaluation of transgene expression after intrathecal injection of pDNA/PAsp(DET) polyplex and other carriers

The gene introduction capacity and safety was evaluated by administering luciferase-expressing pDNA using cationic polymers (PAsp(DET) or LPEI), lipid (Lipofectamine), or naked pDNA. For each condition, an identical amount of pDNA was administered **into normal mice** by intrathecal injection from the interlaminar space between L4 and L5, followed by evaluation of luciferase expression by IVIS™ Imaging System.

One day after administration, the luciferase expression was well detected for polyplexes with PAsp(DET) and LPEI (Fig.1A). PAsp(DET) showed significantly higher expression than LPEI (Fig.1B), and the expression was observed along the spinal canal, suggesting **that the polyplexes were distributed** in the cerebral spinal fluid of subarachnoid space. **Despite a marked decrease a few days after administration, the luciferase expression level was still considerably higher than the background level 7 days after injection of PAsp(DET) polyplex ($4.91 \pm 0.73 \times 10^5$ photons/sec in PAsp(DET), $n = 5$, $2.52 \pm 0.06 \times 10^4$ photons/sec in untreated background mice, $n = 3$).**

Fig.1. Luciferase expression at spinal cord of normal mice after intrathecal injection of luciferase-expressing pDNA using PAsp(DET), LPEI, Lipofectamine, or in the form of naked pDNA. (A) Representative IVIS images of mice 1 day after injection. (B) Time course of luciferase expression quantified from the IVIS images. Data are expressed as means $\pm$ SEMs ($n = 5$), $**p < 0.01$, $*p < 0.05$ vs. LPEI.

In Fig. 1A, the expression from PAsp(DET)/pDNA and LPEI/pDNA polyplexes was intensely observed at thoracic region. To investigate the polyplex distribution more in details, we injected trypan blue solution intrathecally between L4 and L5. The blue staining was widely distributed in the spinal canal but accumulated more intensely at thoracic level than the other parts of spine (Fig. S1A). The cross-sections of thoracic spinal cord (Th9) obtained immediately after the injection revealed that the blue staining was localized in meninges (Fig. S1B). In addition, by injecting Cy5-labelled

pDNA/PAsp(DET) polyplexes to evaluate the distribution histologically, the Cy5 signals were observed in meninges and partly in parenchyma for both immediately or 24 h after the injection (Fig. S2). These results well consistent with previous reports of injecting cationic polyplexes intrathecally [34, 35]. Thus, it is strong suggested that the solution rapidly passed in the subarachnoid space and widely distributed in the spinal tissue. Since the accumulation at thoracic region was observed regardless of polyplex types, it is reasonably assumed to be due to gravitational redistribution of fluid reflecting the spinal curvature.

Supplementary figure S1 Distribution of trypan blue solution in spinal cord

10 μ l of trypan blue solution (0.4%, Invitrogen, Carlsbad, CA, USA) was injected from the level of lumbar cord (L4 – 5). Immediately after the injection, blue staining along spinal canal was evaluated in the frontal plane (A) and in cross section at Th9 level (B). Accumulation of solution in thoracic cord was indicated with a white circle in (A). Localization of the solution (blue) in meninges was indicated with white arrows in (B).

Supplementary figure S2 Distribution of fluorescence-labeled polyplexes in cross section of spinal cord

Labeling of pDNA with Cy5 was performed with Label IT Tracker Intracellular Nucleic Acid Localization Kits (Mirus, Madison, WI) following manufacturer's protocol. PAsp(DET)/pDNA polyplexes prepared from Cy5-labeled pDNA (red) were intrathecally injected from the interlaminar space between L4 and L5. Frozen sections at the level of Th9 were obtained immediately (A) or 24 h after the injection (B, C). A magnified image of a square region in (B) was shown in (C). Cell nuclei were stained with DAPI (blue). Polyplexes in parenchymal tissue were indicated with white arrows.

Safety was investigated by analyzing proinflammatory cytokines after administration of PAsp(DET)/pDNA (luciferase) polyplexes into the SCI region in the mice receiving contusion at the level of the 9th thoracic vertebra (see Materials and Methods). The messenger RNA (mRNA) expression of TNF- α , IL-6, and IL-1 β in the lower thoracic spinal cord region was significantly elevated with contusion, however, the intrathecal injection of the polyplex did not induce further cytokine production (Fig. 2A, B, C). Note that the injection did not affect the spontaneous recovery of motor function that was evaluated by BMS scoring for four weeks (Fig. 2D). Thus, it is

confirmed that intrathecal injection of the polyplex provided sufficient transgene expression without harmful tissue injury and inflammation.

Fig.2. Proinflammatory cytokines gene expression 24 h after intrathecal injection of PAsp(DET)/pDNA (luciferase) polyplexes. The messenger RNA (mRNA) expression of TNF- α (A), IL-6 (B), and IL-1 β (C) in the lower thoracic spinal cord region was evaluated by real-time quantitative PCR. Data are expressed as a relative value to that from intact spinal cord region. Data are expressed as means $\pm$ SEMs (intact control: $n = 3$, the others: $n = 5$), * $p < 0.05$. (D) Spontaneous recovery of walking motion that was evaluated by BMS scoring after inducing experimental SCI ($n = 6$). Intrathecal injection did not affect the recovery.

3.2. Therapeutic effect of BDNF-expressing pDNA on the recovery of walking motion

Using the PAsp(DET)/pDNA polyplex system, we applied BDNF-expressing pDNA for SCI mice. To evaluate the recovery of voluntary motor function, we did scoring of BMS and its subscore. This is a widely-used locomotor measurement with validity and reliability [31].

After administration of BDNF-expressing pDNA using PAsp(DET)/pDNA polyplexes, the BMS showed more rapid recovery compared with other controls of BDNF administration using LPEI/pDNA polyplexes, or negative control pDNA (Luciferase) (Fig. 3A). The higher scores for PAsp(DET) polyplexes were obtained throughout the period from 3 to 28 days after administration of the polyplexes. In line with these results, the BMS subscore which reflects the footstep and its coordination showed remarkably higher scores for the mice receiving PAsp(DET)/pDNA polyplexes compared with the other controls after seven days of administration (Fig. 3B).

Fig.3. Analysis of walking motion by BMS scoring after intrathecal injection of BDNF-expressing pDNA using PAsp(DET) (close square), LPEI (triangle), or luciferase-expressing pDNA using PAsp(DET) (open square), concomitantly with the induction of experimental SCI. (A) BMS, (B) BMS subscore. Data are expressed as means $\pm$ SEMs (n = 6), * p < 0.05.

For a more detailed and quantitative assessment of walking motion, we did an analysis using the CatWalk system, which allows a comprehensive examination of walking motion by assessing the parameters shown in Table 1. These parameters are

reported to precisely reflect the stepping coordination of the hindlimbs [36-38]. As shown in Fig. 4A-E, the mice receiving BDNF-expressing pDNA using the PAsp(DET)/pDNA polyplex generally showed early recovery compared with other groups. Among the parameters, “print area” and “mean intensity”, representing the size and intensity of each footprint, showed significantly higher values one week after administration, suggesting the early recovery of walking motion with sufficient weight bearing (Fig.4A,B). Other parameters of “stride length” and “print positions”, reflecting stepping pattern and coordination, needed two weeks to show significant differences between the PAsp(DET)/pDNA polyplex group and others. It is important to note is that there is an accuracy limitation in the latter two parameters especially during the early period of recovery, because the stride length of the hind foot would be falsely measured once a footstep is missed. In any case, the significant differences in the parameters were observed up to four weeks after administration, strongly suggesting that the BDNF-expressing pDNA introduced by the PAsp(DET)/pDNA polyplex provided sustainable therapeutic effect on the recovery of walking for more than four weeks.

Fig.4. Analysis of walking motion by CatWalk system. The parameters listed in Table 1 were evaluated; (A) print area, (B) mean intensity, (C) Stride length of hindlimb, (D) Base of support in hindlimbs, and (E) Print positions. Data are relative values to the average of non-injured intact control, expressed as means $\pm$ SEMs (n = 6), * $p < 0.05$.

3.3. Therapeutic effect of BDNF-expressing pDNA on neural cell survival and preservation

To investigate the mechanisms of walking motion recovery, the neural cell survival was determined histologically in the injured spinal cord by the TUNEL assay to calculate the number of apoptotic cells (Fig. 5A). On days 3 and 7 after SCI, the number of TUNEL-positive (apoptotic) cells was significantly lower for the mice receiving BDNF-expressing pDNA using PAsp(DET)/pDNA polyplex compared with the control mice receiving luciferase-expressing pDNA (Fig. 5B, C). Since there was no difference in

the total number of cells between these two groups, it is suggested that the BDNF-expressing pDNA effectively decreased apoptotic cell death in the injured spinal cord.

Fig.5. Analysis of cell apoptosis by a TUNEL assay after experimental SCI. **(A)** Schematic representation for obtaining images for apoptotic cell counting. **(B)** Representative images of apoptotic cells that were labeled with TMR red around SCI region. Images were obtained under $\times 200$ magnification using a fluorescence microscope (AxioVision: Carl Zeiss, Oberkochen, Germany) 3 days after induction of SCI and concomitant intrathecal injection of BDNF or luciferase-expressing pDNA using PAsp(DET). Scale bars represent 20 μm . **(C)** Counting of apoptotic cells from section images using image analyzer software (WinROOF: Mitani Co., Tokyo, Japan). Data are expressed as average cell number per image $\pm$ SEMs ($n = 6$), * $p < 0.05$.

The size of damaged spinal cord tissue was evaluated in the injured spinal cord by Luxol fast blue (LFB) staining, a selective staining of myelinated axons. We analyzed

five serial horizontal sections with the range of 400 μ m rostrally and caudally from the epicenter of injury (Fig.6A). As shown in Fig. 6B, the LFB-negative area indicating the damaged spinal cord was apparently smaller in the mice receiving BDNF-expressing pDNA compared with the control mice. The planimetry of the sections revealed that the BDNF provided neuroprotective effects in maintaining the axons in an intact myelinated state after SCI, displayed by the significantly increased LFB-positive areas in the sections of both rostral and caudal regions after two weeks of administration (Fig. 6C). It is reasonable to assume that the anti-apoptotic, neuroprotective effect in the nerve tissue contributed to the rapid recovery of walking motion as early as one week after administration (Fig. 4). On the other hand, at four weeks after administration, the significantly larger LFB-positive area was still observed in the BDNF group (Fig. 6D), strongly suggesting the sustained effect of BDNF-expressing pDNA. However, for BDNF and control groups, the rates of LFB positive area also showed higher values at 4 weeks compared with two weeks, thus a certain degree of spontaneous motor function recovery should be included in the results.

Fig.6. Evaluation of tissue sparing after experimental SCI. (A) Scheme of evaluating the tissue sparing in the longitudinal sections of spinal cord. Transverse spinal cord sections were serially obtained from 400 μm rostral (R400) to 400 μm caudal (C400) to the epicenter with 200 μm intervals. (B) Representative images of transverse spinal cord sections at the epicenter with Luxol fast blue (LFB) staining, 2 weeks after SCI and concomitant intrathecal injection of BDNF or luciferase-expressing pDNA using PAsp(DET). **Scale bars: 500 μm.** (C, D) Quantification of LFB positive area 2 weeks (C) or 4 weeks (D) after SCI and the pDNA injection. Data are expressed as means ± SEMs (n = 6), * $p < 0.05$.

4. Discussion

The aim of this study was to demonstrate the feasibility of the PAsp(DET)/pDNA polyplex system which can introduce genes with high efficacy and safety for the treatment of SCI. While a number of therapeutic agents including BDNF have been identified for SCI, there remains a severe problem of how to deliver these agents to the target region of the injured spinal cord. In the case of peptides and proteins, the effects tend to be too short due to limited duration in the tissues. Therefore, repeated administration or a sustained-release formulation is needed, but these techniques are likely to induce various complications such as scarring and secondary tissue damage [29, 30, 39].

Gene delivery is promising in overcoming these issues. Besides the sustained manner of protein production, the gene can express, in principle, any protein and peptide by modifying its base sequences. Thus, gene introduction potentially applies well to various situations in the complex pathology of SCI. When a secretory factor(s) is applied, the target cells for gene introduction would not be restricted to neural cells. Any cells surrounding the SCI region are capable of secreting the factor(s) into the region.

In this sense, the advantages of non-viral systems such as their safety and ease of preparation deserve attention for SCI treatment. We chose BDNF for the therapeutic agent of SCI. BDNF was discovered in the early 1980s and has been gaining particular interest among several neurotrophic factors because it promotes neuronal function and activity in the CNS [25, 40-42]. BDNF has been tried recently in the treatment of SCI where diverse effects have been reported, including elongation of neural cell survival, promotion of axonal regeneration, plasticity, and re-myelination [26, 43]. In acute SCI, BDNF exhibited anti-apoptotic effects on neurons and oligodendrocytes within two weeks after SCI with BDNF peptide-continuous infusion or transfection of a BDNF-expressing adenoviral vector [44-46].

We introduced BDNF-expressing pDNA into neural tissue using the PAsp(DET)/pDNA polyplex concomitantly with the induction of experimental SCI. As expected, BDNF protein expressed from the pDNA showed remarkable anti-apoptotic effects in neural cells (Fig. 5) and preservation of normally myelinated axons (Fig. 6).

Eventually, walking function showed a remarkably early recovery. Compared with protein administration which shows very rapid clearance in CSF [29], pDNA was capable of providing transgene expression for more than a week, and it is reasonable to assume that BDNF-expressing pDNA was advantageous in managing the complex pathology of SCI that would last for more than several weeks.

It is interesting that the mice receiving BDNF by LPEI/pDNA polyplex also showed considerable recovery of motor function compared with the negative control mice treated with luciferase-expressing gene using PAsp(DET), although the degree of recovery was generally lower than that of the group receiving BDNF by PAsp(DET)/pDNA (Fig. 3, 4). The reason could be partly due to the difference in efficiency of transgene expression between PAsp(DET) and LPEI (Fig. 1B). Indeed, although LPEI is known to have high transfection efficiency due to its endosome-buffering capacity, PAsp(DET) has a more sophisticated manner of pH-selective membrane destabilization to disrupt endosomal membranes, resulting in more efficient gene introduction with smooth transport of the polyplexes into cytoplasm [21, 47].

In addition, a critical problem about the safety of gene introduction should be raised; even after good transgene expression is obtained, cell viability may gradually decrease with fluctuations in endogenous gene expressions [24]. For example, despite the high efficiency of in vitro and even in vivo gene introduction, LPEI still has a problem of long-term safety mostly due to its non-degradability inside the cells, resulting in the regrettable fact that PEI has never been used for clinical purposes. On the other hand, other non-viral systems, especially lipid-based systems, generally have disadvantages in low efficiency of in vivo gene introduction compared with the remarkably high capacity for cultured cells in some cases, comparable to capacities of viral vectors [48, 49]. Indeed, for SCI, there are few reports in animal studies showing sufficient improvement of neural function by introduction of therapeutic molecules, although a considerable number of systems achieved in vitro transgene expression in neural cells such as neurons and astrocytes [50].

PAsp(DET) is advantageous both in safety due to its spontaneous biodegradability into a non-toxic form, and high transfection efficiency due to the

definite mechanism of endosome escape as mentioned previously. As a proof-of-concept study, it is demonstrated that the administration of BDNF-expressing pDNA using PAsp(DET) polyplexes provided high BDNF expression in an effective manner to enhance the motor function recovery after SCI. As far as we know, this is the first report of gene introduction using a non-viral system to confirm the improved neural function with extensive assessment of walking motion. The sustained effect of BDNF in protecting neural cell death, which lasted for a few weeks even after a single administration of BDNF-expressing pDNA, is likely to be attributed to the biocompatible manner of the PAsp(DET) system to minimize toxic effects that might hamper the recovery of neural function.

In conclusion, gene introduction into a spinal cord region using the PAsp(DET)/pDNA polyplex system provided sufficient transgene expression without harmful tissue injury and inflammation. For experimental SCI, the introduction of BDNF-expressing pDNA significantly enhanced the recovery of neural function with neuroprotective effects on neural cells in preventing cell death. We believe this approach has therapeutic use for treating spinal cord injury.

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Figure legends

Fig.1. Luciferase expression at spinal cord of normal mice after intrathecal injection of luciferase-expressing pDNA using PAsp(DET), LPEI, Lipofectamine, or in the form of naked pDNA. (A) Representative IVIS images of mice 1 day after injection. (B) Time course of luciferase expression quantified from the IVIS images. Data are expressed as means $\pm$ SEMs ($n = 5$), $**p < 0.01$, $*p < 0.05$ vs. LPEI.

Fig.2. Proinflammatory cytokines gene expression 24 h after intrathecal injection of PAsp(DET)/pDNA (luciferase) polyplexes. The messenger RNA (mRNA) expression of TNF- α (A), IL-6 (B), and IL-1 β (C) in the lower thoracic spinal cord region was evaluated by real-time quantitative PCR. Data are expressed as a relative value to that from intact spinal cord region. Data are expressed as means $\pm$ SEMs (intact control: $n = 3$, the others: $n = 5$), $*p < 0.05$. (D) Spontaneous recovery of walking motion that was evaluated by BMS scoring after inducing experimental SCI ($n = 6$). Intrathecal injection did not affect the recovery.

Fig.3. Analysis of walking motion by BMS scoring after intrathecal injection of BDNF-expressing pDNA using PAsp(DET) (close square), LPEI (triangle), or luciferase-expressing pDNA using PAsp(DET) (open square), concomitantly with the induction of experimental SCI. (A) BMS, (B) BMS subscore. Data are expressed as means $\pm$ SEMs ($n = 6$), $*p < 0.05$.

Fig.4. Analysis of walking motion by CatWalk system. The parameters listed in Table 1 were evaluated; (A) print area, (B) mean intensity, (C) Stride length of hindlimb, (D) Base of support in hindlimbs, and (E) Print positions. Data are relative values to the average of non-injured intact control, expressed as means $\pm$ SEMs ($n = 6$), $*p < 0.05$.

Fig.5. Analysis of cell apoptosis by a TUNEL assay after experimental SCI. (A) Schematic representation for obtaining images for apoptotic cell counting. (B) Representative images of apoptotic cells that were labeled with TMR red around SCI region. Images

were obtained under $\times 200$ magnification using a fluorescence microscope (AxioVision: Carl Zeiss, Oberkochen, Germany) 3 days after induction of SCI and concomitant intrathecal injection of BDNF or luciferase-expressing pDNA using PAsp(DET). Scale bars represent 20 μm . (C) Counting of apoptotic cells from section images using image analyzer software (WinROOF: Mitani Co., Tokyo, Japan). Data are expressed as average cell number per image $\pm$ SEMs ($n = 6$), * $p < 0.05$.

Fig.6. Evaluation of tissue sparing after experimental SCI. (A) Scheme of evaluating the tissue sparing in the longitudinal sections of spinal cord. Transverse spinal cord sections were serially obtained from 400 μm rostral (R400) to 400 μm caudal (C400) to the epicenter with 200 μm intervals. (B) Representative images of transverse spinal cord sections at the epicenter with Luxol fast blue (LFB) staining, 2 weeks after SCI and concomitant intrathecal injection of BDNF or luciferase-expressing pDNA using PAsp(DET). Scale bars: 500 μm . (C, D) Quantification of LFB positive area 2 weeks (C) or 4 weeks (D) after SCI and the pDNA injection. Data are expressed as means $\pm$ SEMs ($n = 6$), * $p < 0.05$.

Table 1. CatWalk parameters.

CatWalk parameter	Explanation
Print area	Total surface area contacted by the hindpaw during the complete stance duration
Mean intensity	Mean intensity of the pixels forming the maximum area
Stride length	Distance between the placement of a hindpaw and the subsequent placement of the same paw
Base of support(BOS)	Distance between two hindpaws, as measured perpendicular to the waking direction
Print positions	Distance between the placement of a hindpaw and the ipsilateral frontpaw placed just before

Graphical Abstracts

Treatment of spinal cord injury
using BDNF-expressing DNA

Efficient expression along spinal canal

Early recovery of motor function (gait analysis)