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Abstract. Nonviral, site-specific gene delivery to deep tissue is required for gene therapy of a spinal cord injury. However, an efficient method achieving these requirements has not been established. This study demonstrates efficient and targeted gene transfer into the spinal cord by using photomechanical waves (PMWs), which were generated by irradiating a black laser absorbing rubber with 532-nm nanosecond Nd:YAG laser pulses. After a solution of plasmid DNA coding for enhanced green fluorescent protein (EGFP) or luciferase was intraparenchymally injected into the spinal cord, PMWs were applied to the target site. In the PMW application group, we observed significant EGFP gene expression in the white matter and remarkably high luciferase activity only in the spinal cord segment exposed to the PMWs. We also assessed hind limb movements 24 h after the application of PMWs based on the Basso-Beattie-Bresnahan (BBB) score to evaluate the noninvasiveness of this method. Locomotor evaluation showed no significant decrease in BBB score under optimum laser irradiation conditions. These findings demonstrated that exogenous genes can be efficiently and site-selectively delivered into the spinal cord by applying PMWs without significant locomotive damage.

Keywords: central nervous system; gene delivery; gene therapy; functional evaluation; laser-induced stress waves; naked DNA; spinal cord injury.

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1 Introduction

Spinal cord injury (SCI) causes loss of autonomic, sensory and motor functions, and recovery of these functions cannot be expected due to the difficulty in tissue regeneration in the central nervous system (CNS). Thus, SCI patients are left with serious residual disabilities, such as respiratory difficulty, chronic pain, urinary problems, and neurologic decline leading to a considerable decrease in quality of life. Unfortunately, no effective treatment for SCI has been established. Gene therapy has received much attention as a promising treatment for SCI because various growth factor genes, including the nerve growth factor (NGF),1,2 brain-derived neurotrophic factor,3–5 vascular endothelial growth factor,6 glial cell line-derived neurotrophic factor7,8 (GDNF) and hepatocyte growth factor,9 have been found to be effective for SCI treatment. Viral vectors, enabling high transduction efficiency and persistent gene expression, were used in most of those studies. However, the use of viruses has serious problems such as unexpected inflammatory responses and immunogenic complications, which have been hampering clinical application.10–14

Nonviral vectors using naked DNA have advantages in terms of simplicity of use and ease of large-scale production, as well as lack of specific immune response.15–18 However, transfection efficiency obtained by naked DNA injection alone is generally low; there is plenty of scope for improvement in efficiency.16,18 Physical gene transfer methods, such as electroporation19–21 and microbubble ultrasound,22–24 have recently been used to improve the efficiency of naked DNA-based gene delivery into the spinal cord. In these methods, however, targeting characteristics are limited, because the site of gene expression is considerably affected by the distribution of electrical impedance or microbubbles in the tissue. In ultrasound-mediated gene transfer into the spinal cord, for instance, transfected cells were limitedly observed on the dorsal surface of spinal tissue due to the nonuniform distribution of microbubbles;23,24 gene expression in the spinal cord parenchyma is needed for gene therapy of SCI. High spatial controllability is also important for safety, since unnecessary gene expression in intact tissue should be avoided. The development of a nonviral, site-controllable gene delivery method is needed for realizing safe and efficient gene therapy of SCI.

In our previous studies, we demonstrated efficient gene delivery to the skin and brain in rodents by using photomechanical waves (PMWs) or laser-induced stress waves.25–30 Although the detailed mechanism of PMW-based gene transfer has not been fully elucidated, laser-induced impulsive pressures can increase cell membrane fluidity in tissue, followed by uptake of plasmid DNA. Importantly, high spatial controllability of laser energy to generate PMWs enables targeted gene transfer. In addition,
PMWs can efficiently propagate in tissue, and deeply-located tissue can, therefore, be treated with PMWs. These characteristics enable a site-specific gene delivery into the spinal cord parenchyma, which would be advantageous for gene therapy of SCI. However, plasmid DNA-based gene transfer to nervous tissue is generally difficult because nerve tissue is quite sensitive and vulnerable to damage. Optimum conditions should be carefully investigated for efficient and safe gene delivery into the spinal cord by using PMWs.

In this study, we examine the optimum laser parameters for efficient and safe PMW-based gene transfer into spinal cords in rats. We first delivered an enhanced green fluorescent protein (EGFP) gene and investigated the distribution and extent of gene expression in the spinal tissue with various laser fluences and pulses. We then delivered a luciferase gene under the optimum laser irradiation conditions for EGFP gene delivery and measured luciferase activity in each spinal cord segment to assess the targeting characteristics for a transferred site. In addition, since spinal tissue is exposed to high peak pressures in PMW-based gene transfer, we performed locomotive functional tests for rats based on the locomotor BBB (Basso-Beattie-Bresnahan) scale to evaluate the noninvasiveness of this method.

2 Materials and Methods
2.1 Animal Preparation
Female Sprague-Dawley rats (Japan SLC) weighting 180 to 270 g were used in the present study under the protocol approved by the Committee on the Ethics of Animal Experiments in the National Defense Medical College. Before the operation, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg animal weight).

2.2 Generation and Measurement of Pressure Characteristics of Photomechanical Waves
PMWs were generated by irradiating a target, a 0.5-mm-thick black natural rubber disk of 5 mm in diameter, with 6-ns (FWHM) laser pulses from the second harmonic wave (532-nm) of a Q-switched Nd:YAG laser (Brilliant b, Quantel, Les Ulis Cedex, France). The laser pulses were focused with a plano-convex lens (f = 200 mm) to a diameter of 3 mm on the rubber. A 1.0-mm-thick transparent polyethylene terephthalate sheet was bonded on the rubber to confine the laser-produced plasma to increase the peak pressure and impulse of the PMWs.

Before the gene delivery experiment, the pressure profiles of PMWs were measured using a needle-type PZT hydrophone. PMWs were carefully investigated for efficient and safe gene delivery into the spinal cord by using PMWs.

2.3 Construction of Plasmid DNA
Plasmid DNA encoding EGFP (pEGFP-C1, Palo Alto, California), which was driven by the cytomegalovirus (CMV) promoter, was purchased from Clontech. Plasmid DNA encoding firefly luciferase driven by the CMV promoter was provided by Dr. Kaneda. The plasmids were transformed into Escherichia coli competent cells and amplified and purified with a Qiagen column. The vector used as a control vector was a CMV vector plasmid not containing luciferase cDNA. The plasmid vectors were prepared at a final concentration of 2.0 μg/μl in a TE solution (10 mM Tris and 1 mM EDTA, pH 8.0).

2.4 Photomechanical Wave-based Gene Transfer to the Spinal Cord
The spinal cord in a rat was exposed by removing the dorsal part of the tenth thoracic vertebra. A 27-gauge needle of a Hamilton syringe was stereotactically inserted into the spinal cord at a point 1.0 mm distant from the midline and 2.0 mm in depth from the surface. The plasmid solution (10 μl) was slowly injected to minimize tissue damage in the spinal cord and a laser target was placed at the injection point on the spinal cord surface (Fig. 1). Ultrasound conductive gel was used between the target bottom surface and the spinal surface to ensure acoustic impedance matching. The target was irradiated with a single laser pulse to generate a PMW. For multi-pulse irradiation, the target was replaced for each pulse to keep efficient confinement of the laser-produced plasma. For the experiment on EGFP gene transfer, three laser irradiation conditions to generate PMW(s) were examined: three pulses with a laser fluence of 0.3 J/cm², ten pulses with 0.3 J/cm², and one pulse with 0.9 J/cm².

2.5 Gene Expression Assay
At 48 h post-transfection, animals transfected with the EGFP gene were deeply anesthetized and perfused with 50 ml physiological saline and 200 ml of 4% paraformaldehyde. The spinal cord was removed, post-fixed in the same fixative as that used for perfusion for 16 h, and stored in 0.1 M phosphate-buffered saline for 24 h. The tissue was embedded in an optimal cutting temperature compound (Tissue-Tek) and then frozen and sectioned with a cryostat microtome to 10 μm in thickness to observe the fluorescence of EGFP, for which a fluorescent microscope (Eclipse E600, Nikon Corporation, Tokyo, Japan) was used. For the digital fluorescent images obtained, EGFP-derived green pixels were discriminated on the basis of RGB values. For semi-quantitative evaluation of gene expression, the total numbers of EGFP-derived green pixels in the white matter, and the
gray matter were counted. Histological analyses were carried out for four rats in each gene transfer condition.

To investigate the targeting characteristics of PMW-based gene delivery into spinal tissue, we evaluated luciferase activity for the tissue in each spinal segment by using a standard luciferase assay kit (E1500, Promega Corporation, Madison, Wisconsin). The harvested spinal tissues were homogenized with fine surgical scissors in 500 μl lysis buffer solution. The homogenates were centrifuged at 15,000 rpm for 15 min at 4°C. The homogenate supernatant (20 μl) was added to 100 μl luciferin in a 5-ml polystyrene round bottom tube and the luminescence was measured for 10 s with a luminometer (Flash and Glow LB955, Berthold Technologies, Bad Wildbad, Germany).

Analysis of luciferase activities in each spinal segment under the dorsal part of 9th to 11th thoracic vertebra (n = 5).

2.6 Evaluation of Locomotive Function

To evaluate the noninvasiveness of PMW-based gene transfer, hind limb movements at 24 h after the operation were observed in an open field and scored based on the locomotor BBB scale. The criteria for rating on the 21-point score were adopted from Basso et al.31 a score of zero means no spontaneous movement, while a score of 21 indicates normal locomotion. Evaluations were carried out for four to six rats in each EGFP gene transfer condition. For comparison, sham-treated animals were subjected to laminectomy but received neither weight-drop injury nor PMWs (n = 4).

2.7 Statistical Analysis

Analyses of EGFP gene expression in the tissue and luciferase activities in each spinal segment were based on Kruskal-Wallis test with Scheffe’s method for multiple comparisons. Comparisons of temporal changes in luciferase expression were performed using a nonparametric Mann-Whitney’s U-test. Statistical analysis of locomotive functional evaluation was performed using Kruskal-Wallis test followed by Steel’s method for multiple comparisons, including the sham control: laminectomy alone. A value of P < 0.05 was regarded as statistically significant in these analyses.

3 Results

3.1 Pressure Characteristics of Photomechanical Waves

Figure 2 shows pressure temporal profiles of PMWs used for gene transfer to rat spinal cords. The pressure waveforms were dominated by positive pressure, although a negative pressure component, which can cause cavitation, was observed at 0.3 J/cm². However, biological tissues are generally much less susceptible to compressive stress than tensile stress. In addition, the pulse widths of the PMWs were as short as 170 ns (FWHM), resulting in relatively small values of impulse despite the high peak pressures. These characteristics would be associated with the low invasiveness of the present gene transfection.

With laser fluences of 0.3 and 0.9 J/cm², the peak pressures were about 45 and 135 MPa, respectively; the rise times (rise time defined as the time from 10 to 90% of peak pressure) were about 42 and 34 ns, respectively. Thus, the stress gradient (peak pressure divided by rise time) with a laser fluence of 0.9 J/cm² (4.0 MPa/ns) was about 3.7 times higher than that with a laser fluence of 0.3 J/cm² (1.1 MPa/ns).

3.2 Distributions of Enhanced Green Fluorescent Protein Gene Expression

Figure 3 shows fluorescence images of EGFP gene expression in sagittal sections of the rat spinal cords treated under the four different conditions at 48 h post-transfection. In the negative control section, for which the spinal cord was only injected with physiological saline, no fluorescence was observed (data not shown). For the rats with plasmid DNA injection only (no PMWs), EGFP expression was observed in the gray matter with almost no expression in the white matter [Fig. 3(a)]. For the rats with PMW application after plasmid injection, on the other hand, EGFP fluorescence was observed in both the gray and white matters [Figs. 3(b)–3(d)]. Figure 4 shows the results of semi-quantitative evaluation of
EGFP expression in the sections, for which the number of pixels showing green fluorescence was counted for each image. Pixels showing fluorescence of lower intensity than the background level (fluorescence intensity level in the negative control) were excluded in this evaluation. The level of EGFP gene expression in the white matter was higher for all of the PMW application groups than for the group with plasmid injection only, suggesting that PMW-based gene transfection is more effective for cells in the white matter than for those in the gray matter. However, the type of cells showing EGFP gene expression, e.g., neurons or glial cells, was not determined in the present study.

The expression level would be dependent of the laser irradiation conditions. In the white matter, a significantly higher EGFP gene expression level was observed with a laser fluence of 0.3 J/cm² and a pulse number of 10 than that in the control group, although there was no significant difference in gene expression level in the gray matter among the groups. The highest averaged EGFP expression level was observed in spinal tissue exposed to 10 pulses of PMWs generated at a laser fluence of 0.3 J/cm², the total laser energy being highest in all of the groups. In the two groups exposed to the same total laser energy of 0.9 J/cm² (0.3 J/cm² × 3 pulses and 0.9 J/cm² × 1 pulse), there was no significant difference between gene expression levels in the white matter or between gene expression levels in the gray matter. Based on these results, we employed 10 pulses of PMWs generated at 0.3 J/cm² as the optimum laser irradiation conditions in the following experiments.

### 3.3 Targeting Characteristics

Figure 5 shows expression levels of luciferase gene expressed as relative light units (RLU) per milligram of tissue protein for each thoracic spinal segment at two days after gene transfer. In this experiment, plasmid DNA was stereotaxically injected into the spinal cord under the 10th thoracic vertebra (Th 10). The average length and width of the Th-10 vertebra was approximately 6 and 5 mm, respectively. The negative control (control vector injection) shows luciferase activity in the spinal cord injected with the control vector without PMW application (background level of luciferase activity), which was less than 10² RLU per milligram of tissue protein. For Th 10, the level of luciferase activity obtained by PMW application after plasmid DNA injection (PMW application group) was significantly higher (by 16-fold) than that of the negative control. The level of luciferase activity obtained by plasmid DNA injection alone (plasmid DNA injection alone group) was also higher than that of the negative control, but there was no significant difference. For Th 9 and Th 11, the level in the PMW application group was also higher than the levels in other groups, but there were no significant differences.

Figure 6 shows time courses of luciferase gene expression levels integrated for Th 9, Th 10, and Th 11, where the level before any treatment is shown for comparison. Luciferase activity decreased with elapse of time both in the PMW application group and the plasmid injection alone group. At two and five days after gene transfection, however, the luciferase activity levels in the PMW application group were significantly higher than those in the control vector injection group and the plasmid injection alone group. (a) 0.3 J/cm² × 3 pulses, (b) 0.3 J/cm² × 3 pulses, (c) 0.3 J/cm² × 10 pulses, and (d) 0.9 J/cm² × 1 pulse. Values are expressed as means ± S.E.M (n = 4). ** depicts *P < 0.01 versus plasmid injection only.
higher than those in the plasmid injection alone group. For the plasmid injection alone group, the level of luciferase activity at five days after plasmid injection was comparable to that before gene transfection. At 10 days after gene transfection, the luciferase expression levels in both groups were similar to that before gene transfection.

3.4 Locomotive Function

Locomotive damage to the spinal cord after PMW-based gene transfer was examined based on the BBB scores. Figure 7 shows BBB scores for the rats at 24 h after EGFP gene transfer. The averaged locomotive score in the plasmid injection alone group was 20.3, which was nearly the same as the score in the sham operation group (20.9, not shown Fig. 7). This indicates that intrathecal injection was noninvasively conducted in the present experiments. There was also no significant difference in BBB scores between the group with naked plasmid DNA injection alone and the two groups with PMW application (0.3 J/cm² × 3 pulses and 0.3 J/cm² × 10 pulses), the averaged scores in these three groups being 20.0, 20.0, and 20.1, receptively. For the irradiation condition of 0.9 J/cm² × 1 pulse, on the other hand, the averaged score was significantly low (12.6) compared to that in the plasmid injection alone group.

4 Discussion

In the present study, we delivered reporter genes to rat spinal cords using PMWs generated by irradiation with nanosecond laser pulses and investigated transfection efficiency, distribution of gene expression and targeting characteristics. Although various physical methods for transferring naked plasmid DNA into spinal cords have been developed, it is difficult to efficiently and noninvasively transfected the targeted tissue with foreign genes. The findings obtained in this work demonstrated the feasibility of PMW-based gene delivery into the targeted spinal tissue without functional damage.

Our previous studies demonstrated that the efficiency of gene transfer depended on both peak pressure and pulse number of PMWs, while an excessive increase in peak pressure caused mechanical damage in the tissue. The results of the EGFP gene transfer experiment showed that the laser irradiation with 10 laser pulses at 0.3 J/cm² was the appropriate condition to generate PMWs for noninvasive and efficient gene transfer into rat spinal cords (Figs. 3, 4, and 7).

The results of the EGFP gene transfer experiment also showed that intraparenchymal injection of plasmid DNA alone can induce a certain level of gene expression in the gray matter, but there was little expression in the white matter [Figs. 3(a) and 4]. This is presumably due to the higher plasmid concentration in the gray matter than in the white matter by the present plasmid injection method. The application of PMWs after plasmid injection remarkably increased the gene expression level, especially in the white matter, under all of the laser irradiation conditions [Figs. 3(b)–3(d), and 4]. This is partially attributable to the fact that the white matter interacts with higher pressure PMW(s) than the gray matter does since the pressure is decreased by propagation.

It has been reported that in ultrasound microbubbles-mediated transfection of spinal cords, the transfected cells were mainly meningeal cells in the dorsal surface of the spinal cord; no gene expression was observed in neurons or glial cells. This would be due to the distribution of microbubbles in the spinal cord. In our PMW-based gene delivery, substantial gene expression was observed in the parenchyma, both in the gray and white matters. Since the gene therapies for many spinal-related disorders require efficient gene expression in the parenchyma, PMW-mediated gene transfer would be advantageous over ultrasound microbubbles-based transfection from practical aspects. Since the white matter can support long-distance regeneration of axon tracts, the expression of genes such as NGF and GDNF in the white matter would be closely associated with nerve regeneration.37–39

In general, gene delivery based on physical methods using naked plasmid DNA is considered to be safe due to the lack of the immune response and ease of handling compared with methods using viral vectors, but physical energy can cause damage to tissue. For instance, Kondoh et al. stated in their report on gene delivery to the periventricular region in rats by electroporation that tissue damage caused by electrical shock was inevitable and that electroporation is not suitable for gene delivery for neural regeneration. In the present study, there was no significant difference in locomotive scores between the plasmid injection alone group and the PMW application group (0.3 J/cm², three or 10 pulses) (Fig. 7). Under these conditions, peak pressure of the PMW on the spinal cord surface was found to be as high as 45 MPa. However, duration of the PMW was very short (less than one microsecond) (Fig. 2). Thus, the impulse of the PMW used for the present gene transfer was quite small (9.7 Pa·s), and this would be associated with the fact that no functional damage was observed in the rats. Moreover, the PMW was dominated by positive pressure components. In general, biological tissues are mechanically more susceptible to tensile stress than to compressive stress, which would also be a factor contributing to no detectable locomotive damage in the rats under these conditions. When the laser fluence was increased to 0.9 J/cm², the application of only a single-pulse PMW caused a significant decrease in the functional score (Fig. 7). In this case, the peak pressure reached about 135 MPa (Fig. 2) and the level of EGFP expression was decreased (Fig. 4). With the same total laser fluence of 0.9 J/cm², no functional damage was observed when three pulses of a PMW generated at 0.3 J/cm² were applied to the rats (Fig. 7).

![Figure 7](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics October 2011 r Vol. 16(10)108002-5)
results indicate that locomotive function is more sensitive to the fluence of each laser pulse or peak pressure of each PMW than to the total laser fluence under the conditions examined in this study. Since it is important to promote the extension of the axon tracts for therapy of spinal injury, detailed histological and immunological examinations of the nerve fiber pathways will be needed for practical application of our method to the treatment of spinal cord trauma.

For gene therapy of the CNS, site-specific gene delivery is particularly important to avoid unnecessary gene expression in intact sites. Although virus vectors have been widely used for gene delivery to the CNS, it is difficult to spatially control the region of gene expression. In this work, we demonstrated that a gene can be selectively delivered to the targeted spinal segment by applying PMWs (Fig. 5), due to spatially highly controllable energy and directionality of PMWs. In addition, PMW-based gene delivery has a potential for optical fiber-mediated endoscopic application to deep spinal cord tissues.

Gene therapy for spinal cord injury would be one of the most important applications of the PMW-based gene delivery. Following spinal injury, glial cells are activated by inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Within the first 24 h after injury, activation and hypertrophy of microglia are observed. This is followed by the proliferation of microglia; the proliferation is maximized at around three days after injury. In the meantime, such reactive astrocytes provide essential protection and support for neural cells in response to injury, resulting in glial scar formation by over-activation of glial cells from 24 to 48 h after injury. The activation of glial cells is known to be involved in the formation of a glial scar, which is both a physical and a biochemical barrier for axonal regeneration. The duration of plasmid gene expression was relatively short (<10 days) in the present study (Fig. 6), and this short duration is a common characteristic in physical gene transfer methods for spinal cords. Therefore, this method is considered to be suitable to transfer small interfering RNA for transient overexpressed glial proteins within a few days after spinal cord injury. A more direct approach is gene silencing on proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and TNF-α, which would be followed by endogenous axonal plasticity and enhanced tissue repair. In addition, recent studies have shown that transient expression of a neurotrophic factor within the lesion site is sufficient to induce axonal growth, but it is not required to sustain regenerated axons within the site of spinal cord injury. Thus, genetic modification at early stages of SCI could greatly contribute to the alteration of cellular reactions in the lesion site, for which our method would be useful.

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