이학석사 학위논문

Polyamidoamine dendrimer-conjugated triamcinolone acetonide attenuates nerve injury-induced mechanical allodynia by inhibiting spinal cord microglia activation

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ABSTRACT

Polyamidoamine dendrimer-conjugated triamcinolone acetonide attenuates nerve injury-induced mechanical allodynia by inhibiting spinal cord microglia activation

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Neuropathic pain is a pathological pain with allodynia and hyperalgesia that is caused by sensory neuron damage such as peripheral nerve injury (PNI). The activation of spinal cord microglia is critical for the development and maintenance of neuropathic pain after PNI. Previous study showed that triamcinolone acetonide (TA) inhibits microglia activation. However, TA has a limitation in clinical application due to its off-target side effects. To obviate this problem, I developed polyamidoamine (PAMAM) dendrimer-conjugated TA (D-TA), which supposedly delivers TA specifically into microglia. PAMAM dendrimer is a sphere-shape nano-

molecule. In this study, I show that PAMAM-dendrimer is delivered selectively into spinal cord microglia. Intratheal D-TA injection inhibited nerve injury-induced spinal cord microglia activation. D-TA administration reduced mRNA expression of proinflammatory cytokines, such as Nox2, IL-1β, TNFα, and IL-6 in spinal cord after PNI. In addition, D-TA administration significantly attenuated PNI-induced mechanical allodynia. Conclusively, my data demonstrate that D-TA attenuates neuropathic pain after PNI by inhibiting spinal cord microglia activation, suggesting a therapeutic implication for the treatment of neuropathic pain.

Key Words:

Dendrimer-Triamconolone acetonide, neuropathic pain, peripheral nerve injury, spinal cord microglia, proinflammatory cytrokines

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CONTENTS

I. Introduction

Neuropathic pain is pathological chronic pain caused by damage in the nervous system. It evokes dysfunctional pain including allodynia, which is evoked by stimuli that normally do not cause pain sensation, and hyperalgesia enhanced pain sensation caused by noxious stimuli. Traditionally, neuropathic pain was thought to come from the hyperexcitability of sensory neuron caused by a lesion of neurons (Woolf 2010). However, previously available drugs targeting neurons are ineffective in controlling such pain.

Recently, it has been recognized that glial cells are important modulators of neuropathic pain and could be a new target for pain treatment (Watkins et al. 2003). Over the past decade, it has been reported that microglia play important roles in neuropathic pain development and the maintenance. After a peripheral nerve injury, spinal cord microglial cells are activated and lead to the production of proinflammatory cytokines such as IL-1β, TNFα and IL-6 ((Kawasaki et al. 2008). These cytokines' expression is correlated with a pain behavior in response to nerve injury. After microglia activation, it transforms to an ameboid morphology which is mediated by transmembrane receptors, such as fractalkine receptor CX3CR1 and an ATP receptor P2X4 (Tsuda et al. 2003, Verge et al. 2004). It was also proposed that the toll-like receptors 2, 3 and 4 on microglia are involved in nerve injury

induced microglial activation in the spinal cord (Tanga et al. 2005, Kim et al. 2007, Obata et al. 2008)).

Corticosteroid treatments for neuropathic pain have been clinically used and much studied (Kingery et al. 2001, Lee et al. 2010). Localized corticoid triamcinolone acetonide injections decreased DRG satellite glia and spinal cord microglia activation in a rat spinal nerve ligation model (Li et al. 2011). A previous study showed that Triamcinolone acetonide (TA) inhibits microglia activation by blocking the NF-κB signaling pathway (Hong et al. 2012). However, corticosteroid drugs including TA have limitations in clinical application due to their off-target side effects. For decades, the neurotoxic side effects of corticosteroid treatments have been discussed (Koide et al. 1986, Sapolsky et al. 1990). Although some studies demonstrated the safety of intrathecal TA administration, serious side effects which may occur from neurotoxicits, such as arachnoiditis, meningitis, and paralysis still existed. Polyamidoamine (PAMAM) dendrimer is a synthetic polymer with a sphere-like shape in nanoscale size. It is used as a drug delivery vector, and its functional characteristics vary by generation number or terminal application (Dai et al. 2010). Recently it was reported that dendrimers were localized within microglia in rabbits with cerebral palsy by subarachnoid administration (Dai et al. 2010). It is also determined that PAMAM dendrimers selectively delivered drugs to activated retinal microglia in retinal degeneration rat models (Iezzi et al. 2012).

These current studies reached the conclusion that PAMAM dendrimer could be an effective drug carrier for TA. In the present study, I used an L5 peripheral nerve injury (PNI) mouse model to explore the PAMAM dendrimer-conjugated TA (D-TA) effect for neuropathic pain. I found that dendrimer specifically localized in spinal cord microglia and D-TA attenuates neuropathic pain after PNI by inhibiting spinal cord microglia activation.

II. Materials and Methods

Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University. The animal treatments were performed in accordance with the guidelines of the International Association for the Study of Pain. Experiments were carried out in male C57BL/6 mice aged 8-12 weeks. Mice were housed in plastic cages and were allowed to access food and water *ad libitum*. They were under a 12-h dark/light cycle and a constant room temperature of 23±2℃.

Neuropathic pain model and intrathecal injection

Mice were anaesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium and peripheral nerve injury was induced by transecting the L5 spinal nerve (SNT) as descried previously (Kim et al., 2010). A surgical incision was made to the skin and paraspinal muscles were separated. Then the L6 transverse process was partially removed and L5 spinal nerve was transected carefully. The wound was closed with surgical skin staples.

For the administration of D-Rho, D-FITC or D-TA mice were injected under pentobarbital sodium anesthesia (25 mg/kg). Using a 10 μl Hamilton syringe

(Hamilton Company, Reno, NV, USA) with a 30-gauge one-half-inch needle, D-TA (10 μl of 1 μg/μl in Saline) or Saline alone was intrathecally injected in C57BL/6 mice.

Cell culture

Primary mouse brain mixed glial cells were prepared from one-day-old C57BL/6 mice as previously established procedures (Lee et al., 2000). Briefly, after anesthetiszed, meninges were removed from the cerebral hemisphere, tissue was dissociated into a single-cell suspension by gentle repetitive pipetting. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 mM HEPES, 10% FBS, 2 mM L-glutamie, 1X NEAA and 1X antibiotic/antimycotic in 75 cm^2 flasks at 37℃ in a 5% CO_2 incubator, and the medium was changed every 5 days.

Real-time RT-PCR

Real-time RT-PCR was performed using the SYBR Green PCR Master Mix and an ABI Prism 7500 sequences detection system (Applied Biosystems, Foster City, CA) as described previously (Kim et al., 2010). The following PCR primer sequences were used: GAPDH (forward), 5'- AGG TCA TCC CAG AGC TGA ACG-3'; GAPDH reverse, 5'-CAC CCT GTT GCT GTA GCC GTA-3'; Nox2 forward, 5'-GAC CCA GAT GCA GGA AAG GAA-3' ; Nox2 reverse, TCA TGG TGC ACA GCA AAG TGA-3'; IL1-β forward, 5'-GTG CTG TCG GAC CCA

TAT GA-3'; IL1-β reverse, 5'-TTG TCG TTG CTT GGT TCT CC ; TNFα forward, 5'-AGC AAA CCA CCA AGT GGA GGA-3'; TNFα reverse, 5'-GCT GGC ACC ACT AGT TGG TTG-3'; IL-6 forward, 5'-CCA CGA TTT CCC AGA GAA CAT-3'; IL-6 reverse, 5'-TCC ATC CAG TTG CCT TCT TGG-3'. The mRNA level of each gene was normalized to the mRNA levels of GAPDH gene and represented as a fold induction. The fold induction was calculated using the $2^{-\Delta\Delta CT}$ method, as previously descried (Livak and Schmittgen, 2001). All real-time RT-PCR experiments were performed at least three times, and are the mean ± SEM values have been presented unless otherwise noted.

FACS analysis

The mice were deeply anesthetized by pentobarbital sodium and intracardially perfused with ice-cold saline. Lumbar 1 to sacral 3 spinal cord were extracted and homogenized mechanically to a single cell-suspension. Cells were washed with ice-cold 2% fetal bovine serum (FBS) in PBS, and incubated with Fc Blocker™ (BD Bioscience, San Jose, CA) for 10 min at 4℃ prior to staining with CD11b-APC (Biolegend Inc, San Diego, CA). BD FACSCalibur flow cytometer (BD Bioscience) was used to measure the microglia as $CD11b^+$ and non-microglia as $CD11b^-$. Data were acquired with analyzed with BD CellQuest ™system (BD Biosciences).

Immunohistochemistry

Male mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4-5 spinal cord was removed and postfixed in 4% PFA at 4℃ overnight, and transferred to 30% sucrose in PBS for 48 hr. Spinal cord were transected in 30-μm-thick and and prepared on gelatin-coated slide glass using a cryocut microtome. The sections were blocked in solution containing 5% normal donkey serum (Jackson ImmunoResearch, Bar Harbor, ME, USA), 2% BSA (Sigma, St. Louis, MO, USA) and 0.1% Triton X-100 (Sigma) for 1 hr at room temperature. The sections were then incubated overnight at 4℃ with primary antibody for rabbit-anti-Iba-1 (1:1000, Wako, Osaka, Japan) and mouse-anti-GFAP (1:5000, Millipore, MA, USA), rabbit-anti-MAP2 (1:400, Millipore, MA, USA). After rinsing in 0.1 M PBS, the sections were incubated for 1-h at room temperature with a mixture of Cy3- or FITC-conjugated secondary antibodies (1:200, Jackson ImmunoResearch). The sections were mounted, and fluorescent images were obtained using a confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

Behavioral testing

All mice were allowed to acclimate to the testing apparatus and environment twice for 2 hr before testing. Each mouse was placed alone in a plexiglass cage upon elevated mesh in order to access the paws for observation. On each test day, mice were briefly habituated to the test environment for 30 minutes. Baseline thresholds were obtained 2 days before surgical treatment. Mechanical allodynia was assessed by measuring foot withdrawal thresholds in response to mechanical stimuli to the hind paw. All behavior experiments were performed using the up-down method (Chaplan et al.,1994) with von Frey filaments. Mechanical sensitivity was calculated by mean threshold.

Statistical analysis

Statistically differences between two groups were determined by two tailed Student's *t-test*. Differences among multiple groups were made using one-way ANOVA followed by Bonferroni correction. All data are resented as the mean ± SEM, and differences were considered significant when the P-value was less than 0.05.

III. Results

Dendrimer specifically localize at spinal cord microglia.

Previous research examined dendrimer localize in microglial cells (Dai et al. 2010, Iezzi et al. 2012). Using the Rhodamine labeled dendrimer (D-Rho), I tested synthesized dendrimer localized in microglia from a mouse brain. Pure microglial cell cultures were treated with D-Rho for 1 hr and were fixed with 4% PFA. I confirmed that a dendrimer fluorescence signal is detected in microglia regions (Fig.1). To identify the dendrimer specifically localize at microglia cells, I injected D-Rho mice intrathecally. Three days after injection, a rhodamine signal was detected in the lumbar spinal cord. And these signals were co-localized with Iba1 positive cells, which was expressed in microglial cells. However, neither GFAP positive cells, astroglial cells, nor MAP2 positive cells, neuronal cells, were merged with the rhodamine signal (Fig.2). To quantify the rate of dendrimer localized microglia, from lumbar 1 to sacral 3 region spinal cord was measured by flow cytometry at 3 days after dendrimer conjugated FITC intrathecal injection. I confirmed that the FITC fluorescence signal about 57% of the CD11b positive cells. Taken together, these data show dendrimer delivery to microglia specifically *in vivo.*

Synthesis of dendrimer-TA conjugate, D-TA

D-TA was synthesized using a two-step process with glutaric acid as spacer

as shown in the synthetic scheme (Scheme 1). Hydroxy terminal PAMAM Generation 4.0 dendrimer (PAM-G4-OH) is relatively lower cytotoxicity compared to the cationic dendrimers due to the absence of surface primary amines. A glutaric acid spacer was used to reduce the steric hindrance and to enable better drug release ((Khandare et al. 2005, Perumal et al. 2009). The first step involved reaction of glutaric anhydride to triamcinolone acetonide (TA) resulting in a carboxylic acid derivate, which is further reacted with PAM-G4-OH through a coupling reaction to get the second-step conjugate, D-TA. TA was performed with glutaric anhydride dissolved in anhydrous DMF/DMA (80:20) in presence of triethanolamine (TEA) to get acid derivatives of TA. TA-glutarate was characterized by ^{1}H NMR(not shown) and the molecular weight was determined by LC/MS. TA-glutarate was further conjugated with the hydroxyl group of PAMAM G4 dendrimer (PAM-G4-OH) using HOBt, HBTU as coupling reagent and DIPEA as base to get final conjugate in anhydrous DMF. The synthesis of the D-TA was confirmed from ^{1}H NMR spectra (Bruker DPX-400 NMR spectrometer, DMF-d_7) with the conjugation of TA-glutarate. In ^{1}H NMR spectrum, peaks in between 0.9 and 1.8ppm represent methyl protons, and peaks in between 4.6 and 7.4 ppm represent aromatic protons of TA along with PAM-G4-OH peaks confirms the formation of the ester bond. D-TA conjugate was evaluated by NMR data indicates that 5 molecules were reacted per molecule of PAM-G4-OH (Iezzi et al. 2012). D-TA was provided from School of Chemistry and Molecular Engineering, Seoul National University.

D-TA attenuates LTA-induced proinflammatory cytokines in mouse brain mixed glial cells.

Previous studies have reported that proinflammatory genes TNFα, IL-1β and IL-6 were induced in activated glial cells and that they were down regulated by TA treatment (Kawasaki et al. 2008, Zheng et al. 2011, Hong et al. 2012). I tested TA downregulates of these gene expressions when conjugated with dendrimer. To investigate this, I cultured primary mixed glial cells from a mouse brain. Primary mixed glial cells were pretreated with DTA for 1hr then stimulated by LTA, microglia activating molecule, for 3 hr. Upon stimulation with LTA, the mRNA expression of IL-1β, TNFα and IL-6 genes upregulated about 115-, 70- and 75- fold, respectively. The gene induction by LTA, however, was downregulated by treatment DTA in a dose-dependent manner (Fig. 3). These data indicate that D-TA inhibits proinflammatory glial cell activation.

Intrathecal administration of D-TA reduces nerve injury induced morphological activation in spinal cord microglia.

Microglia morphological activation has been documented in the spinal cord after peripheral nerve injury. Here I tested how D-TA inhibit nerve injury induced microglial morphological change. Directly after L5 spinal nerve transection, D-TA was administrated by intrathecal. At 3 days after injury, I extracted L4-5 spinal cord

tissue and immunostained it with cell type-specific antibodies such as Iba1 and GFAP. The Iba-1 immunoreactive signals were strongly activated in the ipsilateral dorsal horn. However, the activation of microglia was significantly decreased in the D-TA treated mice, not the vehicle treated mice (Fig.4). These data demonstrate that D-TA attenuates nerve injury induced spinal cord microglia activation.

D-TA suppresses cytokines at spinal cord in neuropathic pain model.

It is well known that the pro-inflammatory cytokines such as IL-1β and TNFα are involved in spinal cord glial cells by peripheral nerve injury in the neuropathic pain model (Arruda et al. 1998, Sweitzer et al. 2001, Zheng et al. 2011)). These pro-inflammatory cytokines are produced in activated microglial cells in CNS (Smith et al. 2012). Upon spinal cord transection, the mRNA expression of IL-1β, TNFα, Nox2 and IL-6 genes was upregulated by 2.3-, 2.6-, 2.2- and 5.0-fold, respectively. The gene induction by SNT, however, was decreased to almost 1.2-, 1.3-, 1.5- and 1.7- fold, respectively in D-TA treated mice (Fig 5).

Mechanical allodynia suppressed by DTA intrathecal injection.

In order to test whether D-TA inhibits nerve injury-induced microglia activation, its role in attenuating pain was tested. To confirm this, I tested the susceptibility of the D-TA treated mice to nerve injury-induced pain hypersensitivity. Upon L5 spinal nerve transection, wild type mice showed

increased sensitivity to mechanical stimuli. The paw withdrawal threshold to the mechanical stimuli decreased from 1 day to 7 days after nerve injury. The threshold remained below 0.1 g on day 7. The paw withdrawal threshold of the D-TA treated mice, however, significantly reduced on day 1 and 3 compared to the vehicle treated group (Fig 6).

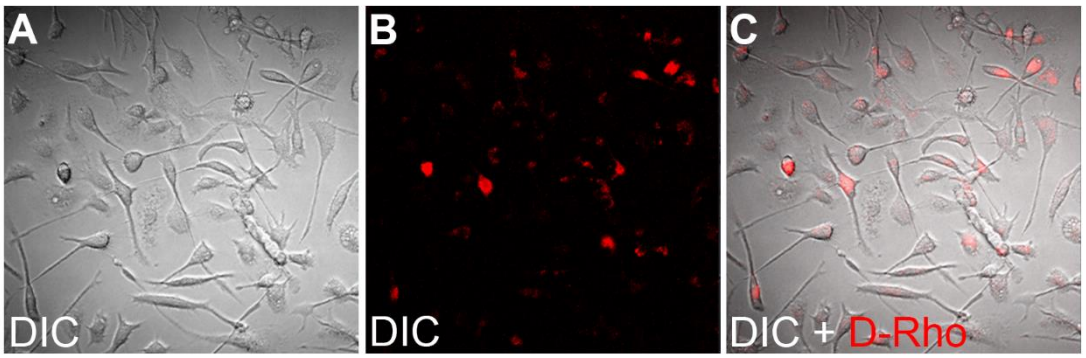

A
DIC
B
DIC
C
DIC + D-Rho

Figure 1. Localization of dendrimer-Rho within the microglial cells in vitro.

Microglial cells prepared from mice brain were seed to PDL coated cover slide. 4 days after, mixed glial cells were treated with Rhodamine conjugated dendrimer for 1-h. Cells were mounted and rhodamine signal detected under confocal microscopy. (A) DIC image of pure microglial cells from mouse brain mixed glial cells. (B) Rhodamine conjugated dendrimer were detected by red signals. (C) Dendrimer-Rho co-localized with microglial cells.

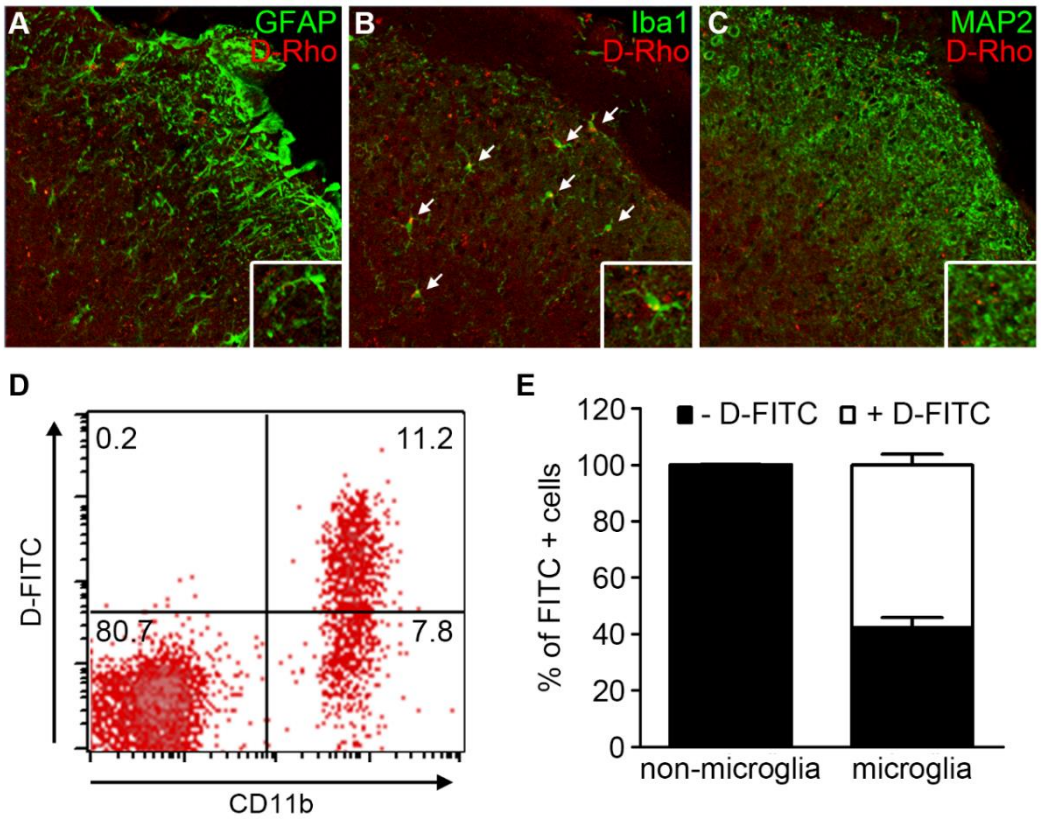
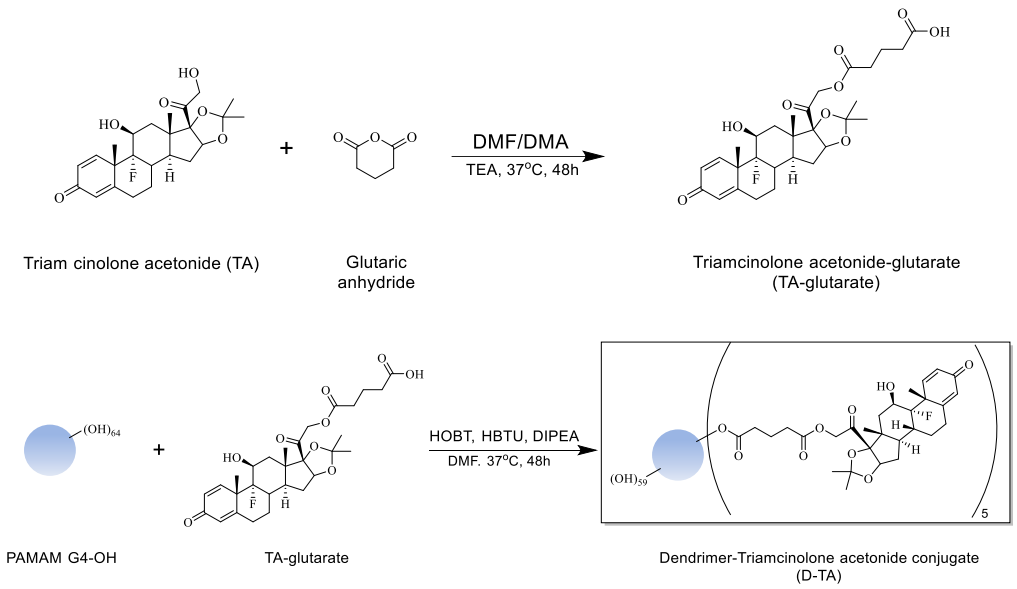

A
GFAP
D-Rho
B
Iba1
D-Rho
C
MAP2
D-Rho
D
0.2
11.2
80.7
7.8
D-FITC
CD11b
E
- D-FITC
+ D-FITC
% of FITC + cells
120
100
80
60
40
20
0
non-microglia
microglia

Figure 2. Microglia-specific delivery of dendrimer in vivo.

(A-C) Mice were received with 5 μg/μl of D-Rho by intrathecal injection. 3 days after, L4-5 spinal cord were obtained and sections were prepared. Each section was stained with GFAP, Iba1 and MAP2. Dendrimer conjugated rhodamine were detected in Iba1 positive cells at 3 days after intrathecal injection (arrows). (D and E) 3 days after D-Rho administration. Cells were isolated from Lumbar 1 to sacral 3 and stained with anti-CD11b-APC. CD11b positive cells were detected by FACScalliber^{TM}. Representative dot plots were showed in (D) and graph were showed in (E). Microglia were defined as CD11b.



Scheme 1. Synthesis of D-TA conjugate

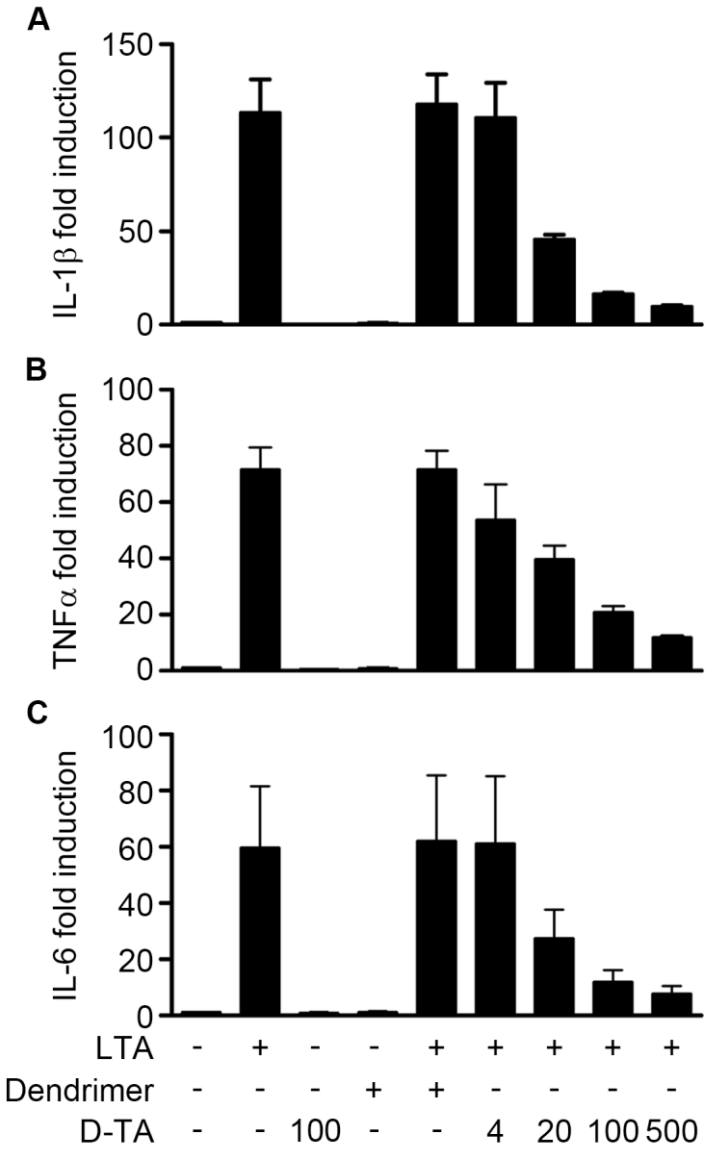

A
IL-1β fold induction
150
100
50
0
B
TNFα fold induction
100
80
60
40
20
0
C
IL-6 fold induction
100
80
60
40
20
0
LTA - + - - + + + + +
Dendrimer - - - + + - - - -
D-TA - - 100 - - 4 20 100 500

Figure 3. D-TA attenuates proinflammatory cytokines in mouse brain mixed glial cells.

Primary glial cells (5 x 10^5 cells/well) were incubated with D-TA in culture for 1 hr, and then exposed to LTA for 3 hrs. IL-1β (A), TNFα (B) and IL-6 (C) expressions were measured by real-time RT-PCR.

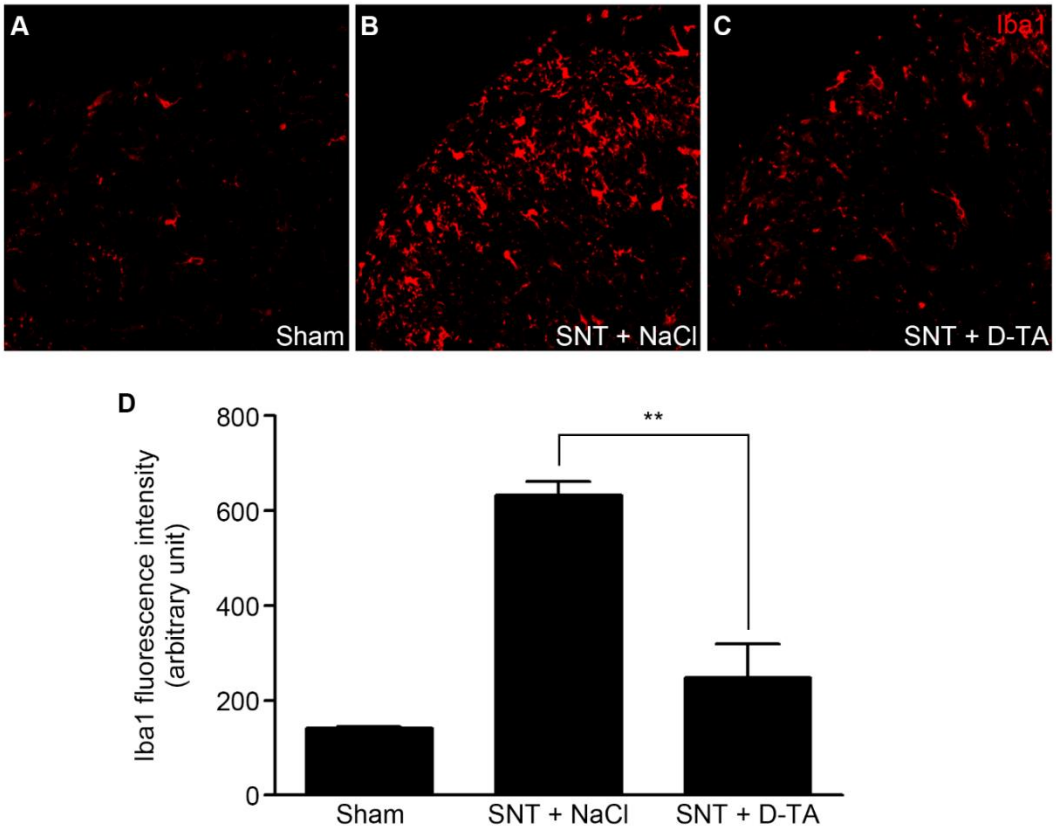

A
Sham
B
SNT + NaCl
C
Iba1
SNT + D-TA
D
Iba1 fluorescence intensity
(arbitrary unit)
800
600
400
200
0
**
Sham
SNT + NaCl
SNT + D-TA

Figure 4. D-TA reduced morphological activation in spinal cord microglia.

Mice were received with 5 μg/μl of D-TA by intrathecal injection right after SNT. After 3 days, L4-5 spinal cord were obtained and sections were prepared. Each section was stained with Iba1. (A) Iba1 was barely detectable in sham injury mouse spinal cord. (B) Spinal cord microglial activation measured by Iba1 staining at 3 days after SNT. (C) D-TA administration reduced microglial activation induced by SNT. (D) Quantitative summary of microglial activation data exemplified in (A-C).

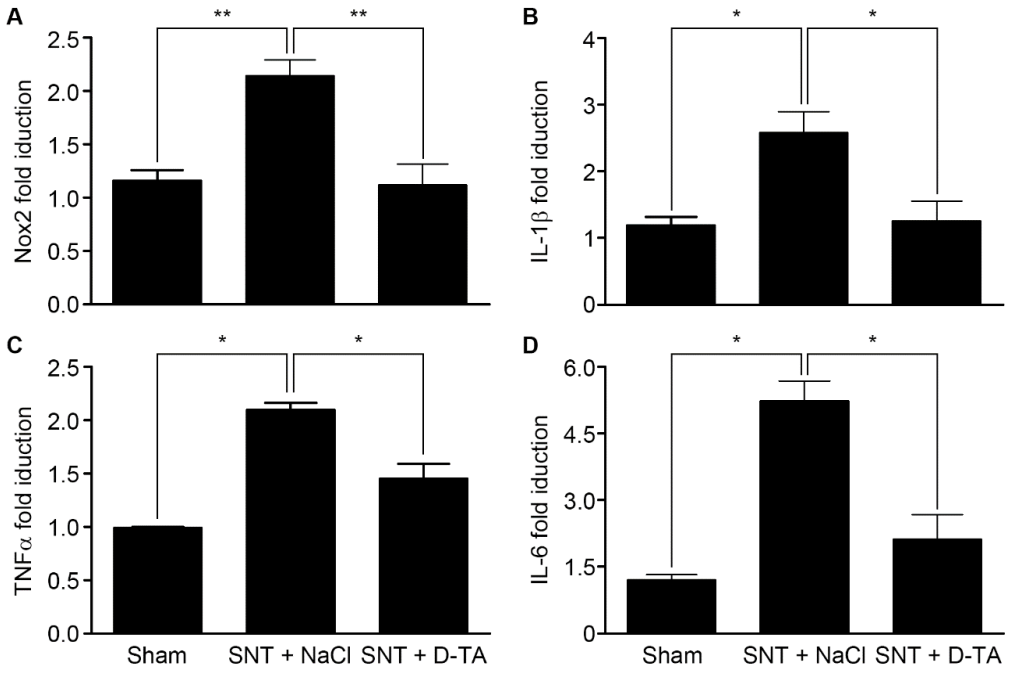

A
B
C
D
Nox2 fold iduction
IL-1β fold iduction
TNFα fold iduction
IL-6 fold iduction
**
**
*
*
*
*
*
*
2.5
2.0
1.5
1.0
0.5
0.0
4
3
2
1
0
6.0
4.5
3.0
1.5
Sham
SNT + NaCl
SNT + D-TA

Figure 5. Proinflammatory cytokines mRNA expression in L4-5 spinal cord at 3 days after L5 SNT.

Mice were received with 5 μg/μl of D-TA by intrathecal injection right after SNT. After 3 days, L4-5 spinal cord were prepared for total RNA isolation and cDNAs were prepared. Then cDNAs were used to measure the mRNA expression of Nox2 (A), IL-1β (B), TNFα (C), and IL-6 (D) by real-time RT-PCR.

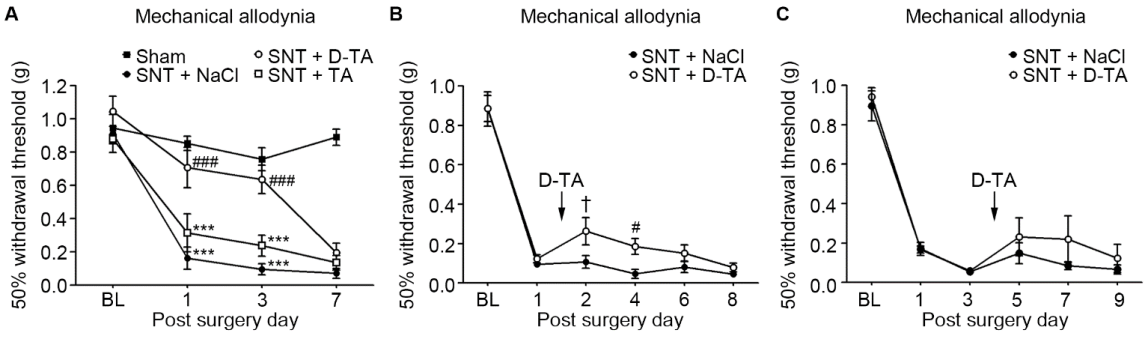

A
Mechanical allodynia
Sham
SNT + NaCl
SNT + D-TA
SNT + TA
50% withdrawal threshold (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
###
###
***
***
***
***
BL
1
3
7
Post surgery day
B
Mechanical allodynia
SNT + NaCl
SNT + D-TA
50% withdrawal threshold (g)
1.0
0.8
0.6
0.4
0.2
0.0
D-TA
†
#
BL
1
2
4
6
8
Post surgery day
C
Mechanical allodynia
SNT + NaCl
SNT + D-TA
50% withdrawal threshold (g)
1.0
0.8
0.6
0.4
0.2
0.0
D-TA
BL
1
3
5
7
9
Post surgery day

Figure 6. Changes in the ipsilateral 50% mechanical threshold of D-TA i.t injection after L5 SNT.

Effect of D-TA administration pain behavior induced by spinal nerve transection. D-TA injected immediately (A), 1 day (B), and 3 days (C) after surgery.

IV. Discussion

There are various drugs for the treatment of chronic pain. Traditionally, opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) were used to control pain. However, their effects are limited and they cause side effects. Opioids tend to be less effective or require higher doses in controlling pain. Also, they have side effects such as cognitive impairment, drowsiness and nausea. NSAIDs, another typical type of medicine used for the treatment of pain, have negative effects on the kidneys and gastrointestinal system (Bhatt et al. 2008, Chou et al. 2015). Additionally, these medications cannot treat chronic pain completely. Therefore, a new strategy is needed for neuropathic pain treatment.

Several studies revealed that glial cells activation in the spinal cord is important in the development and maintenance of neuropathic pain and this suggests that it could be a new target for controlling neuropathic pain (Watkins et al. 2003). Because microglia contribute to neuropathic pain by inflammatory response after a nerve injury, it is expected that some steroid anti-inflammatory drugs will be able to reduce neuropathic pain caused by microglia activation. The anti-inflammatory effect of TA has been previously tested in glial cells treated with necrotic neuronal cells (NNC). TLR-mediated proinflammatory genes are expressed through the NF-κB signaling pathway. TA inhibits NF-κB reporter activity in NNC-activated microglial cells. Because steroid drugs influence a wide range of tissues and cells,

however, the studies developing steroids as analgesic drugs, focused on decreasing their side effects.

Therapeutic methods targeting glial cells are not well developed. A recent study showed that polyamaidoamine (PAMAM) dendrimer is uptaken within retinal microglia (Dai et al. 2010, Iezzi et al. 2012). In this study, I applied dendrimer as a TA carrier to deliver drugs to microglia specifically. As shown in data using florescence conjugated dendrimer, dendrimer was preferred to transfer to microglia rather than other cells in the spinal cord. FACS analysis also showed a 57% FITC signal, dendrimer conjugated fluorescence, in $CD11b^{+}$cells. This data suggest that dendrimer are very specifically uptaken within microglial cells (Fig. 2).

It is well known that the roles of microglia in CNS are similar to activated macrophages in the peripheral system. They enlarge, migrate, proliferate and produce proinflammatory cytokines that are involved in pain induction. In this study, D-TA reduced proinflammatory cytokines induction such as IL-1β, TNFα and IL-6 mRNA expression triggered by LTA in vitro and PNI in vivo (Fig. 3 and 5). In particular, data also showed that D-TA administration inhibits SNT induced microglial activation in the spinal cord (Fig. 4).

These results indicate that D-TA has anti-inflammatory effects in a nerve injury model. Then I prepared a mice behavior test to assess whether D-TA reduced pain behavior. I observed that the treatment of mice with intrathecal D-TA injection immediately after the PNI caused a significant decreased in the mechanical

allodynia (Fig. 6A). However, there was slightly decreased pain sensation in mice administrated with D-TA on day 1 and day 3 after injury (Fig. 6B-C).

Clinically, it was reported that patients who have poor results after lumbar spine surgery undergo chronic pain (Fager et al. 1980). Spinal cord microglia are rapidly activated in response to nerve injury and have important roles in developing chronic pain (Austin et al. 2010). Because my behavior data showed that D-TA works effectively when injected immediately after a nerve injury by modulating early microglia activation, this treatment may be helpful in predicting postoperative pain.

Although my study revealed that D-TA reduced pain sensations by inhibiting microglia through specific targeting, it is still not clear what the delivery mechanism is to the microglia. It was suggested that dendrimer enter the cell by clathrin-mediated endocytosis pathways (Wang et al. 2015). Many molecules are internalized by clathrin mediated endocytosis in microglia (Liu et al. 2007, Luther et al. 2013). It may enable D-TA internalization to microglial cells through clathrin dependent pathway.

In conclusion, I found that dendrimer deliver TA to spinal cord microglia specifically and attenuate neuropathic pain after PNI by inhibiting spinal cord microglia activation. It is suggested that D-TA can be a treatment for neuropathic pain.

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VI. 국문초록

신경병증성 통증은 말초신경 손상에 의한 감각세포의 데미지에 의해 발생하는 이질통증및 통각과민을 동반한 질병을 의미한다. 이러한 말초신경 손상에 의한 신경병증성 통증 발병 및 유지에는 척수조직 내에 존재하는 소교세포의 활성화가 매우 중요한 역할을 한다. 선행 연구결과를 통해 트리암시놀론 아세토니드가 소교세포의 활성화를 조절한다는 역할이 보고된 바 있다. 그러나 트리암시놀론 아세토니드는 다양한 부작용이 존재하여 임상적으로 적용이 어려운 단점이 있다. 이러한 문제를 해결하고자 소교세포 특이적으로 약물을 전달하기위해 폴리아미도아민 덴드리머에 결합된 트리암시놀론 아세토니드(D-TA)를 고안하였다. 폴리아미도아민 덴드리머는 구모양의 나노물질로 약물의 전달체의 역할을 수행한다. 본 연구를 통하여, 폴리아미도아민 덴드리머가 척수강내 주사를 통해 척수소교세포에 특이적으로 위치한다는 것을 확인하였다. D-TA 를 척수강 주사하였을 때, 말초신경 손상에의한 소교세포의 활성이 억제되는 것을 확인하였다. 또한 D-TA 투여에 의하여 신경손상 후 증가되었던 Nox2, IL-1b, TNFa 및 IL-6 의 mRNA 수준이 유의미하게 감소하는 것을 확인하였다. 뿐만아니라 D-TA 척수강내 주사에 의하여 신경손상에 의한 통증이 경감되는 것을 확인하였다. 이를 통해, D-TA 가 척수소교세포의 활성을 억제함으로써

신경손상성 통증을 억제할 수 있음을 보여주었고, 이는 신경병증성 통증의 잠재적인 치료책으로 활용할 수 있다.

주요어: 덴드리머-트리암시놀론 아세토니드, 신경병증성 통증, 말초 신경 손상, 척수 소교세포, 염증성 사이토카인

이학석사 학위논문

Polyamidoamine dendrimer-conjugated triamcinolone acetonide attenuates nerve injury-induced mechanical allodynia by inhibiting spinal cord microglia activation

폴리아미도아민 덴드리머 결합 트라이암시놀론

아세토나이드의 소교세포 활성 저해를 통한

신경손상에 의한 이질 통증 완화

2016 년 2 월

서울대학교 대학원

협동과정 뇌과학전공

김 휘 성

ABSTRACT

Polyamidoamine dendrimer-conjugated triamcinolone acetonide attenuates nerve injury-induced mechanical allodynia by inhibiting spinal cord microglia activation

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Neuropathic pain is a pathological pain with allodynia and hyperalgesia that is caused by sensory neuron damage such as peripheral nerve injury (PNI). The activation of spinal cord microglia is critical for the development and maintenance of neuropathic pain after PNI. Previous study showed that triamcinolone acetonide (TA) inhibits microglia activation. However, TA has a limitation in clinical application due to its off-target side effects. To obviate this problem, I developed polyamidoamine (PAMAM) dendrimer-conjugated TA (D-TA), which supposedly delivers TA specifically into microglia. PAMAM dendrimer is a sphere-shape nano-

molecule. In this study, I show that PAMAM-dendrimer is delivered selectively into spinal cord microglia. Intratheal D-TA injection inhibited nerve injury-induced spinal cord microglia activation. D-TA administration reduced mRNA expression of proinflammatory cytokines, such as Nox2, IL-1β, TNFα, and IL-6 in spinal cord after PNI. In addition, D-TA administration significantly attenuated PNI-induced mechanical allodynia. Conclusively, my data demonstrate that D-TA attenuates neuropathic pain after PNI by inhibiting spinal cord microglia activation, suggesting a therapeutic implication for the treatment of neuropathic pain.

Key Words:

Dendrimer-Triamconolone acetonide, neuropathic pain, peripheral nerve injury, spinal cord microglia, proinflammatory cytrokines

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CONTENTS

I. Introduction

Neuropathic pain is pathological chronic pain caused by damage in the nervous system. It evokes dysfunctional pain including allodynia, which is evoked by stimuli that normally do not cause pain sensation, and hyperalgesia enhanced pain sensation caused by noxious stimuli. Traditionally, neuropathic pain was thought to come from the hyperexcitability of sensory neuron caused by a lesion of neurons (Woolf 2010). However, previously available drugs targeting neurons are ineffective in controlling such pain.

Recently, it has been recognized that glial cells are important modulators of neuropathic pain and could be a new target for pain treatment (Watkins et al. 2003). Over the past decade, it has been reported that microglia play important roles in neuropathic pain development and the maintenance. After a peripheral nerve injury, spinal cord microglial cells are activated and lead to the production of proinflammatory cytokines such as IL-1β, TNFα and IL-6 ((Kawasaki et al. 2008). These cytokines' expression is correlated with a pain behavior in response to nerve injury. After microglia activation, it transforms to an ameboid morphology which is mediated by transmembrane receptors, such as fractalkine receptor CX3CR1 and an ATP receptor P2X4 (Tsuda et al. 2003, Verge et al. 2004). It was also proposed that the toll-like receptors 2, 3 and 4 on microglia are involved in nerve injury

induced microglial activation in the spinal cord (Tanga et al. 2005, Kim et al. 2007, Obata et al. 2008)).

Corticosteroid treatments for neuropathic pain have been clinically used and much studied (Kingery et al. 2001, Lee et al. 2010). Localized corticoid triamcinolone acetonide injections decreased DRG satellite glia and spinal cord microglia activation in a rat spinal nerve ligation model (Li et al. 2011). A previous study showed that Triamcinolone acetonide (TA) inhibits microglia activation by blocking the NF-κB signaling pathway (Hong et al. 2012). However, corticosteroid drugs including TA have limitations in clinical application due to their off-target side effects. For decades, the neurotoxic side effects of corticosteroid treatments have been discussed (Koide et al. 1986, Sapolsky et al. 1990). Although some studies demonstrated the safety of intrathecal TA administration, serious side effects which may occur from neurotoxicits, such as arachnoiditis, meningitis, and paralysis still existed. Polyamidoamine (PAMAM) dendrimer is a synthetic polymer with a sphere-like shape in nanoscale size. It is used as a drug delivery vector, and its functional characteristics vary by generation number or terminal application (Dai et al. 2010). Recently it was reported that dendrimers were localized within microglia in rabbits with cerebral palsy by subarachnoid administration (Dai et al. 2010). It is also determined that PAMAM dendrimers selectively delivered drugs to activated retinal microglia in retinal degeneration rat models (Iezzi et al. 2012).

These current studies reached the conclusion that PAMAM dendrimer could be an effective drug carrier for TA. In the present study, I used an L5 peripheral nerve injury (PNI) mouse model to explore the PAMAM dendrimer-conjugated TA (D-TA) effect for neuropathic pain. I found that dendrimer specifically localized in spinal cord microglia and D-TA attenuates neuropathic pain after PNI by inhibiting spinal cord microglia activation.

II. Materials and Methods

Animals

All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University. The animal treatments were performed in accordance with the guidelines of the International Association for the Study of Pain. Experiments were carried out in male C57BL/6 mice aged 8-12 weeks. Mice were housed in plastic cages and were allowed to access food and water *ad libitum*. They were under a 12-h dark/light cycle and a constant room temperature of 23±2℃.

Neuropathic pain model and intrathecal injection

Mice were anaesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium and peripheral nerve injury was induced by transecting the L5 spinal nerve (SNT) as descried previously (Kim et al., 2010). A surgical incision was made to the skin and paraspinal muscles were separated. Then the L6 transverse process was partially removed and L5 spinal nerve was transected carefully. The wound was closed with surgical skin staples.

For the administration of D-Rho, D-FITC or D-TA mice were injected under pentobarbital sodium anesthesia (25 mg/kg). Using a 10 μl Hamilton syringe

(Hamilton Company, Reno, NV, USA) with a 30-gauge one-half-inch needle, D-TA (10 μl of 1 μg/μl in Saline) or Saline alone was intrathecally injected in C57BL/6 mice.

Cell culture

Primary mouse brain mixed glial cells were prepared from one-day-old C57BL/6 mice as previously established procedures (Lee et al., 2000). Briefly, after anesthetiszed, meninges were removed from the cerebral hemisphere, tissue was dissociated into a single-cell suspension by gentle repetitive pipetting. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 mM HEPES, 10% FBS, 2 mM L-glutamie, 1X NEAA and 1X antibiotic/antimycotic in 75 cm^2 flasks at 37℃ in a 5% CO_2 incubator, and the medium was changed every 5 days.

Real-time RT-PCR

Real-time RT-PCR was performed using the SYBR Green PCR Master Mix and an ABI Prism 7500 sequences detection system (Applied Biosystems, Foster City, CA) as described previously (Kim et al., 2010). The following PCR primer sequences were used: GAPDH (forward), 5'- AGG TCA TCC CAG AGC TGA ACG-3'; GAPDH reverse, 5'-CAC CCT GTT GCT GTA GCC GTA-3'; Nox2 forward, 5'-GAC CCA GAT GCA GGA AAG GAA-3' ; Nox2 reverse, TCA TGG TGC ACA GCA AAG TGA-3'; IL1-β forward, 5'-GTG CTG TCG GAC CCA

TAT GA-3'; IL1-β reverse, 5'-TTG TCG TTG CTT GGT TCT CC ; TNFα forward, 5'-AGC AAA CCA CCA AGT GGA GGA-3'; TNFα reverse, 5'-GCT GGC ACC ACT AGT TGG TTG-3'; IL-6 forward, 5'-CCA CGA TTT CCC AGA GAA CAT-3'; IL-6 reverse, 5'-TCC ATC CAG TTG CCT TCT TGG-3'. The mRNA level of each gene was normalized to the mRNA levels of GAPDH gene and represented as a fold induction. The fold induction was calculated using the $2^{-\Delta\Delta CT}$ method, as previously descried (Livak and Schmittgen, 2001). All real-time RT-PCR experiments were performed at least three times, and are the mean ± SEM values have been presented unless otherwise noted.

FACS analysis

The mice were deeply anesthetized by pentobarbital sodium and intracardially perfused with ice-cold saline. Lumbar 1 to sacral 3 spinal cord were extracted and homogenized mechanically to a single cell-suspension. Cells were washed with ice-cold 2% fetal bovine serum (FBS) in PBS, and incubated with Fc Blocker™ (BD Bioscience, San Jose, CA) for 10 min at 4℃ prior to staining with CD11b-APC (Biolegend Inc, San Diego, CA). BD FACSCalibur flow cytometer (BD Bioscience) was used to measure the microglia as $CD11b^+$ and non-microglia as $CD11b^-$. Data were acquired with analyzed with BD CellQuest ™system (BD Biosciences).

Immunohistochemistry

Male mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4-5 spinal cord was removed and postfixed in 4% PFA at 4℃ overnight, and transferred to 30% sucrose in PBS for 48 hr. Spinal cord were transected in 30-μm-thick and and prepared on gelatin-coated slide glass using a cryocut microtome. The sections were blocked in solution containing 5% normal donkey serum (Jackson ImmunoResearch, Bar Harbor, ME, USA), 2% BSA (Sigma, St. Louis, MO, USA) and 0.1% Triton X-100 (Sigma) for 1 hr at room temperature. The sections were then incubated overnight at 4℃ with primary antibody for rabbit-anti-Iba-1 (1:1000, Wako, Osaka, Japan) and mouse-anti-GFAP (1:5000, Millipore, MA, USA), rabbit-anti-MAP2 (1:400, Millipore, MA, USA). After rinsing in 0.1 M PBS, the sections were incubated for 1-h at room temperature with a mixture of Cy3- or FITC-conjugated secondary antibodies (1:200, Jackson ImmunoResearch). The sections were mounted, and fluorescent images were obtained using a confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

Behavioral testing

All mice were allowed to acclimate to the testing apparatus and environment twice for 2 hr before testing. Each mouse was placed alone in a plexiglass cage upon elevated mesh in order to access the paws for observation. On each test day, mice were briefly habituated to the test environment for 30 minutes. Baseline thresholds were obtained 2 days before surgical treatment. Mechanical allodynia was assessed by measuring foot withdrawal thresholds in response to mechanical stimuli to the hind paw. All behavior experiments were performed using the up-down method (Chaplan et al.,1994) with von Frey filaments. Mechanical sensitivity was calculated by mean threshold.

Statistical analysis

Statistically differences between two groups were determined by two tailed Student's *t-test*. Differences among multiple groups were made using one-way ANOVA followed by Bonferroni correction. All data are resented as the mean ± SEM, and differences were considered significant when the P-value was less than 0.05.

III. Results

Dendrimer specifically localize at spinal cord microglia.

Previous research examined dendrimer localize in microglial cells (Dai et al. 2010, Iezzi et al. 2012). Using the Rhodamine labeled dendrimer (D-Rho), I tested synthesized dendrimer localized in microglia from a mouse brain. Pure microglial cell cultures were treated with D-Rho for 1 hr and were fixed with 4% PFA. I confirmed that a dendrimer fluorescence signal is detected in microglia regions (Fig.1). To identify the dendrimer specifically localize at microglia cells, I injected D-Rho mice intrathecally. Three days after injection, a rhodamine signal was detected in the lumbar spinal cord. And these signals were co-localized with Iba1 positive cells, which was expressed in microglial cells. However, neither GFAP positive cells, astroglial cells, nor MAP2 positive cells, neuronal cells, were merged with the rhodamine signal (Fig.2). To quantify the rate of dendrimer localized microglia, from lumbar 1 to sacral 3 region spinal cord was measured by flow cytometry at 3 days after dendrimer conjugated FITC intrathecal injection. I confirmed that the FITC fluorescence signal about 57% of the CD11b positive cells. Taken together, these data show dendrimer delivery to microglia specifically *in vivo.*

Synthesis of dendrimer-TA conjugate, D-TA

D-TA was synthesized using a two-step process with glutaric acid as spacer

as shown in the synthetic scheme (Scheme 1). Hydroxy terminal PAMAM Generation 4.0 dendrimer (PAM-G4-OH) is relatively lower cytotoxicity compared to the cationic dendrimers due to the absence of surface primary amines. A glutaric acid spacer was used to reduce the steric hindrance and to enable better drug release ((Khandare et al. 2005, Perumal et al. 2009). The first step involved reaction of glutaric anhydride to triamcinolone acetonide (TA) resulting in a carboxylic acid derivate, which is further reacted with PAM-G4-OH through a coupling reaction to get the second-step conjugate, D-TA. TA was performed with glutaric anhydride dissolved in anhydrous DMF/DMA (80:20) in presence of triethanolamine (TEA) to get acid derivatives of TA. TA-glutarate was characterized by ^{1}H NMR(not shown) and the molecular weight was determined by LC/MS. TA-glutarate was further conjugated with the hydroxyl group of PAMAM G4 dendrimer (PAM-G4-OH) using HOBt, HBTU as coupling reagent and DIPEA as base to get final conjugate in anhydrous DMF. The synthesis of the D-TA was confirmed from ^{1}H NMR spectra (Bruker DPX-400 NMR spectrometer, DMF-d_7) with the conjugation of TA-glutarate. In ^{1}H NMR spectrum, peaks in between 0.9 and 1.8ppm represent methyl protons, and peaks in between 4.6 and 7.4 ppm represent aromatic protons of TA along with PAM-G4-OH peaks confirms the formation of the ester bond. D-TA conjugate was evaluated by NMR data indicates that 5 molecules were reacted per molecule of PAM-G4-OH (Iezzi et al. 2012). D-TA was provided from School of Chemistry and Molecular Engineering, Seoul National University.

D-TA attenuates LTA-induced proinflammatory cytokines in mouse brain mixed glial cells.

Previous studies have reported that proinflammatory genes TNFα, IL-1β and IL-6 were induced in activated glial cells and that they were down regulated by TA treatment (Kawasaki et al. 2008, Zheng et al. 2011, Hong et al. 2012). I tested TA downregulates of these gene expressions when conjugated with dendrimer. To investigate this, I cultured primary mixed glial cells from a mouse brain. Primary mixed glial cells were pretreated with DTA for 1hr then stimulated by LTA, microglia activating molecule, for 3 hr. Upon stimulation with LTA, the mRNA expression of IL-1β, TNFα and IL-6 genes upregulated about 115-, 70- and 75- fold, respectively. The gene induction by LTA, however, was downregulated by treatment DTA in a dose-dependent manner (Fig. 3). These data indicate that D-TA inhibits proinflammatory glial cell activation.

Intrathecal administration of D-TA reduces nerve injury induced morphological activation in spinal cord microglia.

Microglia morphological activation has been documented in the spinal cord after peripheral nerve injury. Here I tested how D-TA inhibit nerve injury induced microglial morphological change. Directly after L5 spinal nerve transection, D-TA was administrated by intrathecal. At 3 days after injury, I extracted L4-5 spinal cord

tissue and immunostained it with cell type-specific antibodies such as Iba1 and GFAP. The Iba-1 immunoreactive signals were strongly activated in the ipsilateral dorsal horn. However, the activation of microglia was significantly decreased in the D-TA treated mice, not the vehicle treated mice (Fig.4). These data demonstrate that D-TA attenuates nerve injury induced spinal cord microglia activation.

D-TA suppresses cytokines at spinal cord in neuropathic pain model.

It is well known that the pro-inflammatory cytokines such as IL-1β and TNFα are involved in spinal cord glial cells by peripheral nerve injury in the neuropathic pain model (Arruda et al. 1998, Sweitzer et al. 2001, Zheng et al. 2011)). These pro-inflammatory cytokines are produced in activated microglial cells in CNS (Smith et al. 2012). Upon spinal cord transection, the mRNA expression of IL-1β, TNFα, Nox2 and IL-6 genes was upregulated by 2.3-, 2.6-, 2.2- and 5.0-fold, respectively. The gene induction by SNT, however, was decreased to almost 1.2-, 1.3-, 1.5- and 1.7- fold, respectively in D-TA treated mice (Fig 5).

Mechanical allodynia suppressed by DTA intrathecal injection.

In order to test whether D-TA inhibits nerve injury-induced microglia activation, its role in attenuating pain was tested. To confirm this, I tested the susceptibility of the D-TA treated mice to nerve injury-induced pain hypersensitivity. Upon L5 spinal nerve transection, wild type mice showed

increased sensitivity to mechanical stimuli. The paw withdrawal threshold to the mechanical stimuli decreased from 1 day to 7 days after nerve injury. The threshold remained below 0.1 g on day 7. The paw withdrawal threshold of the D-TA treated mice, however, significantly reduced on day 1 and 3 compared to the vehicle treated group (Fig 6).

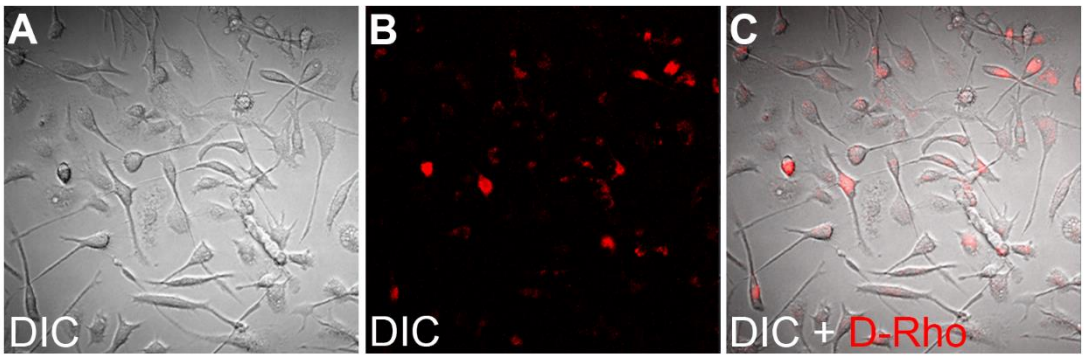

A
DIC
B
DIC
C
DIC + D-Rho

Figure 1. Localization of dendrimer-Rho within the microglial cells in vitro.

Microglial cells prepared from mice brain were seed to PDL coated cover slide. 4 days after, mixed glial cells were treated with Rhodamine conjugated dendrimer for 1-h. Cells were mounted and rhodamine signal detected under confocal microscopy. (A) DIC image of pure microglial cells from mouse brain mixed glial cells. (B) Rhodamine conjugated dendrimer were detected by red signals. (C) Dendrimer-Rho co-localized with microglial cells.

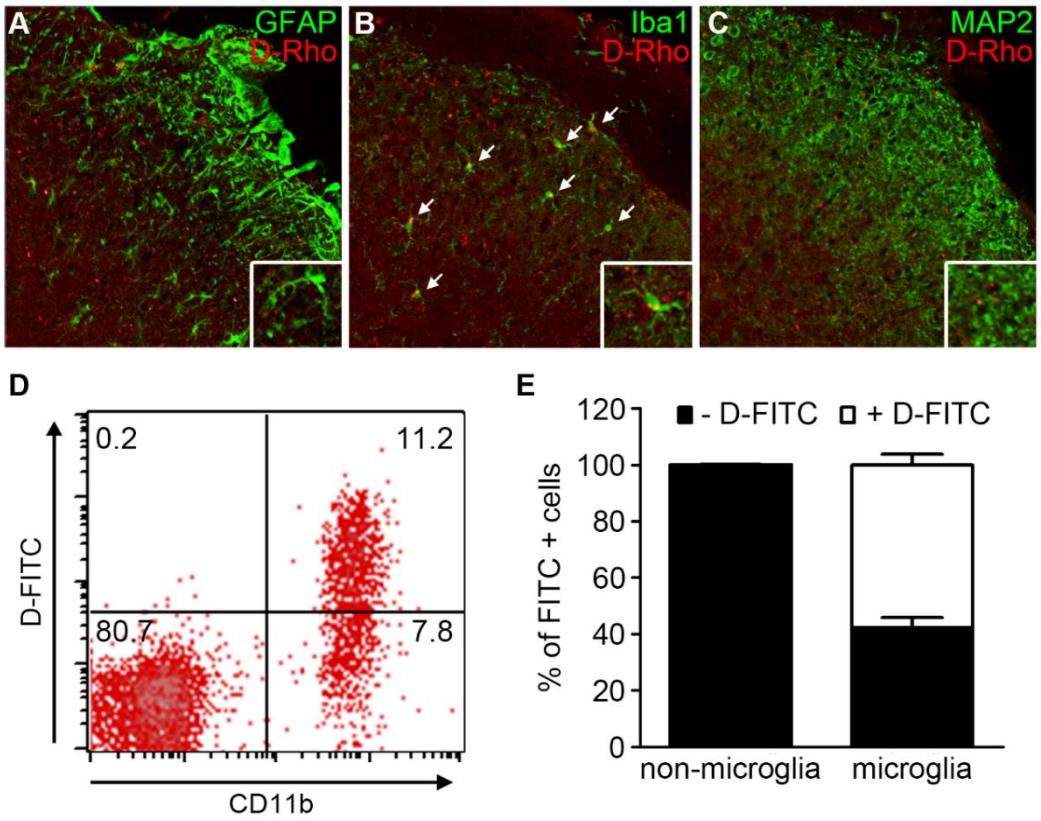
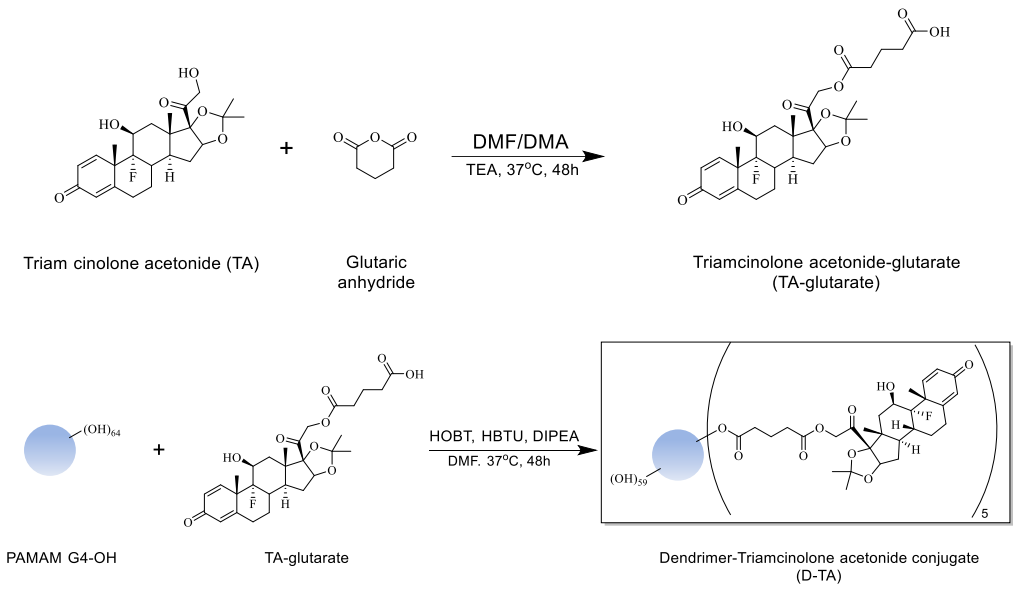

A
GFAP
D-Rho
B
Iba1
D-Rho
C
MAP2
D-Rho
D
0.2
11.2
80.7
7.8
D-FITC
CD11b
E
- D-FITC
+ D-FITC
% of FITC + cells
120
100
80
60
40
20
0
non-microglia
microglia

Figure 2. Microglia-specific delivery of dendrimer in vivo.

(A-C) Mice were received with 5 μg/μl of D-Rho by intrathecal injection. 3 days after, L4-5 spinal cord were obtained and sections were prepared. Each section was stained with GFAP, Iba1 and MAP2. Dendrimer conjugated rhodamine were detected in Iba1 positive cells at 3 days after intrathecal injection (arrows). (D and E) 3 days after D-Rho administration. Cells were isolated from Lumbar 1 to sacral 3 and stained with anti-CD11b-APC. CD11b positive cells were detected by FACScalliber^{TM}. Representative dot plots were showed in (D) and graph were showed in (E). Microglia were defined as CD11b.



Scheme 1. Synthesis of D-TA conjugate

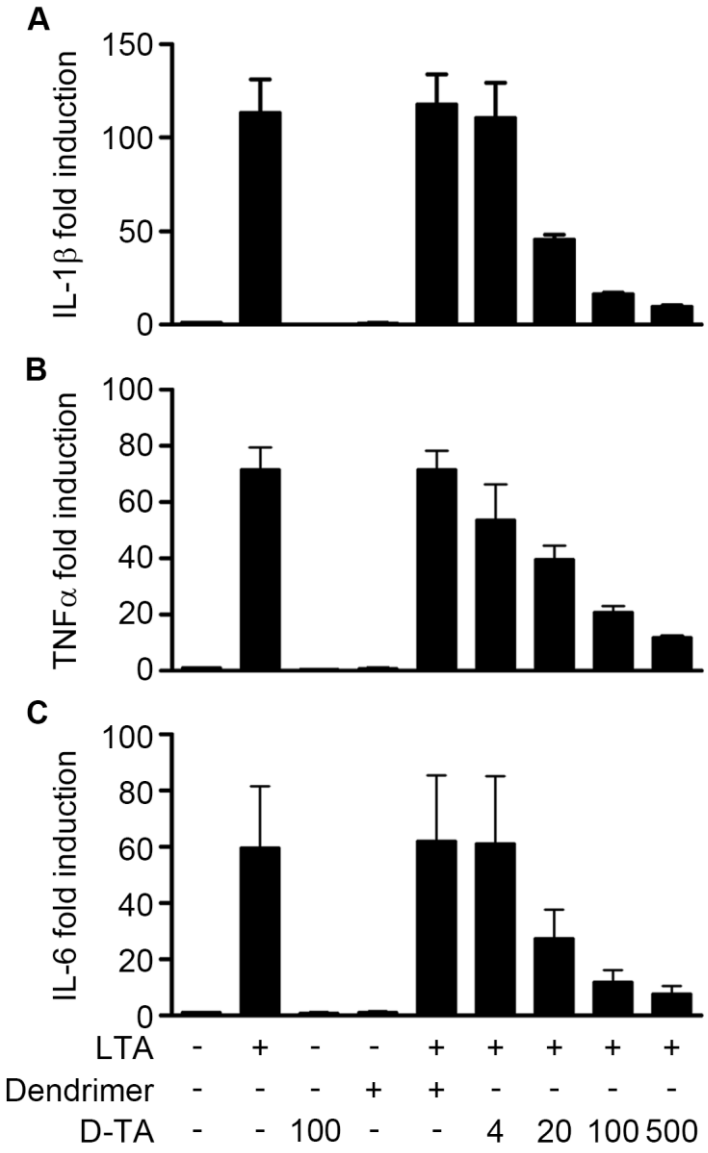

A
IL-1β fold induction
150
100
50
0
B
TNFα fold induction
100
80
60
40
20
0
C
IL-6 fold induction
100
80
60
40
20
0
LTA - + - - + + + + +
Dendrimer - - - + + - - - -
D-TA - - 100 - - 4 20 100 500

Figure 3. D-TA attenuates proinflammatory cytokines in mouse brain mixed glial cells.

Primary glial cells (5 x 10^5 cells/well) were incubated with D-TA in culture for 1 hr, and then exposed to LTA for 3 hrs. IL-1β (A), TNFα (B) and IL-6 (C) expressions were measured by real-time RT-PCR.

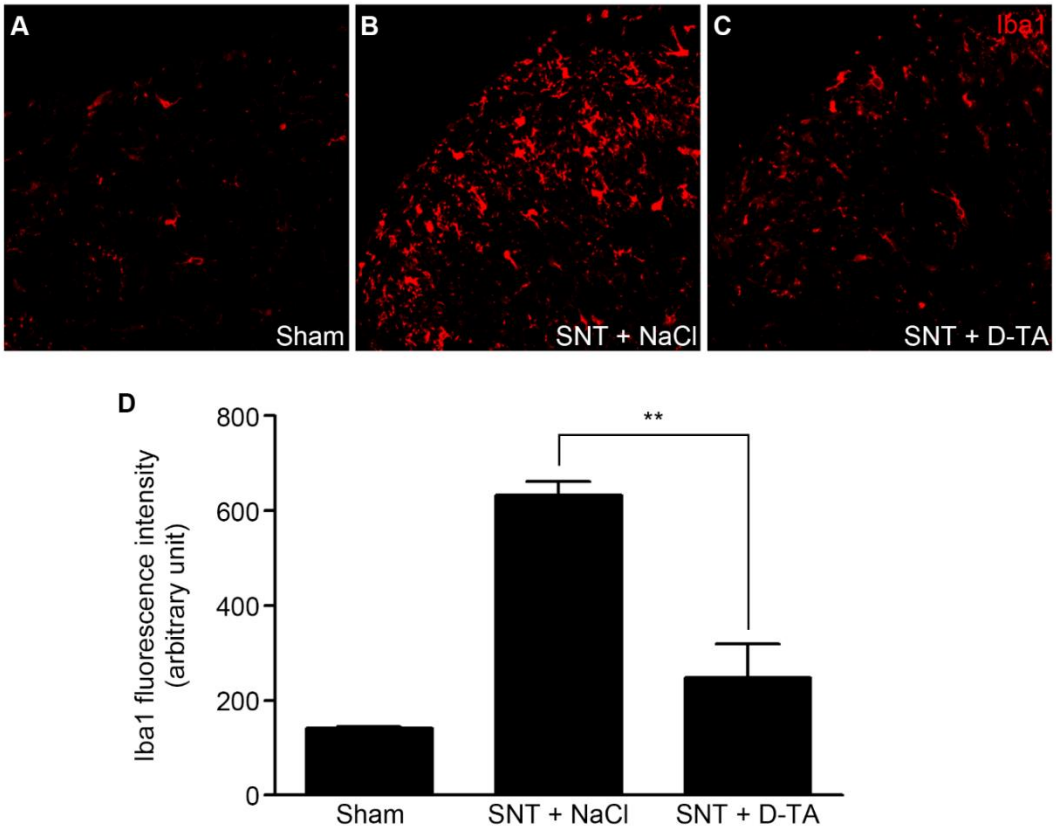

A
Sham
B
SNT + NaCl
C
Iba1
SNT + D-TA
D
Iba1 fluorescence intensity
(arbitrary unit)
800
600
400
200
0
**
Sham
SNT + NaCl
SNT + D-TA

Figure 4. D-TA reduced morphological activation in spinal cord microglia.

Mice were received with 5 μg/μl of D-TA by intrathecal injection right after SNT. After 3 days, L4-5 spinal cord were obtained and sections were prepared. Each section was stained with Iba1. (A) Iba1 was barely detectable in sham injury mouse spinal cord. (B) Spinal cord microglial activation measured by Iba1 staining at 3 days after SNT. (C) D-TA administration reduced microglial activation induced by SNT. (D) Quantitative summary of microglial activation data exemplified in (A-C).

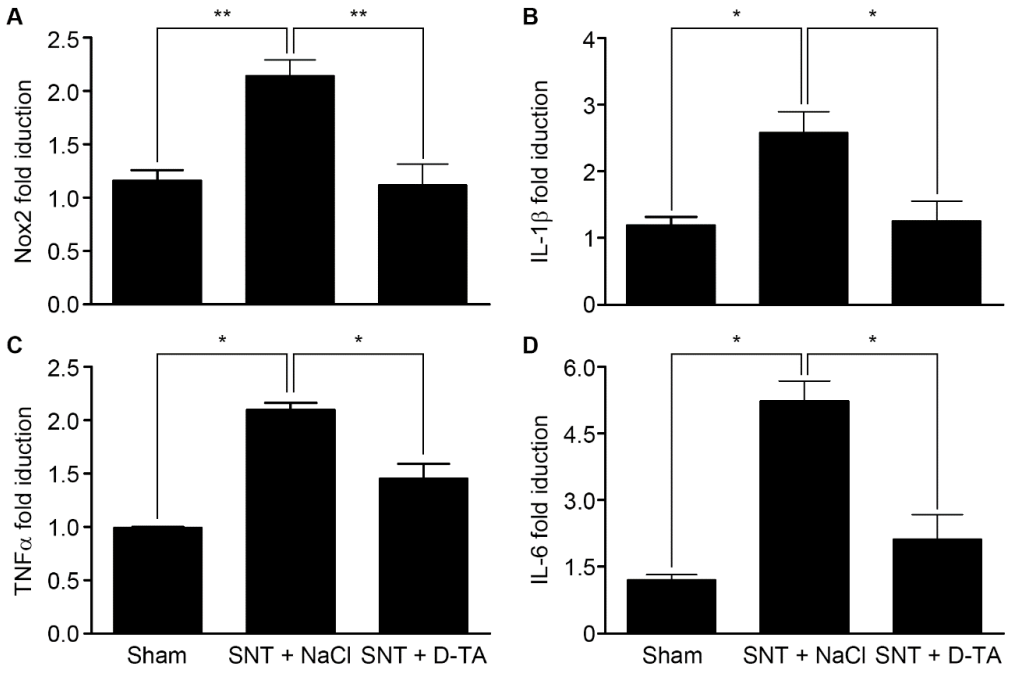

A
B
C
D
Nox2 fold iduction
IL-1β fold iduction
TNFα fold iduction
IL-6 fold iduction
**
**
*
*
*
*
*
*
2.5
2.0
1.5
1.0
0.5
0.0
4
3
2
1
0
6.0
4.5
3.0
1.5
Sham
SNT + NaCl
SNT + D-TA

Figure 5. Proinflammatory cytokines mRNA expression in L4-5 spinal cord at 3 days after L5 SNT.

Mice were received with 5 μg/μl of D-TA by intrathecal injection right after SNT. After 3 days, L4-5 spinal cord were prepared for total RNA isolation and cDNAs were prepared. Then cDNAs were used to measure the mRNA expression of Nox2 (A), IL-1β (B), TNFα (C), and IL-6 (D) by real-time RT-PCR.

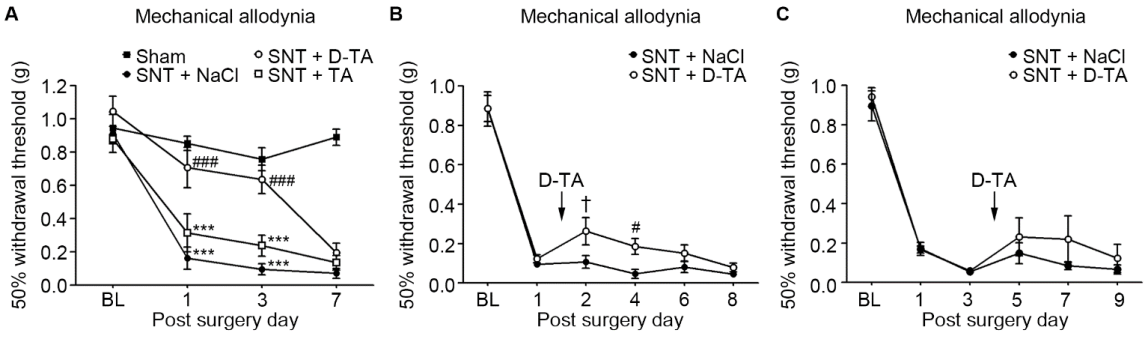

A
Mechanical allodynia
Sham
SNT + NaCl
SNT + D-TA
SNT + TA
50% withdrawal threshold (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
###
###
***
***
***
***
BL
1
3
7
Post surgery day
B
Mechanical allodynia
SNT + NaCl
SNT + D-TA
50% withdrawal threshold (g)
1.0
0.8
0.6
0.4
0.2
0.0
D-TA
†
#
BL
1
2
4
6
8
Post surgery day
C
Mechanical allodynia
SNT + NaCl
SNT + D-TA
50% withdrawal threshold (g)
1.0
0.8
0.6
0.4
0.2
0.0
D-TA
BL
1
3
5
7
9
Post surgery day

Figure 6. Changes in the ipsilateral 50% mechanical threshold of D-TA i.t injection after L5 SNT.

Effect of D-TA administration pain behavior induced by spinal nerve transection. D-TA injected immediately (A), 1 day (B), and 3 days (C) after surgery.

IV. Discussion

There are various drugs for the treatment of chronic pain. Traditionally, opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) were used to control pain. However, their effects are limited and they cause side effects. Opioids tend to be less effective or require higher doses in controlling pain. Also, they have side effects such as cognitive impairment, drowsiness and nausea. NSAIDs, another typical type of medicine used for the treatment of pain, have negative effects on the kidneys and gastrointestinal system (Bhatt et al. 2008, Chou et al. 2015). Additionally, these medications cannot treat chronic pain completely. Therefore, a new strategy is needed for neuropathic pain treatment.

Several studies revealed that glial cells activation in the spinal cord is important in the development and maintenance of neuropathic pain and this suggests that it could be a new target for controlling neuropathic pain (Watkins et al. 2003). Because microglia contribute to neuropathic pain by inflammatory response after a nerve injury, it is expected that some steroid anti-inflammatory drugs will be able to reduce neuropathic pain caused by microglia activation. The anti-inflammatory effect of TA has been previously tested in glial cells treated with necrotic neuronal cells (NNC). TLR-mediated proinflammatory genes are expressed through the NF-κB signaling pathway. TA inhibits NF-κB reporter activity in NNC-activated microglial cells. Because steroid drugs influence a wide range of tissues and cells,

however, the studies developing steroids as analgesic drugs, focused on decreasing their side effects.

Therapeutic methods targeting glial cells are not well developed. A recent study showed that polyamaidoamine (PAMAM) dendrimer is uptaken within retinal microglia (Dai et al. 2010, Iezzi et al. 2012). In this study, I applied dendrimer as a TA carrier to deliver drugs to microglia specifically. As shown in data using florescence conjugated dendrimer, dendrimer was preferred to transfer to microglia rather than other cells in the spinal cord. FACS analysis also showed a 57% FITC signal, dendrimer conjugated fluorescence, in $CD11b^{+}$cells. This data suggest that dendrimer are very specifically uptaken within microglial cells (Fig. 2).

It is well known that the roles of microglia in CNS are similar to activated macrophages in the peripheral system. They enlarge, migrate, proliferate and produce proinflammatory cytokines that are involved in pain induction. In this study, D-TA reduced proinflammatory cytokines induction such as IL-1β, TNFα and IL-6 mRNA expression triggered by LTA in vitro and PNI in vivo (Fig. 3 and 5). In particular, data also showed that D-TA administration inhibits SNT induced microglial activation in the spinal cord (Fig. 4).

These results indicate that D-TA has anti-inflammatory effects in a nerve injury model. Then I prepared a mice behavior test to assess whether D-TA reduced pain behavior. I observed that the treatment of mice with intrathecal D-TA injection immediately after the PNI caused a significant decreased in the mechanical

allodynia (Fig. 6A). However, there was slightly decreased pain sensation in mice administrated with D-TA on day 1 and day 3 after injury (Fig. 6B-C).

Clinically, it was reported that patients who have poor results after lumbar spine surgery undergo chronic pain (Fager et al. 1980). Spinal cord microglia are rapidly activated in response to nerve injury and have important roles in developing chronic pain (Austin et al. 2010). Because my behavior data showed that D-TA works effectively when injected immediately after a nerve injury by modulating early microglia activation, this treatment may be helpful in predicting postoperative pain.

Although my study revealed that D-TA reduced pain sensations by inhibiting microglia through specific targeting, it is still not clear what the delivery mechanism is to the microglia. It was suggested that dendrimer enter the cell by clathrin-mediated endocytosis pathways (Wang et al. 2015). Many molecules are internalized by clathrin mediated endocytosis in microglia (Liu et al. 2007, Luther et al. 2013). It may enable D-TA internalization to microglial cells through clathrin dependent pathway.

In conclusion, I found that dendrimer deliver TA to spinal cord microglia specifically and attenuate neuropathic pain after PNI by inhibiting spinal cord microglia activation. It is suggested that D-TA can be a treatment for neuropathic pain.

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VI. 국문초록

신경병증성 통증은 말초신경 손상에 의한 감각세포의 데미지에 의해 발생하는 이질통증및 통각과민을 동반한 질병을 의미한다. 이러한 말초신경 손상에 의한 신경병증성 통증 발병 및 유지에는 척수조직 내에 존재하는 소교세포의 활성화가 매우 중요한 역할을 한다. 선행 연구결과를 통해 트리암시놀론 아세토니드가 소교세포의 활성화를 조절한다는 역할이 보고된 바 있다. 그러나 트리암시놀론 아세토니드는 다양한 부작용이 존재하여 임상적으로 적용이 어려운 단점이 있다. 이러한 문제를 해결하고자 소교세포 특이적으로 약물을 전달하기위해 폴리아미도아민 덴드리머에 결합된 트리암시놀론 아세토니드(D-TA)를 고안하였다. 폴리아미도아민 덴드리머는 구모양의 나노물질로 약물의 전달체의 역할을 수행한다. 본 연구를 통하여, 폴리아미도아민 덴드리머가 척수강내 주사를 통해 척수소교세포에 특이적으로 위치한다는 것을 확인하였다. D-TA 를 척수강 주사하였을 때, 말초신경 손상에의한 소교세포의 활성이 억제되는 것을 확인하였다. 또한 D-TA 투여에 의하여 신경손상 후 증가되었던 Nox2, IL-1b, TNFa 및 IL-6 의 mRNA 수준이 유의미하게 감소하는 것을 확인하였다. 뿐만아니라 D-TA 척수강내 주사에 의하여 신경손상에 의한 통증이 경감되는 것을 확인하였다. 이를 통해, D-TA 가 척수소교세포의 활성을 억제함으로써

신경손상성 통증을 억제할 수 있음을 보여주었고, 이는 신경병증성 통증의 잠재적인 치료책으로 활용할 수 있다.

주요어: 덴드리머-트리암시놀론 아세토니드, 신경병증성 통증, 말초 신경 손상, 척수 소교세포, 염증성 사이토카인