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Expression of β-1,4-Galactosyltransferase I in Rat Schwann Cells

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ABSTRACT

Glycosylation is one of the most important post-translational modifications. It is clear that the single step of β-1,4-galactosylation is performed by a family of β-1,4-galactosyltransferases (β-1,4-GalTs), and that each member of this family may play a distinct role in different tissues and cells. In the present study, real-time PCR revealed that the β-1,4-GalT I mRNA reached peaks at 2 weeks after sciatic nerve crush and 3 days after sciatic nerve transection. Combined in situ hybridization for β-1,4-GalT I mRNA and immunohistochemistry for S100 showed that β-1,4-GalT I mRNAs were mainly located in Schwann cells after sciatic nerve injury. In conclusion, β-1,4-GalT I might play important roles in Schwann cells during the regeneration and degeneration of the injured sciatic nerve. In other pathology, such as inflammation, we found that LPS administration affected β-1,4-GalT I mRNA expression in sciatic nerve in a time- and dose-dependent manner, and β-1,4-GalT I mRNA is expressed mainly in Schwann cells. These results indicated that β-1,4-GalT I plays an important role in the inflammation reaction induced by intraperitoneal injection of LPS. Similarly, we found that β-1,4-GalT I in Schwann cells in vitro was affected in a time- and concentration-dependent manner in response to LPS stimulation. All these results suggest that β-1,4-GalT I play an important role in Schwann cells in vivo and vitro during pathology. In addition, β-1,4-GalT I production was drastically suppressed by U0126 (ERK inhibitor), SB203580 (p38 inhibitor), or SP600125 (SAPK/JNK inhibitor), which indicated that Schwann cells which regulated β-1,4-GalT I expression after LPS stimulation were via ERK, SAPK/JNK, and P38 MAP kinase signal pathways. J. Cell. Biochem. 108: 75–86, 2009.

KEY WORDS: β-1,4-GALACTOSYLTRANSFERASE I; LPS; SCIATIC NERVE INJURY; REAL-TIME PCR; IN SITU HYBRIDIZATION; SCHWANN CELL; RAT

Illustration of the mechanisms involved in the regeneration of the peripheral nervous system may be a great help to cure peripheral nerve injuries and demyelinating diseases [Ide, 1996]. During the course of the development and regeneration of the mammalian nervous system, the changes of glycosylation in the neuronal surface have been observed by using lectins, anti-carbohydrate antibodies, and carbohydrate-specific toxins [Allendoerfer et al., 1999; Ronn et al., 2000]. These changes suggest that carbohydrates on the cell surface play important roles in the formation of neural tissues. β-1,4-galactosyltransferases (β-1,4-GalTs) [Shur, 1991] can catalyze the galactose from UDP-galactose to the terminal N-acetylglucosamine residues on elongating oligosaccharide chains, which are suggested to be involved in various cellular functions including cell–cell and cell–matrix interactions during embryogenesis [Varki, 1993]. β-1,4-galactosyltransferase I (β-1,4-GalT I) is the first mammalian glycosyltransferase cDNA to be cloned and the first mammalian glycosyltransferase to have its crystal structure resolved [Shaper et al., 1986]. Indirect immunofluorescence staining showed that β-1,4-GalT I localized at the growth cone during neurite outgrowth; blocking β-1,4-GalT I

Abbreviations used: β-1,4-GalTs, β-1,4-galactosyltransferases; Glcβ1,4NAc, galactose-β-1,4-*N*-acetylglucosamine; DIG, digoxigenin; β2-M, β2-microglobulin; HRP, horseradish peroxidase; RCA-I, Ricinus communis agglutinin-I; RT, room temperature; RT-PCR, reverse transcription polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; SD, Sprague–Dawley.

Huiguang Yang and Meijuan Yan contributed equally to this work.

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activity with its antibody or consuming its substrates in laminin by pregalactosylation inhibited neurite outgrowth and elongation from PC12 cells, sensory neurons, and chick dorsal root ganglia (DRG). Overexpression of β-1,4-GalT I in PC12 cells remarkably enhanced neurite formation and elongation [Huang et al., 1995]. All these results suggest that β-1,4 GalT I is involved in the neurite outgrowth. In the present study, we focused on β-1,4 GalT I to study its change in expression during the degeneration and regeneration in a crushed sciatic nerve.

Although some studies have attempted to characterize the function of β-1,4-GalT I in the nervous system, no consensus has been reached. The available data on the difference in expression of β-1,4-GalT I mRNA in peripheral nervous system injuries and cellular synthesis of β-1,4-GalT I mRNAs in the rat is very limited. Therefore, we undertook this study to identify the cellular expression changes of β-1,4-GalT I mRNAs in rats subjected to sciatic nerve injury. In this study, we established a sciatic nerve crush model and a sciatic nerve transection model, and examined the changes of expression levels of the β-1,4-GalT I mRNAs to explore the roles of β-1,4-GalT I during the process of regeneration and degeneration in injured sciatic nerve by using real-time PCR, in situ hybridization, and immunohistochemistry at different post-injury stages.

A variety of signaling pathways have been elucidated in the response to LPS [Diks et al., 2004]. The best described of which is the pathway leading to MAPKs activation. MAPKs are important mediators of cytokine expression. In the present study, specific inhibitors of MAPK subgroups were used to block MAPKs to investigate whether Schwann cells regulated β-1,4-GalT I expression after LPS stimulation was via ERK, SAPK/JNK, and P38 MAP kinase signal pathways.

MATERIALS AND METHODS

ANIMALS

Sprague–Dawley (SD) rats, weighing from 180 to 220 g, were provided by the Experimental Animal Center of Nantong University. All animals were kept under standardized laboratory conditions in an air-conditioned room with free access to food and water. All the animal tests were carried out in accordance with the US National Institute of Health (NIH) Guide for the Care and USE of Laboratory Animals published by the US National Academy of Sciences (http://oacu.od.nih.gov/regs/index.htm) and approved by the Administration Committee of Experimental Animals, Jiangsu Province, China.

CELL CULTURES

A rat Schwann cell line, RSC 96 (Cell Bank, Chinese Academy of Sciences) was grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 2 mM glutamine (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin with 10% fetal bovine serum (Hyclone) in 5% CO_2 at 37°C.

METHODS

All animals were randomly divided into three groups: the normal group (control group), the sciatic nerve crush group, and the sciatic nerve transection group. Inflammatory rats were intraperitoneally injected with 0.5–10 mg/kg LPS. The control group received no injury or LPS stimulation. Six rats were used at each time point after sciatic nerve injury per batch, six rats were used at every different LPS concentration or at different time point at the same concentration. All experiments were repeated at least three times and total three bathes rats were used.

SURGICAL PROCEDURE AND TREATMENTS

Animals of control and injury group were deeply anesthetized with a cocktail of xylazine (10 mg/kg), ketamine (95 mg/kg), and acepromazine (0.7 mg/kg) administered intraperitoneally. In the sciatic nerve crush group, the right sciatic nerves were crushed three consecutive times using the same forceps, the length of each crush time and the interval between each crush were 10 s. The sciatic nerves on the left side were left intact and thus used as internal controls. At the same time, the sciatic nerves of the control group were not operated on. In the sciatic nerve transection group, the right sciatic nerves at the region of mid-thigh were cut, a piece of 0.5 cm nerve tissue was removed. To establish LPS model, rats were intraperitoneally injected with 0.5–10 mg/kg of LPS (Sigma) or sterile normal sodium (NS) from 0.5 to 24 h. All animals were kept in an air-conditioned room with free access to food and water. On post-operative time 6 h, 12 h, 1 day, 2 days, 1 week, 2 weeks, and 4 weeks, the experimental rats were anesthetized with the above cocktail, and the chest chamber exposed. Rats were perfused transcardially with 250 ml normal saline followed by 250 ml of 4% polyfomaldehyde in 0.1 M phosphate buffer (pH 7.4), taking out the sciatic nerves. All tissues were fixed in the same 4% PA for 12 h, and then transferred to 30% sucrose 0.1 M PBS until the tissues sank. The dissected sciatic nerves of three rats in each group were subjected to cryosectioning (cross and longitudinal sections) for in situ hybridization and immunohistochemistry. Cryostat sections were cut at a thickness of 10 μm. The sections were prepared and used for these studies as previously reported [Ma et al., 2000].

For real-time PCR analysis, sciatic nerves were taken from each normal or injured rat. The individual nerves were weighed followed by storing at −80°C until homogenization.

QUANTITATIVE ANALYSIS OF β-1,4-GALT I TRANSCRIPTS IN RAT SCIATIC NERVE AND SCHWANN CELLS BY REAL-TIME PCR

The concept and validation of real-time quantitative PCR have been described previously [Winer et al., 1999]. We have used the relative standard curve method to determine the β-1,4-GalT I transcript levels in sciatic nerves and Schwann cells. β2-microglobulin (β2-M) was used as an endogenous control to normalize the expression level of the transcripts. Standard curves for β2-M were constructed using serial dilutions of cDNA from injured sciatic nerves at different times. Standard curves and experimental conditions were amplified in quadruplet. The standard curves for the β-1,4-GalT I and β2-M (standards) were constructed from respective mean C_t values, and the linear equation was derived using the Sequence Detection Systems (SDS) software (PE Biosystems, Norwalk, CT). The amount of template in the cDNA pool of the respective experimental conditions was then determined by applying the mean C_t value of that reaction in the equation of the standard curve. The expression levels of β-1,4-GalT I were normalized to the respective β2-M value.

TABLE I. Sequences of β-1,4-GalT I and β2-M Primer-Probe TaqMan Set

Targets	Sequence
β-1,4-GalT I-sense	5′-TACAACTGCTTTGTGTTCAGTGATG-3′
β-1,4-GalT I-antisense	5′-GCAGGCTAAACCCGAACTTG-3
β-1,4-GalT I-probe	FAM-TGCTTTTCACAGCCACGGCATATTTCT-TAMRA
β2-M-sense	5′-GTCTTTCTACATCCTGGCTCACA-3′
β2-M-antisense	5′-GACGGTTTTGGGCTCCTTCA-3′
β2-M-probe	FAM-CACCCACCGAGACCGATGTATATGCTTGC-TAMRA

The normalized values of the control and sciatic nerve injured samples were compared.

Primers and probes of the β-1,4-GalT I and β2-M were designed using Primer Premier 5 software by Shanghai Bioengineering Company (Shanghai, China), according to the specification of the TaqMan protocols [Winer et al., 1999]. Primers and probes for the respective β-1,4-GalT I and β2-M are listed in Table I.

PCR and real-time PCR detection of PCR product accumulation were performed using an iCycler (Bio-Rad, Denmark). Primers for β-1,4-GalT I and β2-M were used at a final concentration of 900 and 50 nM, respectively, whereas the probes were used at a final concentration of 200 nM. Amplification was done in a 20 μl final volume, under the following cycling conditions: the PCR cycling was initiated by activating the Taq polymerase for 2 min at 93°C; thereafter, PCR was run for 40 cycles. Each PCR was denatured for 10 s, at 93°C, followed by annealing and elongation for 60°C, 40 s, and 72°C, 10 s. The housekeeping gene β2-M was targeted for an internal control gene. The target genes (β-1,4-GalT I) data were normalized by corresponding β2-M data, and the mean-value of duplicate sample extracts was used for determining the relative RNA levels. The relative RNA levels were calculated as the ratio of the sample mean-value to the baseline sample mean-value. β-1,4-GalT I and β2-M templates were amplified in separate wells.

RNA ISOLATION AND REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION (REAL-TIME PCR)

Total RNA was extracted from Schwann cells by Trizol reagent. RNA concentration was determined by absorption at 260 nm and the 260/280 nm absorption ratio of the samples were >1.9. The ThermoScript RT System (Fermentas) was used for the RT reactions. Each sample contained approximately 5 μg RNA. The cDNA was diluted 1:1 and 2 μl was used in each 25 μl PCR reaction volume.

For PCR amplifications of specific cDNAs derived from β-1,4-GalT I and GAPDH mRNAs, two sets of sense and antisense oligonucleotides were used. These were sense 5′-TACAACTGCT-TTGTGTTCAGTGATG-3′, antisense: 5′-GCAGGCTAAACCCGAACT-TG-3′ for β-1,4-GalT I; the PCR products have predicted sizes of 111 bp. PCR amplification was carried out with the initial denaturing step at 94°C for 3 min, followed by 30 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s), and extension (72°C, 30 s), then a further extension at 72°C for 7 min. The PCR products were electrophoresed in 2% agarose gels in the presence of ethidium bromide, visualized by ultraviolet fluorescence, and recorded by a digital camera connected to a computer (SYNGENE, Gene Genius Imaging System). The widely used cDNA of glyceraldehydes-3-phosphate dehydrogenase (GAPDH, 452 bp) was adopted as an intrinsic standard during reverse transcription polymerase chain reaction (RT-PCR), sense primer: 5′-ACCACAGTCCATGCCATCAC-3′, antisense primer: 5′-TCCACCACCCTGTTGCTGTA-3′.

PREPARATION OF PROBES FOR IN SITU HYBRIDIZATION

The probes for in situ hybridization were prepared by RT-PCR (KIT from TaKaRa Biotech), using rat brain total RNA. The first-strand cDNA fragments were prepared by RT using 5 μg total RNA. The cDNA fragments were amplified by PCR with sense primer 5′-TACAACTGCTTTGTGTTCAGTGATG-3′ and antisense primer 5′-GCAGGCTAAACCCGAACTTG-3′ specific for rat β-1,4-GalT I. The PCR products were sequenced by a laser fluorescent DNA sequencer (Pharmacia). RT-PCR amplified cDNA fragments of β-1,4-GalT I were prepared and used as the probes for in situ hybridization. Amplified cDNA fragments were subcloned into pGEM®-T Easy (Promega). Plasmids were linearized with the restriction enzymes *Apa*I and *Sac*I. The digoxigenin (DIG) labeled sense and antisense β-1,4-GalT I RNA probes were produced using SP6 RNA polymerase. The labeling efficiency was measured by dot blot analysis with a DIG detection kit (Boehringer Mannheim) [Xian and Zhou, 1999].

IN SITU HYBRIDIZATION

In situ hybridization was performed as previously described [Nam et al., 1998]. In brief, sections were incubated in 10 μg/ml proteinase K for 15 min at 37°C and treated with 0.2 M HCl for 10 min, and 0.25% (v/v) acetic anhydride/0.1 M triethanolamine (pH 8.0) for 15 min. The sections were then prehybridized in the following solution: 50% (v/v) formamide, 10% (w/v) dextran sulfate, 5× SSC (standard saline citrate, 150 mM NaCl, 15 mM sodium citrate), 0.5% (w/v) sodium dodecyl sulfate (SDS), 5× Denhardt's reagent, and 200 μg/ml of yeast tRNA in DEPC-treated water at 42°C for 60 min and hybridized at 42°C for 16 h by adding DIG-labeled sense or antisense probe in humidified chamber. After hybridization, the slides were washed in 50% formamide/2× SSC at 42°C for 20 min three times and treated with 10 μg/ml of RNase A in buffer (10 mM Tris–HCl [pH 7.6], 500 mM NaCl, and 1 mM EDTA) at 37°C for 30 min. The sections were washed in 2× SSC at 42°C for 20 min and in 0.2× SSC at 42°C for 20 min twice, and then immersed in 7.5 U/ml of an anti-DIG-FITC-conjugated antibody.

COMBINED IN SITU HYBRIDIZATION FOR β-1,4-GALT I MRNA AND IMMUNOHISTOCHEMISTRY FOR S100

The sciatic nerve sections were air-dried, hybridized with β-1,4-GalT I probe (10 pmol/ml), and then rinsed 30 min in 1× SSC, at 42°C, and in 0.2× SSC at 42°C for 20 min twice, followed by 3 min × 10 min in 0.05 mol/L PBS at room temperature (RT). Next, the sections were preincubated in 1% bovine serum album (BSA with 0.1% Triton X-100) for 30 min at RT, followed by incubation overnight at 4°C with a polyclonal rabbit antibody directed against S100 (Sigma) diluted 1:800 in 1% BSA. Then, the sections were rinsed 4 min × 10 min in 0.05 mol/L PBS at RT and incubated for 1 h at RT with TRITC-conjugated secondary antibody (1:300, Jackson Immunolab). Finally, development of the in situ hybridization signal was performed as described above.

THE MEASUREMENT OF SCIATIC FUNCTION INDEX (SFI)

To do the walking track test, we investigated the activity, wound, claw-spread, and spirit of the rat after the right sciatic nerve crush, and then we calculated the SFI based on the following formula:

$$SF = -38.3\,(EPL - NPL)/NPL + 109.5\,(ETS - NTS)/NTS + 13.3\,(EIT - NIT)/NIT - 8.8$$

The factors that contributed to this formula were print-length factor (PLF), toe-spread factor (TSF), and intermediary toe-spread factor (ITF). E and N in the formula represent experimental side and normal side respectively. EPL, experimental side print-length; NPL, normal side print-length; ETS, experimental side toe-spread; NTS, normal side toe-spread; EIT, experimental side intermediary toe-spread; NIT, normal side intermediary toe-spread. Zero value indicates normal or complete recovery, and −100 accounts for the sciatic nerve being crushed or transected completely. The sciatic functional index offers the peripheral nerve investigator a noninvasive quantitative assessment of hindlimb motor function in the rat with selective hindlimb nerve injury [Bain et al., 1989; Siconolfi and Seeds, 2001].

STATISTICAL ANALYSIS

At least three repetitive real-time PCR assessments were performed, and for each assessment, all groups were tested in quadruplicate. All data were given in terms of relative values and expressed as mean ± standard error. One-way ANOVA was used to compare differences between the operated groups and the control group. All statistical analyses were conducted with a STATA 7.0 software package (Stata Corp., College Station, TX), and all significance levels were set at $P < 0.05$.

RESULTS

THE MEASUREMENT OF SFI POST-SCIATIC NERVE INJURY

Neurological recovery was analyzed by the SFI, which characterized hindlimb use and foot and toe positions, and by the pinch reflex. The SFI evaluates crucial aspects of locomotion involving recovery of hindlimb sensory and motor function; nonlesioned animals have an SFI of 0 (±10). The present study showed that SFI value was normal before lesion, −100 at 6 h, 12 h, and 1 day after crushing the sciatic nerve, then increased gradually from 2 days, reaching near the normal level at 4 weeks (Fig. 1A). By contrast, SFI value was −100 at any time after sciatic nerve transection (Fig. 1B). This phenomenon showed that the sciatic nerve crush and transection models were successfully established and indicated that the sciatic nerve function could recover at the defined time after crush and could not recover after transection.

TIME PROFILE OF β-1,4-GALT I MRNA EXPRESSION IN RAT SCIATIC NERVE USING QUANTITATIVE REAL-TIME PCR

To determine whether the expression of β-1,4-GalT I in injured sciatic nerve is related to the injury time and injury model, real-time PCR analysis was performed to examine the expression of β-1,4-GalT I during sciatic nerve injury. The expression level of rat β-1,4-GalT I mRNA in sciatic nerve was shown as a relative value to the β2-M transcript. In crushed sciatic nerve model, β-1,4-GalT I mRNA increased significantly at 2 weeks after sciatic nerve crush, however, β-1,4-GalT I mRNA was expressed at a low level in the normal and injured sciatic nerves at other different injury times (Fig. 2A). In transected sciatic nerve model, β-1,4-GalT I mRNA increased from 1 day, peaked at 3 days, and then decreased gradually to normal levels at 4 weeks in the distal stumps of transected sciatic nerves. However, β-1,4-GalT I mRNA was expressed at a low level in the normal and transected sciatic nerves at 6 and 12 h (Fig. 2B). The same results were obtained in three independent experiments.

LOCALIZATION OF β-1,4-GALT I MRNA EXPRESSION IN LESION NERVE

The high expression levels of β-1,4-GalT I mRNA were at 2 weeks after sciatic nerve crush. To reveal the distribution of β-1,4-GalT I transcript in the crushed sciatic nerve, in situ hybridization was performed with the sciatic nerve 2 weeks after crush and the normal sciatic nerve to further analyze the β-1,4-GalT I expression. The accurate localization of β-1,4-GalT I mRNA expression in the sciatic

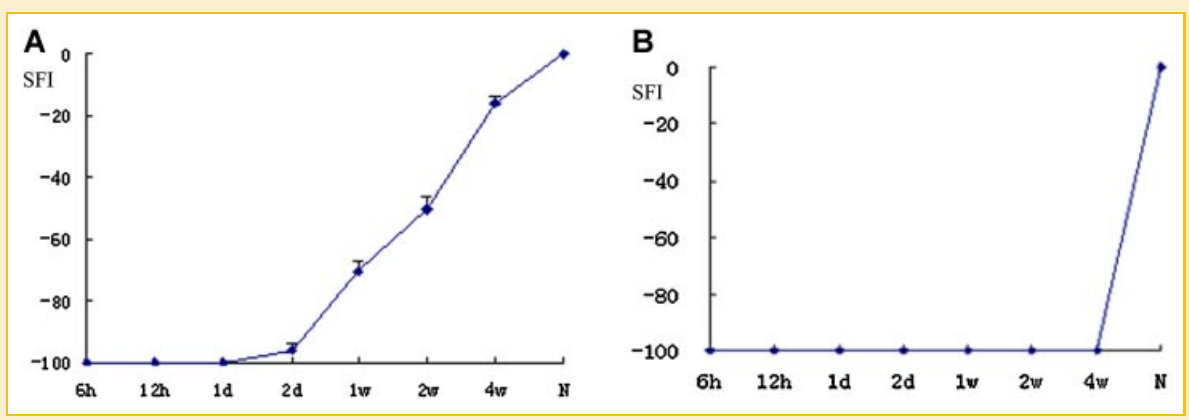

Fig. 1. The sciatic function index (SFI) values of the rats at different time points post-sciatic nerve injury and in normal sciatic nerve. A: Sciatic nerve crush model, (B) sciatic nerve transection model. N, normal sciatic nerve; h, hour; d, day; w, week.

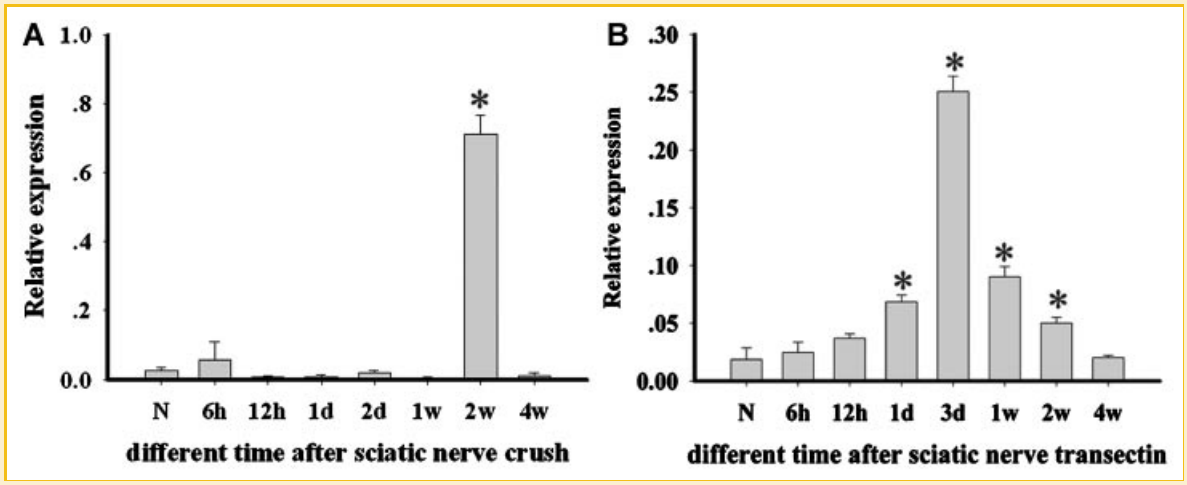

Fig. 2. Quantification of β-1,4-GalT I mRNA in sciatic nerve at various times after crush and transection, respectively. There was a significant increase in β-1,4-GalT I mRNA at 2 weeks after sciatic nerve crush (A), at 1 day, 3 days, 1 week, and 2 weeks in the distal stump after sciatic nerve transection (B) ($P < 0.05$). The data (means ± SEM) were presented as the ratio of β-1,4-GalT I mRNA to β2-M mRNA, and the experiments were tripled under the same condition. $^{*}P < 0.05$ indicates a statistically significant difference compared with any other group. N, normal sciatic nerve (lane 1); 6 h post-injury (lane 2); 12 h (lane 3); 1 day (lane 4); 2 days (lane 5); 1 week (lane 6); 2 and 4 weeks (lane 8). h, hour; d, day; w, week.

nerve, as studied by in situ hybridization, was consistent with the general expression pattern seen by using real-time PCR. In situ hybridization showed that the expression of β-1,4-GalT I mRNA in normal sciatic nerve was present. In order to examine whether β-1,4-GalT I is present in all cell types or in certain cell types of the sciatic nerve, analysis of sections subjected to combining in situ hybridization for β-1,4-GalT I mRNA and immunohistochemistry for Schwann cell marker S100, which consistently revealed β-1,4-GalT I mRNA-expressing cells in the normal sciatic nerve were S100-immunoreactive (Fig. 3). The negative control samples produced no signals above background (Fig. 3).

The expression of β-1,4-GalT I mRNA at 2 weeks after sciatic nerve crush was ubiquitous, and strong hybridization signals were detected both in the longitudinal and cross sections. Combined in situ hybridization for β-1,4-GalT I mRNA and immunohistochemistry for Schwann cell marker S100 consistently revealed β-1,4-GalT I mRNA-expressing cells in the crushed sciatic nerve, which were S100-immunoreactive (Fig. 4).

Similar to the sciatic nerve crush, the signals of β-1,4-GalT I mRNA at 3 days in distal stumps of sciatic nerve after transection were strong both in the longitudinal and cross sections, combined in situ hybridization for β-1,4-GalT I mRNA and immunohistochemistry for Schwann cell marker S100 consistently revealed that β-1,4-GalT I mRNA-expressing cells in the transected sciatic nerve were S100-immunoreactive (Fig. 5). Because S100 is a Schwann cell marker in peripheral nervous system, we could conclude that β-1,4-GalT I was expressed in Schwann cells of sciatic nerves in physiological or in injury pathological conditions.

LPS ADMINISTRATION AFFECTS β-1,4-GALT I MRNA EXPRESSION IN SCIATIC NERVE IN A TIME- AND DOSE-DEPENDENT MANNER

In the present study, we first reported that intraperitoneal administration of LPS affected the expression of β-1,4-GalT I mRNA in rat sciatic nerves. Real-time PCR showed that sciatic nerves expressed β-1,4-GalT I mRNA in a dose-dependent manner (Fig. 6A), however 1 mg/kg LPS had no significant effect on the expression of β-1,4-GalT I mRNA in the sciatic nerve. LPS (0.5 mg/kg) had a significant role in the expression of β-1,4-GalT-I mRNA, so we investigated the time-course expression of β-1,4-GalT I mRNA after 0.5 mg/kg LPS stimulation. Real-time PCR showed that the sciatic nerve expressed β-1,4-GalT I mRNA in a time-dependent manner. We found that β-1,4-GalT I mRNA peaked at 6 h after 0.5 mg/kg LPS administration in rat sciatic nerves (Fig. 6B).

To further confirm these results, we detected β-1,4-GalT I mRNA by using RT-PCR. It was observed that the levels of β-1,4-GalT I mRNAs were low in normal untreated sciatic nerves, enhanced slightly after 1 mg/kg LPS administration and enhanced significantly after 0.5, 5, and 10 mg/kg LPS administration in rat sciatic nerves. These results were consistent with the results from real-time PCR (Fig. 6C).

LOCALIZATION OF β-1,4-GALT I MRNA EXPRESSION IN SCIATIC NERVE AFTER LPS STIMULATION

The high expression level of β-1,4-GalT I mRNA in the sciatic nerve after LPS stimulation was at 6 h after 0.5 mg/kg LPS stimulation. To reveal the distribution of β-1,4-GalT I transcript in the inflammatory sciatic nerve, in situ hybridization was performed. Strong β-1,4-GalT I mRNA signals were found in sciatic nerves after LPS stimulation, and combined in situ hybridization for β-1,4-GalT I mRNA and immunohistochemistry for Schwann cell marker S100 consistently revealed that β-1,4-GalT I mRNA-expressing cells in the sciatic nerve after 0.5 mg/kg LPS stimulation for 6 h were S100-immunoreactive (Fig. 7), which indicated that β-1,4-GalT I mainly located in Schwann cells of sciatic nerve during inflammation.

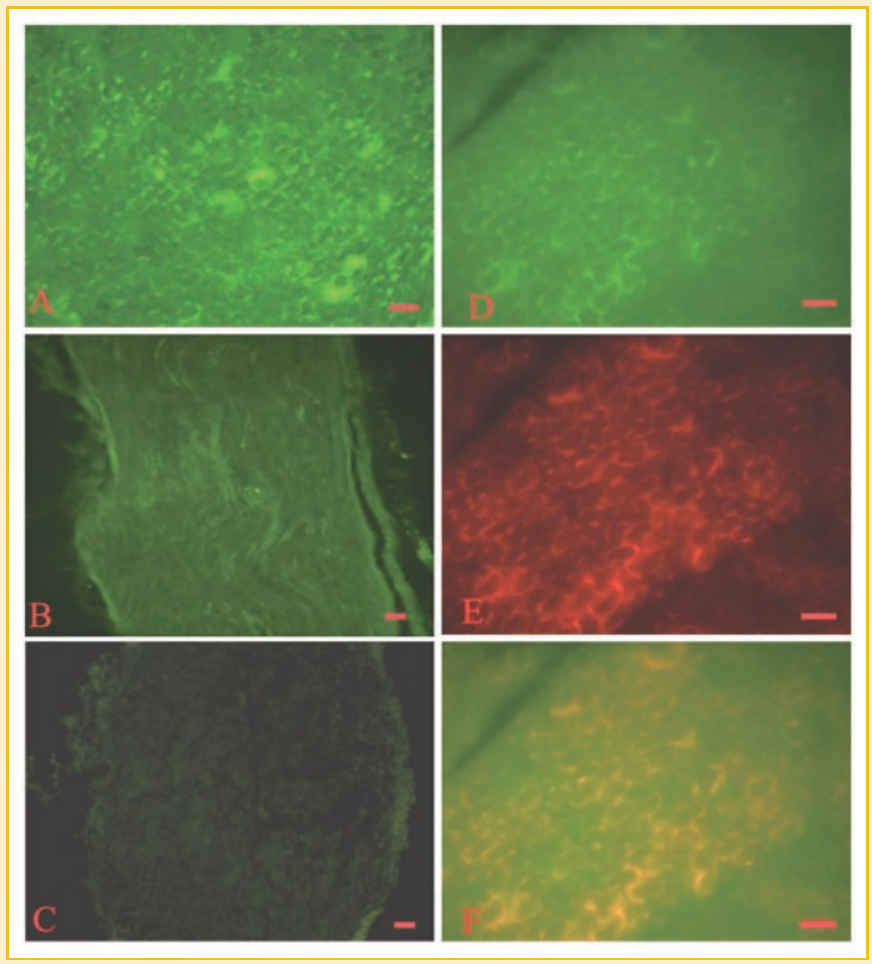

Fig. 3. The expression of β-1,4-GalT I mRNA in normal sciatic nerve Combined in situ hybridization and immunohistochemistry was performed on sections of normal rat sciatic nerve using β-1,4-GalT I cRNA probe (green) and antibody S100 (red) specific for Schwann cells. A: Cross section. B: Longitudinal section. C: Digoxigenin-labeled β-1,4-GalT I sense riboprobe showed background staining level. D–F: A high magnification of sciatic nerve cross section. F: Merged picture indicated that β-1,4-GalT I mRNA (green) was co-localized to S100(red). A: Scale bar = 20 μm. B,C: Scale bars = 10 μm. D–F: Scale bars = 50 μm.

LPS INDUCES β-1,4-GALT I MRNA EXPRESSION IN SCHWANN CELLS IN A TIME- AND DOSE-DEPENDENT MANNER

Since we know that β-1,4-GalT I mRNA is expressed in Schwann cells in vivo depending on physiology or pathology, we wanted to know about its expression in Schwann cells in vitro. Therefore, we investigated the expression of β-1,4-GalT I mRNA in normal and inflammatory Schwann cells in vitro. To establish the kinetics of the LPS mediated expression level of β-1,4-GalT I mRNA, confluent monolayers of Schwann cells were incubated in the presence of increasing concentrations of LPS for different times. Prior to this study, β2-microtubulin (β2-M) was determined as the optimal endogenous reference gene for normalization in the experimental conditions used in the present work. When stimulated with 0.1 ng/ml–10 μg/ml LPS, Schwann cells transcribed mRNA for β-1,4-GalT I which was determined by quantitative real-time PCR. The expression level of β-1,4 GalT I mRNA in cultured Schwann cells was in a dose-dependent manner. Compared with the control group, β-1,4 GalT I mRNA increased from 0.1 ng/ml, reached a peak at 10 ng/ml, and then decreased gradually from 100 ng/ml to 10 μg/ml LPS stimulation (Fig. 8A). In addition, the expression of β-1,4-GalT I mRNA in cultured Schwann cells was in a time-dependent manner. β-1,4-GalT I mRNA increased from 1 h, peaked at 6 h, then decreased gradually to the normal level at 12 h after exposure to 10 ng/ml LPS (Fig. 8B). Real-time PCR revealed that β-1,4-GalT I mRNAs were low in the normal untreated Schwann cells and enhanced after exposure to LPS stimulation, but we do not know about their distributions in these cells. Immunocytochemistry showed that β-1,4-GalT I was expressed weakly in the cytoplasm of normal untreated Schwann cells (Fig. 9A), and distributed mainly in the cytoplasm and processes of Schwann cells and the density of stain increased significantly in Schwann cells after exposure to 10 ng/ml LPS (Fig. 9B).

THE EFFECTS OF MAPK INHIBITORS ON β-1,4-GALT I MRNA SYNTHESIS INDUCED BY LPS

A variety of signaling pathways have been elucidated in the response to LPS [Diks et al., 2004]. The best described of which is the pathway leading to MAPKs activation. MAPKs are important mediators of cytokine expression. To assess whether MAPKs plays an important role in LPS-induced β-1,4-GalT I synthesis, we used

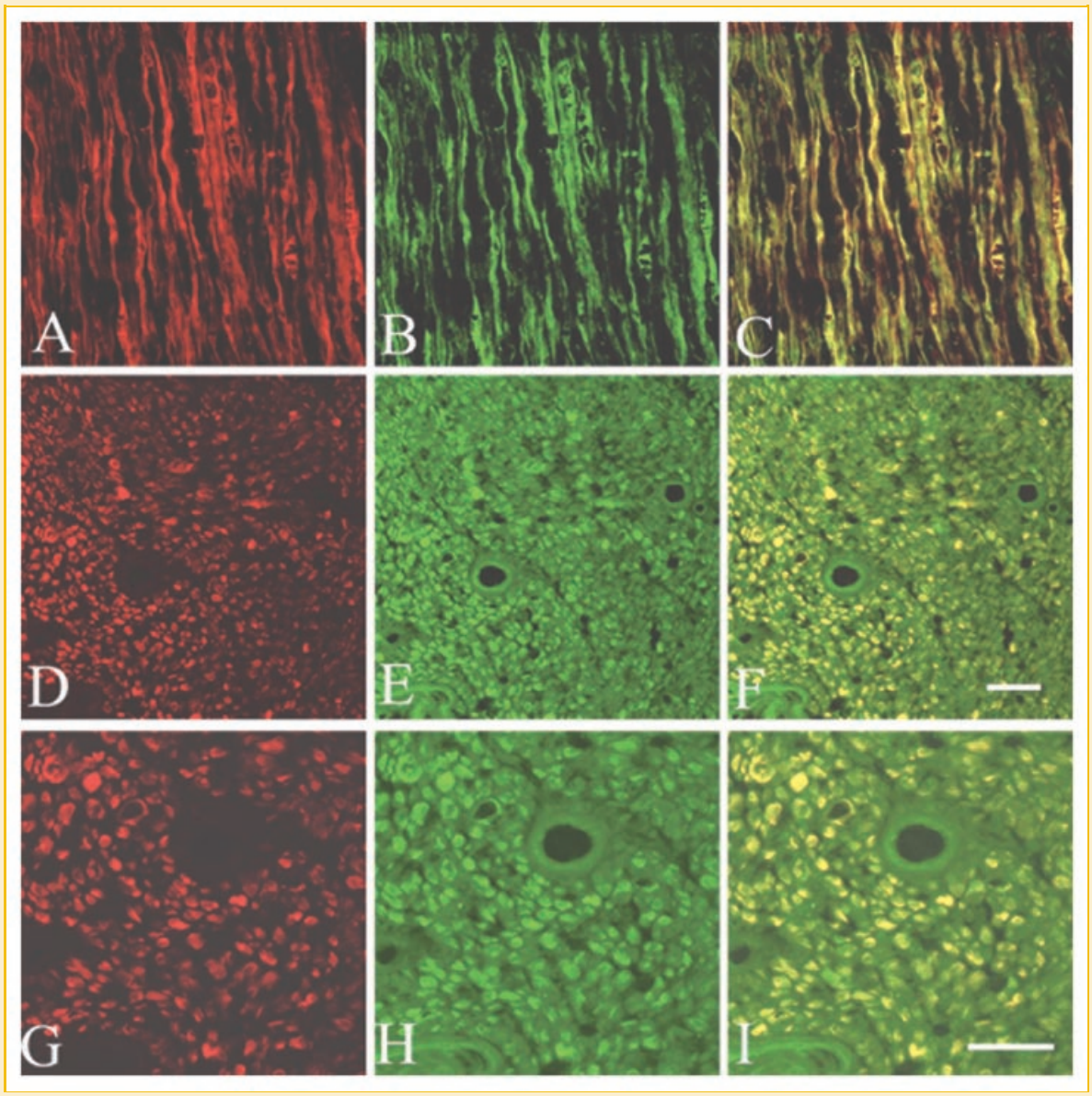

Fig. 4. The distribution of β-1,4-GalT I mRNA in crushed sciatic nerve at 2 weeks. A–C: Longitudinal sections, (D–I) cross sections, (G–I) a high magnification of (D–F). Combined in situ hybridization and immunohistochemistry was performed on sections of 2 weeks sciatic nerve after crush using β-1,4-GalT I cRNA probe (green) and antibody S100 (red) specific for Schwann cells. C,F,I: Merged pictures indicated that β-1,4-GalT I localized in Schwann cells. A–C,G–I: Scale bars = 40 μm. D–F: Scale bars = 20 μm.

specific inhibitors of MAPKs cascades: U0126 (Fig. 10A), SB202190 (Fig. 10B), and SP600125 (Fig. 10C). Real-time PCR showed that all three reagents inhibited β-1,4-GalT I mRNA production induced by LPS. It is reasonable to conclude that the production of β-1,4-GalT I mRNA is regulated by ERK, P38, and SAPK/JNK at the transcriptional level.

DISCUSSION

β-1,4-GalT I is thought to play a multifunctional role in many aspects of cellular physiology and pathology, such as mammalian fertilization, embryonic development, cancer metastasis, and so on [Hinton et al., 1995; Johnson and Shur, 1999; Nixon et al., 2001]. The expression level of β-1,4-GalT I mRNA could be induced in some organs such as liver, lung, lymph node, spleen after LPS administration [Qian et al. 2007]. However, research about β-1,4-GalT I biological functions in pathological sciatic nerves are very few. In the present study, we established sciatic nerve injury models and an inflammatory model to investigate β-1,4-GalT I expressional changes in pathological sciatic nerves.

The sciatic nerve crush, with the continuity of the nerve preserved, allows for complete axonal return and restoration of the nerve–muscle interaction. This leads to nerve regeneration. In contrast, the sciatic nerve transection, with continuity of the nerve destroyed, results in nerve degeneration. In the present study, we

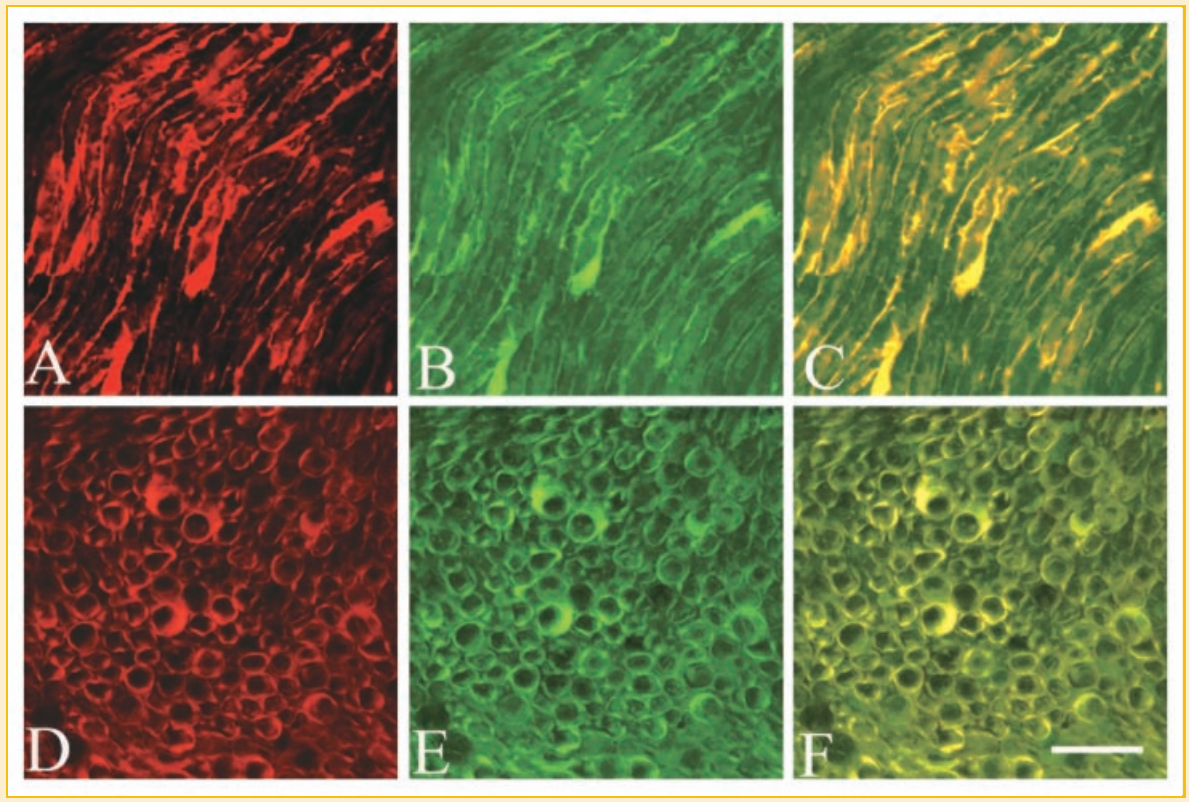

Fig. 5. The expression of β-1,4-GalT I mRNA in transected sciatic nerve at 3 days. A–C: Longitudinal sections of distal stumps. D–F: Cross sections of distal stumps. Combined in situ hybridization and immunohistochemistry were performed on sections of 3 days transected sciatic nerve using β-1,4-GalT I cRNA probe (green) and antibody S100 (red) specific for Schwann cells. C,F: Merged pictures indicated that β-1,4-GalT I localized in Schwann cells. Scale bars = 40 μm.

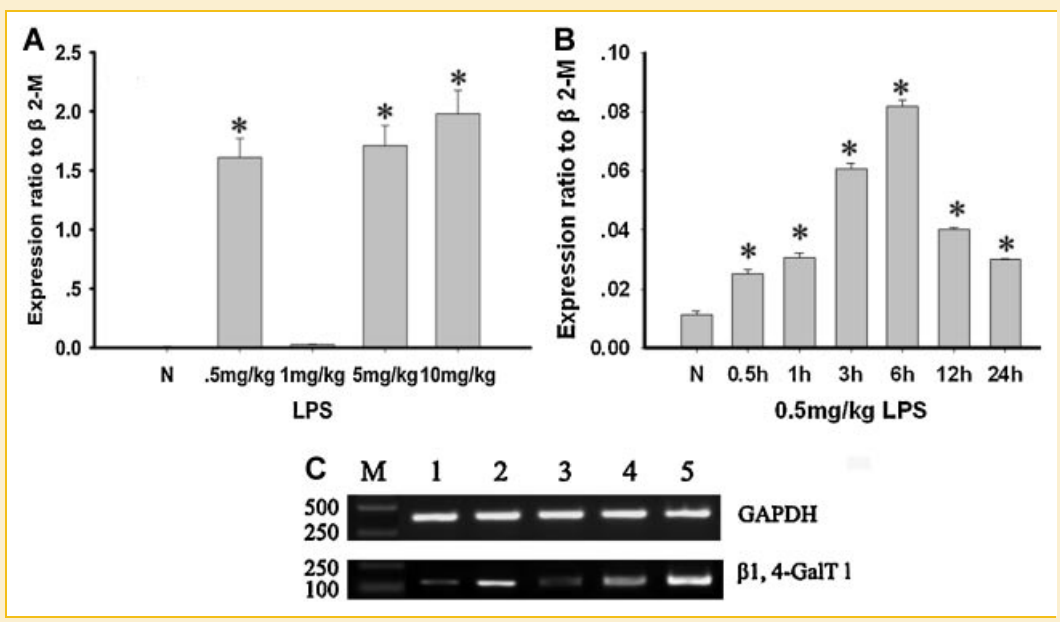

Fig. 6. Quantification of β-1,4-GalT I mRNA in sciatic nerve after intraperitoneal administration of LPS. Rats were treated with LPS (0.5–10 mg/kg) for 0.5–24 h. LPS dose-dependence was measured for a 6 h (A), time-dependence was measured with 0.5 mg/kg LPS (B). β-1,4-GalT I transcripts were measured by real-time PCR. The data (means ± SEM) were presented as the ratio of β-1,4-GalT I mRNA to β2-M mRNA, and the experiments were tripled under the same condition. $^{*}P < 0.05$ indicates a statistically significant difference compared with any other group. C: β-1,4-GalT I mRNA was measured by RT-PCR. (1) Normal sciatic nerve, rats treated with 0.5 mg/kg LPS (2), 1 mg/kg LPS (3), 5 mg/kg LPS (4), 10 mg/kg LPS (5). GAPDH served as internal control. The DNA markers (M) were run on the left of the same gel and the names of PCR bands were indicated on the right.

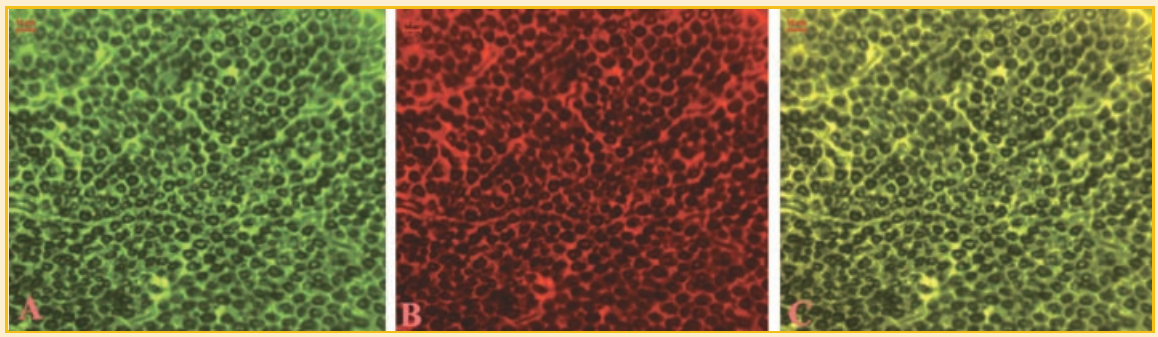

Fig. 7. The distribution of β-1,4-GalT I mRNA in sciatic nerve at 6 h intraperitoneal administration of 0.5 mg/kg LPS. A–C: Cross sections. Combined in situ hybridization and immunohistochemistry was performed on sections of sciatic nerve after LPS treatment using β-1,4-GalT I cRNA probe (green, A) and antibody S100 (red, B) specific for Schwann cells. C: Merged pictures of (A) and (B) indicated that β-1,4-GalT I localized in Schwann cells. Scale bars = 10 μm.

found that a complete dysfunction of the sciatic nerve was observed from 6 h to 1 day after sciatic nerve crush in terms of the SFI value of −100, and then recovered gradually to a relatively normal level at 4 weeks. The result is in agreement with previous reports showing that a complete dysfunction of the sciatic nerve was observed immediately following sciatic nerve crush in terms of the SFI value of −100 [Sun et al., 2006]. Afterwards, a steady recovery of locomotive function of the crushed sciatic nerve of the rats was demonstrated in the light of continuous increases in the SFI value until the SFI value of about −16 was reached at 4 weeks post-sciatic nerve crush [Sun et al., 2006]. These results demonstrated that the sciatic nerve crush models were established successfully, and suggested that sciatic nerve crush could lead to sciatic nerve regeneration. However, in the sciatic nerve transected model, SFI value was −100 from 6 h to 4 weeks after sciatic nerve transection, which indicated that sciatic nerve transection models were established successfully, and suggested that sciatic nerve transection could not lead to sciatic nerve regeneration.

In the central nervous system, β-1,4-GalT I gene is expressed mainly in mid-embryonic stages [Liedtke et al., 2001; Nakamura et al., 2001], but in the peripheral nervous system, the expression of β-1,4-GalT I is unclear. In the present study, we first reported that the expression of β-1,4-GalT I mRNA in crushed sciatic nerves reached a peak at 2 weeks after sciatic nerve crush, and we found that β-1,4-GalT I mRNA reach a peak in distal sciatic nerve on the 3 days after sciatic nerve transection, which is consistent with the results in our previous study [Shen et al., 2002, 2003]. These results showed that β-1,4-GalT I increased much earlier in transected sciatic nerves than those in crushed ones. The difference was probably due to the nature of the injury. In the crushed model, the critical feature of crush injury is that the continuity of basal lamina tubes is protected from injury. In contrast, in the transected model, the continuity of the basal lamina is completely disrupted. Research suggests that disruption of basal lamina tube continuity was essential to induce β-1,4-GalT I expressions earlier. Regenerating axons exhibit a preference for growing along the basal lamina tubes

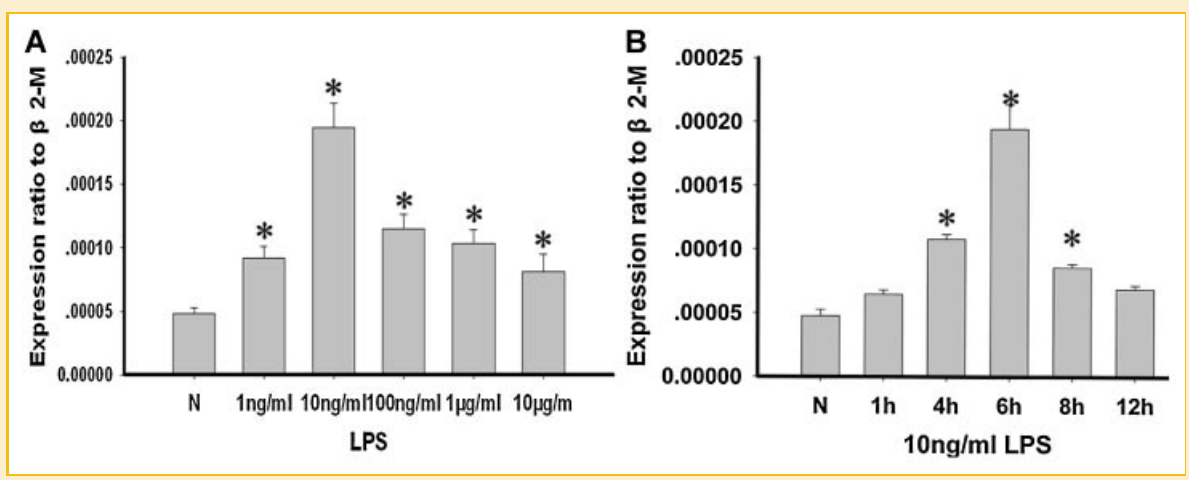

Fig. 8. Quantification of β-1,4-GalT I mRNA in rat Schwann cells after exposure to LPS stimulation. Schwann cells were treated with LPS (1–10 μg/ml) for 1–12 h. LPS dose-dependence was measured for 6 h (A), time-dependence was measured with 10 ng/ml LPS (B). β-1,4-GalT I transcripts were measured by real-time PCR. The data (means±SEM) were presented as the ratio of β-1,4-GalT I mRNA to β2-M mRNA, and the experiments were tripled under the same condition. $^{*}P < 0.05$ indicates a statistically significant difference compared with any other group.

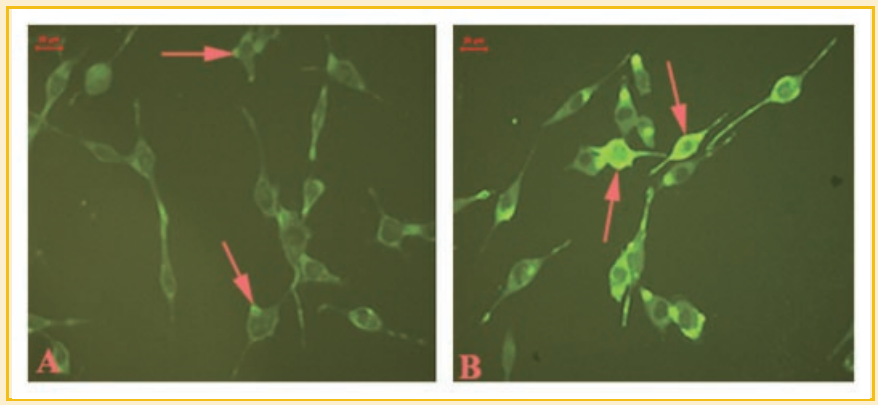

Fig. 9. The distribution of β-1,4-GalT I in Schwann cells by using immunocytochemistry. A: Normal untreated Schwann cells. B: Schwann cells treated with 10 ng/ml LPS for 6 h. A,B: FITC-labeling with anti-β-1,4-GalT I serum. Arrows indicated that these cells were β-1,4-GalT I positive cells. Scale bars = 20 μm.

in the distal nerve stump [Ide et al., 1983]. As long as the basal lamina tubes remain intact, they guide the regenerating axons to the original target. Complete transection of the peripheral nerve under transect injury disrupts the continuity of the basal lamina tubes, resulting in potential misdirection of regenerating axons [Brown and Hardman, 1987]. All these results demonstrated that β-1,4-GalT I gene expression was influenced by the sciatic nerve injury, and it varied at the different injury times and in the different injury models.

Schwann cells are thought to be a crucial factor in peripheral nerve regeneration [Fu and Gordon, 1997]. As we know, Schwann cells de-differentiate and proliferate after axotomy of peripheral nerves [Bunge, 1993]. The denervated Schwann cells are known to upregulate expression of a number of cell surface molecules which are presumed to aid axonal regeneration, including NCAM, L1, CHL1, and p75 [Gupta et al., 1990; LeBlanc and Poduslo, 1990]. β-1,4-GalT I has been found on the plasma membrane of different types of cells where it serves as a cell surface adhesion molecule, mediating various cell–cell and cell–matrix interactions such as sperm–egg binding, cell spreading, migration, and neurite outgrowth [Huang et al., 1995; Lu and Shur, 1997; Shi et al., 2001]. One of the ligands for β-1,4-GalT I during neurite outgrowth is laminin. In transected and regenerating nerves, extracellular matrix molecule, laminin, is increased in the endoneurium. In this study, combined in situ hybridization for β-1,4-GalT I mRNA and immunohistochemistry for S100 showed that β-1,4-GalT I mRNAs were mainly located in Schwann cells after sciatic nerve crush or transection. All these results demonstrated that β-1,4-Gal T I may play an important role in Schwann cells during regeneration and degeneration in the peripheral nervous system.

Asano et al. [2003] reported that acute and chronic inflammatory responses were suppressed in β-1,4-GalT I-deficient mice, and neutrophil infiltration at the inflammatory sites was primarily reduced. Mori et al. also found that skin wound healing was significantly delayed in the β-1,4-GalT $I^{-/-}$ mice. Neutrophil and macrophage recruitment at the wound site in the inflammatory phase was reduced probably because of the reduced expression of selectin ligands in the β-1,4-GalT $I^{-/-}$ mice compared with β-1,4-GalT $I^{+/-}$ mice [Mori et al., 2004]. Wassler et al. [2001] reported that there were functional interaction between the cytoplasmic domain of β-1,4-GalT I and the SSeCKS scaffolding protein, and Kitamura et al. [2002] reported that SSeCKS had been shown to be a major LPS response protein which markedly upregulated in several organs, including lung, heart, kidney, brain etc. indicating a possible role of SSeCKS in inflammatory process. Because the response of β-1,4-GalT I during inflammation in the peripheral nervous system is unknown, we established the same inflammatory model as the Kitamura used to observe the expression of β-1,4-GalT I in sciatic nerves during inflammatory pathology. In the present study, we found that LPS administration affected β-1,4-GalT I mRNA expression in the sciatic nerve in a time- and dose-dependent manner, and β-1,4-GalT I mRNA was expressed mainly in Schwann cells. These results indicated that β-1,4-GalT I played an important role in the inflammation reaction induced by intraperitoneal injection of LPS, however, the exact mechanism of β-1,4-GalT I in inflammation remains to be further elucidated. Garcia-Vallejo et al. reported that β-1,4-GalT I in primary human endothelial cells (HUVECs) was upregulated in a time- and concentration-dependent manner in response to TNF-α stimulation due to an increase in the stability of the mRNA transcript [Garcia-Vallejo et al., 2005].

Schwann cells are glia of the peripheral nervous system. Besides their roles in myelination, trophic support, and regeneration of axons, Schwann cells exhibit potential for some immune functions. Schwann cells can be induced to produce cytokines and chemokines, adhesion molecules, and serve as antigen presenting cells. They produce chemokines and macrophage inflammatory protein-1α (Oliveira et al. 2003). LPS could induce the expression of TNFα mRNA and protein in cultured Schwann cells in a concentration- and time-dependent manner [Cheng et al. 2007]. In the present study, we found that β-1,4-GalT I was mainly located in Schwann cells of sciatic nerves wherever Schwann cells were in normal physiology condition or in pathology conditions including injury and inflammation. In addition, we found that β-1,4-GalT I in cultured Schwann cells was affected in a time- and concentration-dependent manner in response to LPS stimulation. All these results suggest that β-1,4-GalT I play an important role in inflammation wherever they are located in Schwann cells in vitro or in vivo.

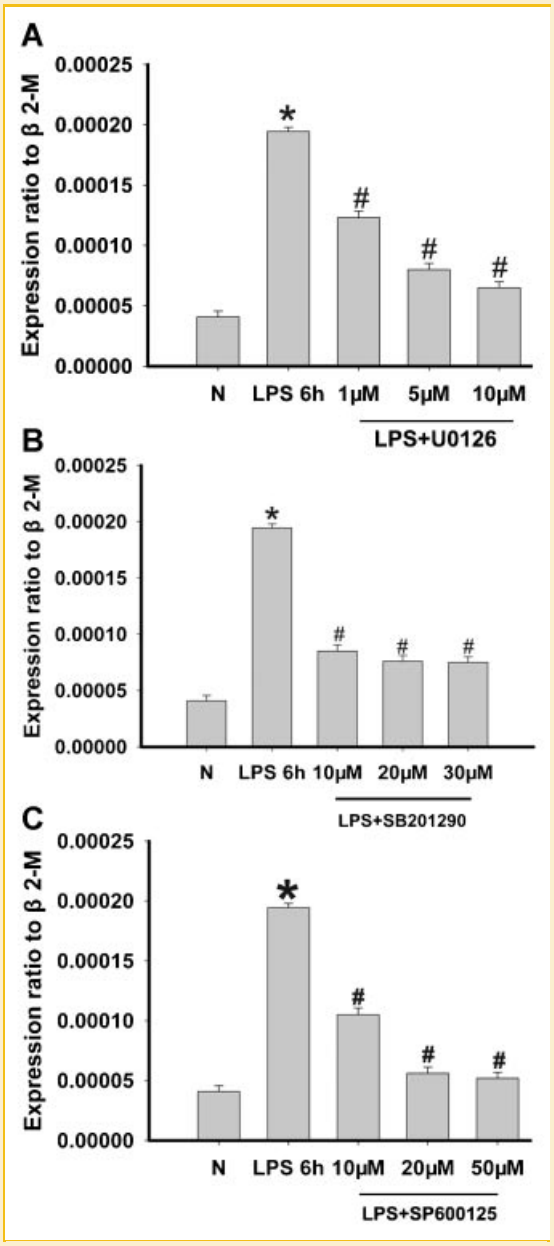

Fig. 10. Inhibition of LPS-induced β-1,4-GalT I mRNA expression by inhibitor of MAPKs in Schwann cells. Schwann cells were treated with LPS in the absence or presence of U0126 (1, 5, and 10 μm) (A), SB202190 (10, 20, and 30 μm) (B), SP600125 (10, 20, and 50 μm) (C). β-1,4-GalT I transcripts were measured by real-time PCR. Data (means ± SEM) were presented as the ratio of β-1,4-GalT I mRNA to β2-M mRNA, and the experiments were tripled under the same condition. $^{*}P < 0.05$ indicates a statistically significant difference compared with control group. $^{\#}P < 0.05$ indicates a statistically significant difference compared with LPS 6 h treatment group.

Real-time PCR showed that specific inhibitors of MAPKs cascades including U0126, SB202190, and SP600125 inhibited β-1,4-GalT I production induced by LPS. These results are consistent with a variety of signaling pathways have been elucidated in the response to LPS [Diks et al., 2004]. The best described of which is the pathway leading to MAPKs activation. MAPKs are important mediators of cytokine expression. So it is reasonable to conclude that Schwann cells regulated β-1,4-GalT I expression after LPS stimulation were via ERK, SAPK/JNK and, P38 MAP kinase signal pathways.

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