Desert Hedgehog-Patched 2 Expression in Peripheral Nerves during Wallerian Degeneration and Regeneration

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ABSTRACT: Hedgehog proteins are important in the development of the nervous system. As Desert hedgehog (Dhh) is involved in the development of peripheral nerves and is expressed in adult nerves, it may play a role in the maintenance of adult nerves and degeneration and regeneration after injury. We firstly investigated the Dhh-receptors, which are expressed in mouse adult nerves. The Dhh receptor patched (ptc)2 was detected in adult sciatic nerves using RT-PCR, however, ptc1 was undetectable under the same experimental condition. Using RT-PCR in purified cultures of mouse Schwann cells and fibroblasts, we found ptc2 mRNA in Schwann cells, and at much lower levels, in fibroblasts. By immunohistochemistry, Ptc2 protein was seen on unmyelinated nerve fibers. Then we induced crush injury to the sciatic nerves of wild-type (WT) and dhh-null mice and the distal stumps of injured nerves were analyzed morphologically at different time points and expression of dhh and related receptors was also measured by RT-PCR in WT mice. In dhh-null mice, degeneration of myelinated fibers was more severe than in WT mice. Furthermore, in regenerated nerves of dhh-null mice, minifascicular formation was even more extensive than in dhh-null intact nerves. Both dhh and ptc2 mRNA levels were down-regulated during the degenerative phase postinjury in WT mice, while levels rose again during the phase of nerve regeneration. These results suggest that the Dhh-Ptc2 signaling pathway may be involved in the maintenance of adult nerves and may be one of the factors that directly or indirectly determines the response of peripheral nerves to injury. © 2005 Wiley Periodicals, Inc. J Neurobiol 66: 243–255, 2006

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INTRODUCTION

The hedgehog (Hh) family of secreted ligands plays a critical role in a variety of developmental processes in both insects and vertebrates (Hammerschmidt et al., 1997). Three homologues of Hh, Sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehog, have been described in mammals. Dhh mRNA is expressed in the Sertoli cells of developing testis and Schwann cells of the peripheral nervous system (PNS) in mouse. In PNS, dhh-null mice show abnormal devel-
opment of the perineurium, which results in minifascicular formation within the endoneurium (Parmantier et al., 1999). Similarly, we found that a missense mutation of dhh gene in a patient resulted in minifascicular neuropathy with 46XY, partial gonadal dysgenesis (Umehara et al., 2000). These findings suggest that Schwann cell-derived Dhhs signals the formation of the connective tissue sheath around peripheral nerves in both mice and humans. Dhhs signaling is mediated by a multicomponent receptor complex involving two transmembrane proteins, Patched (Ptc) and Smoothened (Smo) (Johnson and Scott, 1997; Carpenter et al., 1998; Pathi et al., 2001). The vertebrate Ptc family includes two homologues, identified as Ptc1 and Ptc2 (Takabatake et al., 1997; Carpenter et al., 1998; Motoyama et al., 1998b), which can both bind to all known Hh homologues and also form a complex with Smo (Carpenter et al., 1998). However, the expression patterns of the two Ptc receptors are not completely overlapping, suggesting their distinct properties in Hh signaling (Carpenter et al., 1998; Motoyama et al., 1998a; Rahnama et al., 2004).

Nerve injury leads to dramatic changes in expression of a large number of genes, which recapitulates the pattern seen in early development (Griffin and Hoffman, 1993). Dhhs protein plays an important role in the development of peripheral nerves and it is also expressed in adult nerves (Parmantier et al., 1999). However, little is known about its role in adult nerves. These observations prompted us to characterize the expression of Dhhs and its receptors in mature nerves after injury. In this study, we firstly investigated and localized the main Dhhs receptors, which are expressed in adult nerves of mice. Then we induced crush injury to the sciatic nerves of adult wild-type (WT) mice to observe the mRNA expression changes of Dhhs and its receptors in distal stumps of injured nerves and also compared the histopathological and nerve fiber morphometric changes after injury in injured nerves of WT mice with those of dhh-null mice.

MATERIALS AND METHODS

Animals

Forty-five DDY female WT mice and 18 female dhh-null mice were used throughout the injury study. The dhh-null mice were kindly provided by Dr. A.P. McMahon (Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA), which were obtained by homologous recombination as previously reported (Bitgood et al., 1996). Genotyping of dhh-null mice was also performed as previously described (Parmantier et al., 1999). The mice were 8 weeks old and 26–31 g. Five postnatal day 3 WT mice were used in other experiments. All experimental protocols involving animals were approved by the committee of the life science resource development research center of Kagoshima University.

Nerve Injury Procedures

The right sciatic nerves of mice were exposed under mixed anesthesia using a 0.1 mL intraperitoneal injection of sodium pentobarbital (5 mg/mL) and inhaled ether. The exposed nerves were crushed by pinching the nerve with jeweler forceps for 15 s about 10 mm proximal to the trifurcation. We grouped 20 WT mice for RT-PCR experiments (three to five mice for each time point of sacrifice) and the others (25 WT and 18 dhh-null mice) for light microscopy and immunohistochemical experiments (three to four mice for each time point of sacrifice). The mice were sacrificed 2, 7, 14, 28, and 42 days after injury and approximately 1 cm of the right sciatic nerve distal to the injury and the same portion of left sciatic nerve (as control sample) were harvested.

Preparation of Purified Mouse Schwann Cells and Fibroblasts

Postnatal day 3 nerve fibroblasts and Schwann cells were purified by immunopanning using antibodies to Thy-1 as described previously (Dong et al., 1999). Fibroblasts were grown in 90 mm diameter tissue culture dishes in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum at 37°C in 5% CO2. Schwann cells were plated onto 35 mm poly-d-lysine and laminin-coated tissue culture dishes in defined medium with low insulin (Dong et al., 1999), D-glucose, and 10 ng/mL, neuregulin-β1. Twenty-four hours prior to the experiment, the Schwann cell medium was changed to Dulbecco’s modified Eagle’s medium and 10% fetal calf serum.

Histochemistry

Teased Preparations of Sciatic Nerves. Teased nerves were prepared as previously described (Jessen et al., 1985). The antibodies and blocking reagents were diluted in PBS containing 10% fetal calf serum, 0.1 M lysine, and 0.02% sodium azide. PBS was used for washes. The Fc receptors were blocked with 2% normal horse serum for 10 min. Ptc2 antibody (goat polyclonal, diluted 1:20, N-19; Santa Cruz Biotecnology, CA) was applied for 1 h at room temperature followed by 1 h of incubation with FITC-conjugated rabbit antigoat immunoglobolus (diluted 1:30; Dako Cyto- mation, Denmark A/S). The teased nerves were mounted in Citifluor antifade mounting medium (Citifluor Ltd., London) containing 1/1000 Hoechst 33258 (Sigma-Aldrich Company Ltd., UK) for nuclear staining and viewed on a Nikon fluorescence microscope with epifluorescence or phase-contrast optics.
Nerve Sections

Immunofluorescence Double Staining. Fresh samples of adult mouse intact nerves and injured nerves harvested at days 2 and 28 were frozen in isopentane cooled in liquid nitrogen. Cryostat sections were cut cross sectionally and longitudinally and were dried at room temperature. The blocking and diluting solutions were the same as the solutions that were used for the staining of teased preparations of nerves (above). All incubations with antibodies were done at room temperature.

For double staining with Ptc2 antibody and glial fibrillary acidic acid (GFAP) antibody (marker for nonmyelinating Schwann cells) (Jessen and Mirsky, 1984), nerve sections were fixed in cold acetone for 10 min and then were incubated with blocking solution for another 10 min. The sections were incubated with Ptc2 antibody (goat polyclonal, diluted 1:20; Santa Cruz Biotechnology) for 1 h at room temperature followed by 10 min incubation with GFAP antibody (rabbit polyclonal, ready-to-use; Dako Cytomation).

For double staining with Dhh antibody and S-100 antibody (marker for Schwann cells) (Stefansson et al., 1982), nerve sections were fixed with 4% paraformaldehyde for 20 min and then were incubated for 10 min with blocking solution. The sections were incubated with S-100 antibody (rabbit polyclonal, ready-to-use; Ylem, Italy) for 30 min, followed by 1 h incubation with Dhh antibody (goat polyclonal, diluted 1:20; Santa Cruz Biotechnology).

All sections were then incubated with a mixture of affinity purified FITC-conjugated donkey antigoat IgG (diluted 1:50; Chemicon International) and TRITC-conjugated swine antirabbit IgG (diluted 1:50; Dako Cytomation) for 1 h and were mounted with fluorescent mounting solution (Dako Cytomation).

There was no positive staining in the following negative controls: (1) omission of the primary antibodies; (2) incubation of the sections with either Dhh or Ptc2 antibody followed by incubation with TRITC-conjugated swine antirabbit IgG; (3) incubation of the sections with either GFAP or S-100 antibody followed by incubation with FITC-conjugated donkey antigoat IgG.

Macrophage Staining. Formalin-fixed, paraffin embedded sections from injured sciatic nerves of adult WT and dhh-null mice sacrificed at day 7 postinjury were labeled with biotinylated Ricinus communis agglutinin-1 lectin (RCA-1; diluted 1:5000; Vector Laboratories, Burlingame, CA) to detect the macrophages inside the nerves fascicles as described elsewhere (Eto et al., 2003). Briefly, after blocking with 0.03% hydrogen peroxide for 10 min, the tissue sections were incubated with biotinylated RCA-1 for 1 h. The sections were then incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 5 min. Reactivity was visualized with 3′,3′-diaminobenzidine (DAB) as the chromogen, yielding a brown reaction product. Labeled cells, identifiable morphologically as macrophages in the total number of nerve fascicles were counted.

mRNA Extraction and Semiquantitative RT-PCR

Sciatic Nerves of WT Mice. Total RNA was extracted from freshly frozen sciatic nerves, and precipitated using a Quick Prep mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The RNA sample was reverse transcribed into cDNA in a 13 µL reaction mixture containing 11 µL bulk first-strand cDNA reaction mixture, 1 µL DTT solution, and 1 µL Pd(N)6 primer using a First-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). The amount of cDNA synthesized from each sample was normalized by PCR amplification using specific primers for 18S rRNA. cDNA (3 µL) was used in 50 µL PCR reactions using the hot start amplification method (QIAGEN GmbH, Hilden, Germany). An optimum number of amplification cycles were determined for each gene to be in the exponential phase. The following primer pairs with the related product size, annealing temperature, and optimum number of amplification cycles were used in PCR reactions: dhh primers: forward: 5′-CATGTGGCCGGAGTACGCC-3′, reverse: 5′-CGCTGCA- TACAGCGGCCAGTA-3′, 346 bp, 66°C (Parmantier et al., 1999), 35 cycles; ptc1 primers: forward: 5′-AACAAAT TCAAACAAACCTCT-3′, reverse: 5′-TGTCCTCATCCTCGAT GTGATG-3′, 244 bp, 55°C (Takabatake et al., 1997), 38 cycles; ptc2 primers: forward: 5′-TGCCGACTCATC CGTCAA-3′, reverse: 5′-AAGTTCGAAGGACCACCAT ATG-3′, 112 bp, 60°C (Yamago et al., 2001), 38 cycles; smo primers: forward: 5′-ATCGGATCACACCGAAGTA GG-3′, reverse: 5′-GCAACAGGTTCCATATGG-3′, 214 bp, 54°C (Parmantier et al., 1999), 35 cycles; 18S rRNA: forward: 5′-TGTCCTCATCCTCTGA-3′, reverse: 5′-GGGAAAC GCCGTCATTAT-3′, 341 bp, 50°C (Owens and Boyd, 1991), 26 cycles. After PCR amplification, one-tenth of each reaction was analyzed by 2% gel agarose electrophoresis. The PCR product bands were analyzed by computerized densitometric scanning and the NIH image program. In all instances the resulting products were verified to be of the expected size and the authenticity of each one was confirmed by direct sequencing. In each RT-PCR, the control sciatic nerve from each mouse was examined in parallel to the injured nerve. In addition to the intact control nerves, extracted cDNA from mouse nerve-derived Schwann cell cultures or immortalized Schwann cell line cultures (Watabe et al., 1995) was used as positive controls for dhh (Parmantier et al., 1999) and ptc2 (according to our positive results presented afterward) in the PCR experiments. Moreover, the cultures of mouse nerve-derived fibroblasts were used as positive controls for ptc1 (Parmantier et al., 1999) and smo (Parmantier et al., 1999) in the PCR amplifications.

Purified Cultures of Mouse Schwann Cells and Fibroblasts. Total RNA was extracted using Trizol reagent (Invitrogen Ltd, UK) according to the manufacturer’s instructions. The RNA was quantified and analyzed by agarose gel electrophoresis. In general, 500 ng of total RNA was reversed transcribed into cDNA in a 50 µL reaction using random hexamers and superscript II reverse transcriptase. One microliter of each sample was used to assess the relative amount of cDNA synthe-
sized from each sample by PCR amplification using GAPDH primers. For ptc2 expression, 2.5 μg total RNA was used to generate the cDNA. The amplification conditions were: one initial cycle at 94°C for 3 min, followed by 27 cycles for GAPDH and 35 for ptc2 at 94°C for 30 s; 63°C for 30 s (55°C for ptc2, 1 min), 68°C for 30 s (72°C for ptc2), and one final extension at 68°C for 5 min (72°C for ptc2). The primer pairs and the related product sizes were as follows: GAPDH: forward: 5’-ACCACATGTCATGCTAC-3’, reverse: 5’-TCCACCATCTGTTGCTGA-3’, 452 bp (SSH protocol, www.clontech.com); ptc2: forward: 5’-TGCCTCTCAGGAGGCTTCC-3’, reverse: 5’-CAGTTCTCTGTG-CCAGTGCA-3’, 208 bp (Takabatake et al., 1997).

**Light Microscopy and Morphometric Studies**

Nerves were fixed in 3% glutaraldehyde in 0.125 M cacodylate buffer (pH 7.4), washed, osmicated, and embedded in epon. Semithin transverse sections from both injured and control nerves of WT and dhh-null mice, stained with toluidine-blue, were used for light microscopy and morphometric analysis. For morphometric analysis, the entire nerve cross-section was photographed at a magnification of 400× (totaling 10–12 fields per nerve). Photographic prints with final magnification of 1000× were produced. The myelinated fibers were counted, the diameter of each myelinated nerve fiber was measured, and cumulative histograms were constructed using the Zeiss TGA10 analyzer. The results are explained as the mean ± SD. The nerve fibers were sorted according to their fiber diameter as small (<3 μm in diameter), medium (3–7 μm in diameter), and large (>7 μm in diameter).

The number of minifascicles in the injured nerves (28 days after injury) of dhh-null mice was counted under 1000× magnification. For comparison, the intact sciatic nerves of the contralateral side of the injured nerve were used.

We used OPEN LAB software (Improvision; Scientific Imaging, Lexington, MA) and electron micrographs to measure the axon diameter and fiber diameter (axon plus Schwann cell) of the myelinated nerve fibers in intact nerves of dhh-null and dhh-heterozygous mouse littermates. The G-ratio was calculated as the ratio of axon diameter to total fiber diameter.

**Statistical Analysis**

Data were analyzed by using one-way analysis of variance (ANOVA), Newman-Keuls posthoc test, Student’s t-test, Kolmogorov-Smirnov test, and Mann-Whitney U test where appropriate. A value of \( p < 0.05 \) was considered significant.

**RESULTS**

**Ptc2 Was Expressed in the PNS of Adult WT Mice**

We had previously found by RT-PCR and in situ hybridization that ptc1 was expressed in the perineurial cells of developing mouse nerves, but that expression dropped to much lower levels in adult nerves (Parmantier et al., 1999). To investigate the main Dhh receptors that are expressed in peripheral nerves of adult mice, we studied the mRNA expression of Ptc2 receptor in comparison to Ptc1. We found that ptc2 mRNA was expressed in sciatic nerves of adult mice, while ptc1 mRNA was undetectable, using the same experimental condition as ptc2 (see Materials and Methods) [Fig. 1(A)]. However, using higher amounts of starting mRNA at a higher PCR cycle number, ptc1 mRNA was weakly detected, in agreement with our previous results (data not shown) (Parmantier et al., 1999). To study the cells that express Ptc2, we stained the adult sciatic nerves of WT mice with Ptc2 antibody. Ptc2 protein was confined to unmyelinated fibers in teased preparations of adult sciatic nerves by immunofluorescence, being absent from myelinated fibers [Fig. 1(B,C)]. Moreover, RT-PCR revealed that the ptc2 mRNA was clearly found in purified cultured Schwann cells, and at much lower levels in purified nerve fibroblasts [Fig. 1(A)]. ptc2 mRNA was not detectable in mouse nerve fibroblasts at 35 PCR amplification cycles (identical cycles to PCR on purified Schwann cell cultures), but was weakly detectable at 40 cycles [Fig. 1(A)], implying a lower level of ptc2 mRNA expression in nerve fibroblasts than in Schwann cells.

**dhh and ptc2 mRNA Levels Show Parallel Changes in Degenerating and Regenerating Peripheral Nerves**

After identifying and localizing the main Dhh receptors in adult WT mouse nerves in normal condition, we induced crush injury to the sciatic nerves of adult WT mice, in order to investigate the mRNA expression changes of Dhh and its receptors after injury. Then we performed semiquantitative RT-PCR analysis on nerve stumps distal to the injury at various time points following injury, designed to encompass both the degenerating and regenerating phases of the process (Vaughan, 1992). When compared with control uninjured nerves, RT-PCR revealed a dramatic decrease of both dhh and ptc2 mRNA expression in injured nerves between day 2 and day 7 after injury [Fig. 2(A)]. The dhh and ptc2 mRNA levels increased gradually at days 14, 28, and 42 following injury [Fig. 2(A)]. There was no significant difference in dhh and ptc2 mRNA levels during this period in control nerves (\( p > 0.05 \), ANOVA, Newman-Keuls posthoc test) [Fig. 2(A)]. The dhh mRNA levels in injured nerves were significantly lower than those in control nerves at days 2, 7, 14, and 28 postinjury (\( p < 0.05 \), Mann-Whitney U test)
The ptc2 mRNA levels in injured nerves were also significantly lower than those in control nerves at days 7, 14, and 28 postinjury (*p < 0.05, Mann-Whitney U test) [Fig. 2(C)]. The smo mRNA levels did not change after injury up to day 42 [Fig. 2(A)], and the ptc1 mRNA signal was undetectable in both injured and control nerves at the same PCR amplification cycle number as that used for ptc2 [Fig. 2(A)].

To study the nature of the cells expressing Dhh and Ptc2 molecules before and after injury, we immunofluorescence double stained the intact nerves and injured nerves of postinjury day 2 (from degeneration phase) and day 28 (from regeneration phase) with Schwann cell markers and antibodies against Dhh or Ptc2. In both cross and longitudinal sections of nerves before and after injury, Ptc2 staining was found colocalized with the staining of GFAP antibody, which is the marker of nonmyelinating Schwann cells (Jessen and Mirsky, 1984). The double stained longitudinal sections of intact and injured nerves are shown in Figure 3. The trends of changes of Ptc2 protein staining during degeneration and regeneration were similar to Ptc2 mRNA changes. Ptc2 staining was detectable on intact nerves [Fig. 3(A)], however, it was faintly stained on nerve samples of day 2 [Fig. 3(D)] after injury and appeared to be stained more intensely at day 28 after injury [Fig. 3(G)] compared to day 2 after injury. Weak staining of Dhh protein was detected on Schwann cells, colocalized with most of the S-100 positive Schwann cells, which is compatible with previous studies (Bitgood and McMahon, 1995;
though we could not detect positive staining with Dhh antibody in injured nerves up to day 28 after injury (data not shown).

Small Myelinated Fibers Were Significantly Increased in Intact Nerves of dhh-Null Mice

By studying the histopathological changes in transverse sections of injured nerves, we monitored whether the degeneration and regeneration happened in different time points after injury in our injury model in WT mice. Furthermore, to study the possible differences in degeneration and regeneration caused by loss of Dhh signaling, we took advantage of dhh-null mice (Bitgood et al., 1996). In these mice, we previously showed significant differences in the organization of the nerve connective tissue sheaths in normal nerve (Parmantier et al., 1999), while nerve fiber conduction velocity was slightly but not significantly slowed.

Figure 2 Injury-induced changes in mRNA levels for dhh, ptc1, ptc2, and smo in sciatic nerves of WT mice. (A) Expression levels of dhh and ptc2 mRNA decreased from 2 days after injury. They were undetectable at day 7 and were up-regulated at days 14 to 42 postinjury. In contrast, smo mRNA levels did not change after injury. ptc1 mRNA levels were undetectable in injured and control nerves under the same experimental condition as ptc2. (B) Quantification of mRNA levels showed that dhh/18S mRNA ratios were significantly lower at days 2, 7, 14, and 28 after injury when compared with control nerves. (C) ptc2/18S mRNA ratios were significantly lower at days 7, 14, and 28 following injury as compared with those in controls. In histograms, data are given as mean ± SEM and significant differences (p < 0.05) when compared with controls are indicated by asterisk.
We first compared nerve sections from uninjured nerves of dhh-null mice with those of WT mice. In transverse semithin sections, the myelinated fibers of dhh-null mice did not show obvious morphological differences from those in WT mice [Fig. 4(A,B)]. Similarly, the density of myelinated fibers in the nerves of dhh-null mice (15,925 ± 734 fibers/sq.mm) was not significantly different from that of WT mice (15,288 ± 991 fibers/sq.mm) (p > 0.05, Mann-Whitney U test) [Fig. 5(A)]. Furthermore, the G-ratios (see Materials and Methods for definition) of myelinated nerve fibers in dhh-null mice (0.641 ± 0.096) were not statistically different from those in dhh-heterozygous littermates (0.639 ± 0.098) (p > 0.05, Student’s t-test), indicating that Dhh is not likely to change the myelin sheath thickness relative to axon diameter. However, there was a significant difference in the myelinated fiber size distributions between dhh-null and WT mice (p < 0.05, two-sample Kolmogorov-Smirnov test). A significant reduction in the proportion of large myelinated fibers (>7 μm in diameter) was observed in dhh-null mice [Fig. 5(A)], which was apparently balanced by an equivalent significant increase in the proportion of small myelinated fibers (<3 μm in diameter) (p < 0.05, Mann-Whitney U test) [Fig. 5(A)]. Moreover, the nerve fiber diameters in dhh-null mice (4.58 ± 2.01 μm) were significantly smaller than those in WT mice (5.58 ± 2.3 μm) (p < 0.05, Student’s t-test). The results indicate a modest shift in diameter size towards smaller fibers in dhh-null mice.

**Dhh-Null Mice Showed More Severe Degeneration Compared to WT Mice Following Crush Injury**

To investigate whether the Dhh signaling pathway is involved in nerve degeneration and regeneration after...
injury, we compared the response of WT and dhh-null mice to crush injury. In crushed nerves, differences between dhh-null and WT nerves in response to injury were seen as early as 2 days after crush. Degenerating changes including axonal fragmentation, reduced number of identifiable myelinated fibers, myelin ovoid formation, and infiltrating of macrophages were found in the nerves of both dhh-null and WT mice following crush injury [Fig. 4(C–F)]. The injured nerves of dhh-null mice had fewer identifiable remaining myelinated fibers by days 2 and 7 following injury [Fig. 4(C,E)] compared with those of WT mice [Fig. 4(D,F)]. In order to quantify and compare the density of myelinated fibers in dhh-null and WT nerves in degeneration phase, morphometric measurements were done on injured nerves at day 2 postinjury. The density of myelinated fibers in dhh-null mice (5952 ± 572 fibers/sq.mm) was significantly lower than that in WT mice at day 2 after injury (8485 ± 878 fibers/sq.mm) (p < 0.05, Mann-Whitney U test) [Fig. 5(B)]. As macrophage response to injury is one of the fundamental changes in Wallerian degeneration (Griffin and Hoffman, 1993), we stained and compared the number of macrophages inside the injured nerves of dhh-null and WT mice at day 7 following injury. The macrophages reacted strongly with RCA-1 in injured nerves of both WT and dhh-null mice (Fig. 6). The macrophages inside the total number of nerve fascicles in the dhh-
null mice (98 ± 12) were significantly more numerous than those in WT mice (24 ± 8) at day 7 postinjury (p < 0.05, Mann-Whitney U test) (Fig. 6). Much lower numbers of macrophages were also observed in intact nerves of dhh-null and WT, but the macrophages were more numerous in dhh-null intact nerves (S. Sharghi-Namini, K.R. Jessen, R. Mirsky, unpublished observations).

**Regeneration Occurred in Injured dhh-Null Nerves**

Small regenerating axons appeared in the injured nerves of dhh-null and WT mice at day 28 following injury [Fig. 4(G,H)]. In line with histological findings, the distribution histograms of myelinated fiber diameters in both WT and dhh-null mice were skewed to the left at day 28 postinjury in comparison with intact nerves (p < 0.05, two-sample Kolmogorov-Smirnov test) [Fig. 5(C)], indicating the significant increase in small fibers. The density of total myelinated fibers in the injured nerves of dhh-null mice (14,508 ± 668 fibers/sq.mm) was not significantly different from that in WT mice (11,606 ± 971 fibers/sq.mm) (p > 0.05, Mann-Whitney U test) [Fig. 5(C)]. The density of small myelinated fibers of dhh-null mice (<3 μm in diameter) was, however, significantly greater than that of WT mice (p < 0.05, Mann-Whitney U test) [Fig. 5(C)]. Nevertheless, in comparison with the respective intact nerves, the increase in small myelinating fibers at day 28 postinjury was not significantly different between dhh-null mice and WT mice. This observation indicates that at day 28 post-injury, the greater number of small myelinated fibers in dhh-null mice is not due to more regeneration. The number of minifascicles at day 28 following injury in nerves of dhh-null mice (794 ± 151) was significantly more than that in intact nerves of dhh-null mice (262 ± 74) (p < 0.05, Mann-Whitney U test) [Fig. 4(A,G)].

**DISCUSSION**

In the present study we have shown that: (1) the Dhh-receptor ptc2 is expressed in adult nerves but ptc1 is undetectable under the same experimental condition as ptc2; (2) the expression of both dhh and ptc2 is regulated in degenerating and regenerating nerves; and (3) when injured nerves are compared after crush injury in dhh-null and WT mice, degeneration of myelinated fibers following injury is more severe in dhh-null mice than in WT animals, and that this is accompanied by a large increase in the number of macrophages in dhh-null nerves relative to WT nerves.
Ptc2 Expression in Intact Nerves of Adult WT Mouse

Ptc2, similar to Ptc1, can bind to all Hh proteins and can form a complex with Smo. The expression pattern of Ptc2 does not fully overlap with that of Ptc1 and they appear to have distinct roles in the Hh signaling pathway (Carpenter et al., 1998; Motoyama et al., 1998a). Tissue distribution analysis indicates that ptc2 is preferentially expressed in the skin and testis, where it is likely to mediate the action of the Dhh signal (Carpenter et al., 1998). Ptc2 mRNA is highly expressed in spermatocytes, colocalized with other Dhh signaling mediators like fused and members of Gli family, while only low levels of ptc1 could be detected on the Leydig cells (Carpenter et al., 1998). Moreover, using ptc1-null mouse cells, ptc2 splice variants could reconstitute the Dhh-dependent transcriptional response (Rahnama et al., 2004). Therefore, Ptc2 is likely to have an important role in mediating Dhh function in testis (Carpenter et al., 1998).

We previously found ptc1 mRNA in epineurial/peri-neurial precursors of developing peripheral nerves, although as discussed above, ptc1 mRNA was barely detectable in adult nerves (Parmantier et al., 1999). Similarly, we detected both dhh and ptc2 mRNA in sciatic nerves of adult WT mice, but ptc1 mRNA was not detected under the same experimental condition as ptc2 and it was detected only at a very high cycle number and by using higher amounts of starting mRNA in RT-PCR. We confirmed ptc2 mRNA expression in both Schwann cells and at much lower levels in nerve-derived fibroblasts in vitro. Ptc2 protein reactivity was also found on unmyelinated fibers of mouse sciatic nerves. Taken together, these results raise the possibility that the Dhh-Ptc2 signaling may play a role in the maintenance of the PNS of adult mice, however, the details of the signaling pathway remain to be investigated.

Morphometric Differences of PNS between dhh-Null and WT Mice

We have reported a patient with a dhh missense mutation, who developed polyneuropathy and showed a decreased density of myelinated fibers with mini-fascicular formation in sural nerve biopsy (Umehara et al., 2000). These findings suggest that lack of Dhh may affect the development or stability of myelinated fibers as well as perineurial formation in the PNS. In the present study, dhh-null nerves showed an increase in the proportion of smaller fibers and a concomitant decrease of large myelinated fibers compared with WT mice. Moreover, the myelinated fibers in dhh-null mice were significantly smaller in diameter than fibers in WT mice. These findings might indicate an underlying signaling defect related to the caliber of the myelinated fibers of dhh-null mice. A chronological study of neuropathology in dhh-null mice is now underway.

Serial Changes of dhh- ptc2 mRNA in Nerve Degeneration and Regeneration

In our crush injury model of WT mice, dhh and ptc2 mRNA levels showed a marked decline at days 2 and 7, which coincided with axonal degeneration in the distal part of injured nerves (Vaughan, 1992). Both dhh and ptc2 mRNA levels gradually increased during the regeneration phase, when axonal-Schwann
cell interactions are re-established (days 14 to 42) (Jessen and Mirsky, 2002).

Schwann cells undergo rapid changes in molecular composition and number in response to nerve degeneration and regeneration after injury (Griffin and Hoffman, 1993). The dhh and ptc2 mRNA level changes after injury may be due to one of these major changes in Schwann cells in the distal part of the injury: (1) the changes of Schwann cell numbers after injury underlie the changes in the dhh and ptc2 mRNA levels postinjury; (2) the altered expression levels of dhh and ptc2 in the Schwann cells following injury underlie the observed changes in dhh and ptc2 mRNA levels. Concerning the first possibility, several previous reports have shown that Schwann cells within the degenerating region proliferate rapidly within the first week after injury (Scherer and Slazer, 2001; Jessen and Mirsky, 2004; Clemence et al., 1989). This is not in parallel with a marked decrease of dhh and ptc2 in the first week following injury in our study. Moreover, within 3 to 4 weeks after injury, Schwann cells associated with regenerating axons begin to stop dividing (Pellegrino and Spencer, 1985; Clemence et al., 1989; Chandross, 1998). This is also not consistent with the increase of dhh and ptc2 mRNA levels in the regeneration phase in our study (days 14 to 42). Therefore, it is unlikely that the changes in the number of Schwann cells underlie the observed dhh and ptc2 mRNA signal changes after injury. Accordingly, we supposed that the changes in expression levels of dhh and ptc2 mRNA in Schwann cells underlie their mRNA signal alteration in our RT-PCR experiments.

Similar temporal expression patterns have been reported for ciliary neurotrophic factor (CNTF) (Lee et al., 1995) and myelin proteins (LeBlanc and Poduslo, 1990; Gupta et al., 1993; Jessen and Mirsky, 2002) after crush injuries. Both CNTF and myelin proteins, like Dhh, are produced in Schwann cells. It has been proposed that the expression of CNTF and myelin proteins is controlled by axonal-Schwann cell interactions, which are interrupted by axonal degeneration and are re-established in regeneration (LeBlanc and Poduslo, 1990; Gupta et al., 1993; Lee et al., 1995). Given the similar temporal expression patterns of dhh and ptc2 after crush injury, it is likely that expression of both dhh and ptc2 is also regulated by axon-Schwann cell interactions.

The expression of neurotrophic factors is regulated during Wallerian degeneration and regeneration (Funakoshi et al., 1993). Reciprocal interactions between the Dhh related protein, Shh, and neurotrophic factors such as nerve growth factor (Cotrina et al., 2000; Reilly et al., 2002) and neurotrophin-3 (Dutton et al., 1999) have been reported in neuronal survival and death. It is therefore possible that the expression of dhh and ptc2 may also be affected by the alteration of some neurotrophic factors after injury.

The colocalization of Ptc2 immunofluorescence staining with GFAP staining in intact nerves and after injury in both degeneration and regeneration phase shows that the nature of Ptc2-expressing cells is the same before and after injury. The failure to detect Dhh protein expression in injured nerves might be due to the low expression of Dhh protein up to day 28 after injury.

In our previous study, smo expression levels remained relatively unchanged with developmental age in mouse sciatic nerve (Parmantier et al., 1999). Furthermore, the treatment of nerve-derived fibroblast cultures with Dhh protein also did not change the smo mRNA expression levels (Parmantier et al., 1999). In the present study, the smo mRNA levels were unchanged at different time points after injury. These findings suggest that smo expression levels may not be affected by different levels of Dhh protein in mouse peripheral nerves.

Possible Vulnerability of dhh-Null Mice PNS to Crush Injury

In the degeneration phase after nerve injury, we found more severe myelin and axonal damage associated with increased macrophage infiltration in dhh-null mice compared with WT mice. The perineurium of dhh-null mice is structurally abnormal, and it is functionally defective, allowing the entry of both proteins and inflammatory cells (Parmantier et al., 1999). The large influx of macrophages in dhh-null nerves after injury may be partially related to their defective perineurial barrier, and this in turn may lead to more degeneration. It is well-established that macrophages contribute to Wallerian degeneration after nerve injury (Beuche and Friede, 1984), though the role of macrophages remains to be established definitively in our model.

Although the insult to the nerves in our injury model was equal in WT and dhh-null mice, the structural abnormalities in peripheral nerve sheaths of dhh-null mice (Parmantier et al., 1999) could make them more vulnerable and consequently induce more severe damage. More degeneration in dhh-null mice may also be a result of the absence of Dhh protein in mutant mice. However, both structural abnormalities and the absence of Dhh signaling may participate in severe degeneration in dhh-null injured nerves and these two possibilities are not mutually exclusive. Nevertheless, the Dhh signaling pathway and other
probable related pathways in the process of degeneration and regeneration are not well understood and need further investigation.

Injured nerves of dhh-null mice showed more extensive minifascicle formation in the regeneration phase, compared with intact nerves. Minifascicular formation in peripheral nerves has been reported in injury models when the perineurium is destroyed or resected (Popovic et al., 1994; Terho et al., 2002). Similarly, vulnerability of the perineurium in the PNS of dhh-null mice may result in extensive minifascicular formation after injury.

Despite the lack of Dhh, nerve fiber regeneration was not obviously delayed in dhh-null mice, compared with WT mice. The appearance of regenerating fibers in dhh-null nerves (day 42) suggests that Dhh is not a major player in nerve regeneration at least at later stages. Recent studies have demonstrated that administration of exogenous Shh protein enhanced the speed of nerve recovery after injury at early time points after injury (Pepinsky et al., 2002) and had therapeutic effects in the treatment of diabetic neuropathy (Calcott et al., 2003). Therefore, further studies will be required to determine whether nerve regeneration and reinnervation proceed on schedule in dhh-null mice.

In conclusion, the persistent expression of dhh and ptc2 in the PNS of adult WT mice and the remarkable changes in their mRNA expression following injury suggest that the Dhh-Ptc2 signaling pathway may be involved in the maintenance of adult nerves and may be one of the factors that directly or indirectly determines the response of peripheral nerves to injury. Further investigations on the Dhh signaling pathway, such as studying the outcome after application of Dhh protein to the dhh-null nerves following injury or the blockade of hedgehog signaling with inhibitors like cyclopamine in the injured WT nerves, are warranted to elucidate the mechanism of Dhh signaling in nerve degeneration and regeneration.

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REFERENCES


