Nociceptive nerves innervate the skin and play an important role in the generation of neuropathic pain. However, it remains elusive whether and how nociceptive nerve terminals degenerate in neuropathic pain conditions. To address this issue, we investigated cutaneous innervation in a model of painful mononeuropathy, the chronic constriction injury (CCI). The hind paws of rats were immunocytochemically stained with a pan-axonal marker, protein gene product 9.5 (PGP 9.5). Within 2 days after CCI, rats exhibited thermal hyperalgesia, and there was a partial depletion of epidermal nerves. The extent of reduction in epidermal nerves after CCI was variable with an epidermal nerve density of 3.65 ± 1.97 fibers/mm (compared to 15.39 ± 1.58 fibers/mm on the control side, P < 0.02). There was a mild but concomitant increase in PGP 9.5 (1) Langerhans cells in the epidermis of the skin with CCI (10.19 ± 1.99 vs 7.75 ± 1.36 cells/mm, P < 0.05). In the skin denervated by tight ligation of the sciatic nerve, epidermal nerves were completely depleted (0 fibers/mm vs 12.26 ± 1.44 fibers/mm on the control side, P < 0.001). Animals with tight ligation of the sciatic nerve exhibited thermal anesthesia. These findings suggest that the epidermis is partially denervated in CCI, and that a partial injury of nerves is correlated with the development of neuropathic pain.

Key Words: nerve degeneration; chronic constriction injury; neuropathic pain; Wallerian degeneration; painful neuropathy; skin innervation; epidermal nerves; animal model; small-fiber neuropathy; Langerhans cell.

INTRODUCTION

Nerve degeneration plays an important role in the generation of neuropathic pain, particularly the speed of nerve breakdown (12). However, correlations between neuropathic pain and types of degenerated nerves remain controversial (33, 44). Sensory neurons and their axons are classified into large-diameter nerves and small-diameter sensory nerves according to their functions and cytoskeletal organization (7, 42). Terminals of small-diameter nerves are responsible for nociception and reside in the epidermis of the skin. Multiple mechanisms at different levels of the neural axis contribute to the generation of neuropathic pain (2, 6, 11). The role of large-diameter nerve degeneration in neuropathic pain has been elucidated (33, 43, 44). A further issue is whether the injury of small-diameter sensory nerves is associated with neuropathic pain.

Previous studies were mainly focused on the excitability of dorsal horns, cellular responses in sensory neurons, and degeneration of sensory nerves. The generation of neuropathic pain signals starts from sensory receptors or cutaneous nerve terminals. To date, there have been rare studies addressing the roles of sensory nerve terminals in painful neuropathy (20, 38). This is mainly due to the fact that examinations of small-diameter nerves usually require ultrastructural studies (28, 29). We have demonstrated the epidermal innervation of the skin by immunocytochemistry with various markers under light microscopy (22, 31). These neuronal proteins include calcitonin gene-related peptide, substance P, and protein gene product 9.5 (PGP 9.5). PGP 9.5 is a ubiquitin carboxy hydrolase, which is abundantly present in the nervous system, particularly epidermal nerves (8, 23, 34, 50, 51).

Painful mononeuropathy can be induced by placing loose ligatures on the sciatic nerve, i.e., the chronic constriction injury (CCI) (4). The CCI model produces thermal hyperalgesia, and mimics many aspects of painful neuropathies (3). The sciatic nerve innervates the plantar side (glabrous skin) of the hind paws in rats. Nerves distal to the cut end degenerate in a stereotyped fashion, Wallerian degeneration. Because epidermal nerves are completely depleted after nerve transection (22), these results raise the question of whether epidermal nerves degenerate in CCI? In addition, whether or not degeneration of small-diameter sensory nerve terminals is related to neuropathic pain.

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remains unanswered. Do different kinds of nerve injury result in neuropathic pain of the same degree? To address these issues, we compared the contribution of different nerve injuries to neuropathic pain behaviors, i.e., CCI vs tight ligation of the sciatic nerve.

**MATERIALS AND METHODS**

**Animal Surgery**

We performed two kinds of animal surgery to compare cutaneous innervation, i.e., chronic constriction injury and tight ligation of the sciatic nerve. For each animal, the surgery was performed on one side, with the other side sham-operated. To avoid mechanical damage to the paw skin, animals were housed in plastic cages. The experiments followed the guidelines of the International Association for the Study of Pain (IASP) (1983).

Chronic constriction injury (CCI). The animals with generated neuropathic pain were male 8-week-old Sprague–Dawley rats (200–250 g) following the Bennett model (4). Briefly, four ligatures (4.0 chromic gut) were placed loosely around the sciatic nerve at the midthigh level with a spacing of 1 mm. The ligatures were tightened until they just constricted the sciatic nerve, retarded the circulation through the superficial epineurial vasculature, and sometimes induced a brief twitch in the surrounding muscles.

Tight ligation of the sciatic nerve. This procedure was carried out in male 8-week-old Sprague–Dawley rats, and was similar to what has been established in the sciatic nerve transection model (24). Under chloral hydrate anesthesia, the sciatic nerve on one side was tightly ligated at the thigh level. The sciatures were tightened until they just constricted the sciatic nerve, retarded the circulation through the superficial epineurial vasculature, and sometimes induced a brief twitch in the surrounding muscles.

**Behavioral Testing**

The evaluation of thermal hyperalgesia followed the established method of paw-withdrawal test by Hargreaves et al. (Ugo Basile, Comerio, Italy) (4, 17, 32). Investigators were blinded to the surgical status of each animal. Rats were placed in a plastic box on a 3-mm-thick glass plate. An infrared source was placed directly under the plantar surface of the hind paw of rats. The paw-withdrawal latency was defined as the time elapsed from the onset of radiant heat stimulation to withdrawal of the hind paw. Latencies were measured to the nearest 0.1 s. The radiant heat source was adjusted to result in a baseline latency of 8–10 s, and a cut-off time of 18 s was set to avoid possible tissue damage. Each paw was tested five times, with a 5-min interval between consecutive tests. The five latencies per side were averaged and a difference score was calculated by subtracting the latency of the control side from the latency of the surgery side.

**Immunocytochemistry**

For immunocytochemistry on freezing microtome sections (8), animals were fixed by intracardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The skin areas innervated by the sciatic nerve were fixed for another 6 h, and then changed to PB for storage. After thorough rinsing in PB, samples were cryoprotected with 30% sucrose in PB overnight. Sections perpendicular to the epidermis were cut at 30 μm on a sliding microtome, labeled sequentially, and stored at −20°C. To ensure adequate sampling, every fourth section for each tissue was chosen for immunocytochemistry. The sections were treated with 0.5% Triton X-100 in 0.5 M Tris buffer (pH 7.6) (Tris) for 30 min and processed for immunostaining. Briefly, sections were quenched with 1% H2O2 in methanol, and blocked with 5% normal goat serum in 0.5% nonfat dry milk/Tris. The sections were incubated with rabbit antiserum to PGP 9.5 (UltraClone, UK, 1:1000 diluted in 1% normal serum/Tris) for 16–24 h. After rinsing in Tris, sections were incubated with biotinylated goat anti-rabbit IgG for 1 h, and the avidin–biotin complex (Vector, Burlingame, CA) for another hour. The reaction product was demonstrated by 3,3'-diaminobenzidine (DAB, Sigma, St Louis, MO).

**Quantitation of Epidermal Innervation and Langerhans Cells**

Epidermal innervation was quantified according to modified protocols in a coded fashion (22, 37). PGP 9.5-immunoreactive nerves in the epidermis of each footpad were counted at a magnification of 40× with an Olympus BX40 microscope (Shibuya-ku, Japan). Each individual nerve with branching points inside the epidermis was counted as one. For epidermal nerves with branching points in the dermis, each individual nerve was counted separately. The total length of the epidermis along the upper margin of the stratum corneum in each footpad was measured with the Image-Pro PLUS system (Media Cybernetics, Silver Spring, MD). Epidermal nerve density was therefore derived and expressed as the number of fibers per millimeter of epidermal length. Langerhans cells immunoreactive for PGP 9.5 were counted, and the abundance was expressed as the number of Langerhans cells per millimeter of epidermal length. Every fourth section of each tissue was quantified, and there were three sections for each footpad.

**Statistical Analysis**

The quantitation was performed in a blinded fashion. Groups of five animals were used at various time intervals after surgery. Data were expressed as the mean ± SD. Analyses included t test and Wilcoxon rank sum test between the control side and the injury
difference in withdrawal latency was observed in rats with CCI, indicating thermal hyperalgesia. Beginning from 2 days post-CCI, rats showed significant decreases in withdrawal latency. Data are expressed as the mean ± SD, and the dotted lines correspond to the range of withdrawal latency difference in the controls.

RESULTS

Nociceptive Behaviors in Chronic Constriction Injury

Before surgery, the difference in withdrawal latency between the right and the left sides followed a normal distribution with 0.041 ± 0.825 s (P = 0.9). Within 2 days of CCI, rats exhibited thermal hyperalgesia. The difference in withdrawal latency was −5.920 ± 1.428 s (P < 0.001, Fig. 1). The thermal hyperalgesia persisted during the experimental period (up to 28 days post-CCI). Maximal withdrawal latency differences were found between 7–14 days post-CCI: with −6.783 ± 1.935 s (P < 0.001) at 7 days post-CCI, and −6.263 ± 1.825 s at 14 days post-CCI (Fig. 1). Rats with tight ligation of the sciatic nerve showed complete anesthesia to thermal stimuli immediately after surgery.

Skin Innervation in Painful Mononeuropathy

The epidermis of the normal skin was innervated by PGP 9.5 (+) nerves (Fig. 2A). Epidermal nerves originated from the subepidermal nerve plexus paralleling the epidermal-dermal borders. Individual PGP 9.5 (+) nerves ascended perpendicularly in the epidermis and had typical varicosities (Fig. 2A). Some Langerhans cells, the epidermal antigen-presenting cells, were immunoreactive for PGP 9.5, consistent with previous observations (23).

In the skin of animals with CCI, there were signs suggesting degeneration of cutaneous nerves. Epidermal nerves were moderately depleted, and there was up-regulation of PGP 9.5 in epidermal Langerhans cells (Fig. 2B) (24). In the dermis, some individual dermal nerves either lost PGP 9.5-immunoreactivity or became fragmented. The signs of epidermal nerve degeneration and dermal nerve degeneration began from 2 days post-CCI and persisted throughout the experimental period.

We investigated the effects of sciatic nerve tight ligation on the skin. This procedure produced a total loss of epidermal nerves and a complete degeneration of dermal nerves (Fig. 2C). In the skin of animals with tight ligation of the sciatic nerves, all epidermal nerves disappeared, with up-regulation of PGP 9.5 in epidermal Langerhans cells (Fig. 2C).

Quantitation of Epidermal Innervation in Painful Mononeuropathy

In rats with CCI, there was a loss of epidermal nerves to varying degree, indicating the nature of partial denervation of footpads. The epidermal nerve density was 15.39 ± 1.58 fibers/mm on the control sides. There was a variable loss of epidermal nerves on 14 days post-CCI with an epidermal nerve density of 3.65 ± 1.97 fibers/mm (P < 0.02, Fig. 3). Further analysis by the linear regression model indicated that there was no apparent correlation between epidermal nerve densities and differences in withdrawal latency in CCI animals. Epidermal nerves were totally depleted in the rats with tight ligation of the sciatic nerves (0 fibers/mm compared to 12.26 ± 1.44 fibers/mm on the control side, P < 0.001, Fig. 3). Taken together, these findings suggested that the CCI and tight ligation are distinct groups and the remaining epidermal nerves are related to thermal hyperalgesia.

Phenotypic Changes in Epidermal Langerhans Cells after CCI

There was a reciprocal increase in PGP 9.5 (+) Langerhans cells after CCI. Fourteen days after CCI in Langerhans cell density was increased compared to that on the control side (10.19 ± 1.99 vs 7.75 ± 1.36 cells/mm, P < 0.05, Fig. 4). The increased in PGP 9.5 (+) Langerhans cell density was more significant in sciatic nerve tight ligation (15.84 ± 0.42 vs 9.69 ± 1.13 cells/mm, P < 0.01, Fig. 4). To eliminate variations in Langerhans cell densities among different animals, we also compared the Langerhans cell density difference between the surgical side and the control side. In rats with CCI, the Langerhans cells density difference mildly increased to 2.44 ± 0.66 cells/mm (P < 0.05), consistent with the previous observation that PGP 9.5 was up-regulated in Langerhans cells of completely denervated skin. The difference in Langerhans cell density was 5.59 ± 0.33 cells/mm with tight ligation (P < 0.01).
DISCUSSION

This report demonstrates that (1) epidermal nerves degenerate in the painful mononeuropathy of CCI, (2) only partial nerve injury results in neuropathic pain, and (3) PGP 9.5 is up-regulated in epidermal Langerhans cells of partially denervated skin by CCI.

Role of Nerve Injury in Generation of Neuropathic Pain

The partial nerve injury model by loose ligation of sciatic nerves results in neuropathic pain behaviors, and the consequence of partial nerve injury is demonstrated by reduced epidermal innervation in the skin territory of the sciatic nerves. In contrast, tight ligation of the sciatic nerve results in anesthesia, and total depletion of epidermal nerves. Clinically, the behaviors of animals with CCI and tight ligation differ. In the CCI animals, the hind foot took a guarding posture, i.e., hyper-flexion of the arch of the hind foot with minimal contact with the floor, presumably to avoid the pain on touching the floor. After tight ligation of the sciatic nerves, animals had a gait with the hind foot dragging (26). These findings indicate that the remaining epidermal nerves in CCI are related to hyperalgesia, and suggest that a partial nerve injury is associated with neuropathic pain.

FIG. 2. Skin innervation in chronic constriction injury (CCI). Skin tissues of footpads from hind paws of rats were immunocytochemically stained with protein gene product 9.5 (PGP 9.5). The panel shows sections from the control (A), CCI (B), and ligation (C) 14 days after surgical procedures. (A) The epidermis of footpads on the control side was richly innervated with PGP 9.5 (+) nerves (arrows), with occasional and faintly stained PGP 9.5 (+) Langerhans cells (arrowhead). (B) In the skin of rats with CCI, there was a variable loss of epidermal nerves, with evident PGP 9.5 (+) Langerhans cells. (C) In the epidermis of rats whose skin was denervated by tight ligation of the sciatic nerve, there were no epidermal nerves but with up-regulation of PGP 9.5 in epidermal Langerhans cells.

FIG. 3. Quantitation of epidermal nerves in chronic constriction injury (CCI). The graph shows the comparison of epidermal nerve densities after CCI and, tight ligation of the sciatic nerve (Tight Lig.). The comparison is based on results 14 days after surgical procedures. Filled bars are for the control sides, and open bars for the surgical sides. Data are expressed as the mean ± SD. *P < 0.02; **P < 0.001.
The relations between the types of nerve fibers loss and the development of neuropathic pain remain intriguing issues. The studies by Sommer et al. suggest that myelinated nerve areas in the sciatic nerves significantly reduced beginning from 1 day post-CCI, and very few myelinated nerves remained on 7 days post-CCI. The loss of unmyelinated nerves in the sciatic nerves followed a similar pattern and began from 3 days post-CCI (33, 43, 44). These studies indicate that both myelinated and unmyelinated nerves are lost in CCI. Because the sciatic nerve is a mixed nerve consisting of motor, sensory, and autonomic axons, it is not clear from these studies whether small-diameter sensory nerves are lost in CCI. The present report clearly demonstrates that small-diameter nociceptive nerves terminating in the epidermis degenerate in CCI. Of course, this finding does not exclude the possibilities that the loss of large-diameter nerve fibers and the synaptic plasticity in the dorsal horn of the spinal cord also contribute to thermal hyperalgesia. For example, Bester et al. observed that tactile stimulation induced c-fos expression in the dorsal horn after sciatic nerve crush. This altered behavior was not detected in animals with intact sciatic nerves (5).

A further issue is to what extent the loss of small-diameter nerves results in neuropathic pain. From this study, it is clear that the total depletion of epidermal nerves results in anesthesia. Only partial injury causes thermal hyperalgesia with no apparent correlation between epidermal nerve density and the difference in withdrawal latency. This finding suggests that there is a threshold in epidermal nerve density. Alternatively, the remaining epidermal nerves may change their neurochemical phenotypes, and become the source of neuropathic pain.

Nevertheless, the current study provides evidence of epidermal nerve degeneration in CCI, which extends what have been observed in human painful neuropathy (19, 20). Recently, skin biopsy has become a new approach to evaluate human sensory neuropathy affecting small-diameter nerves terminating in the skin (16, 25, 39). In patients with various types of painful neuropathy, epidermal nerves were reduced in abundance, but they never became totally depleted (18, 30, 36, 38, 41).

Phenotypic Changes of Epidermal Nerves and Langerhans Cells in Painful Neuropathy

The remaining epidermal nerves in CCI animals are potentially responsible for transmitting nociceptive stimuli. Alternatively they are the source of neuropathic pain. Several lines of evidence suggest that sodium channels are up-regulated or became hyperexcitable in degenerating nerves (10, 14, 35, 49). The alterations of sodium currents in the dorsal root ganglion neurons of CCI animals occur mainly in the small-diameter neurons, and different subtypes of sodium channels are differentially regulated in neuropathic pain (9, 27). We speculate that the remaining epidermal nerves contribute the hyperexcitability, and the phenotypic changes of remaining epidermal nerves will be important for understanding the peripheral contribution of neuropathic pain.

The mechanisms of neuropathic pain in CCI could be diverse. Macrophage infiltration and cytokine production are other contributing factors, for example, tumor necrosis factor-α (TNF-α) (45). TNF-α is up-regulated in the CCI model of neuropathic pain, and inhibitors of TNF-α effectively alleviated neuropathic pain, suggesting that TNF-α is an important mediator of neuropathic pain (15). In this report, we also demonstrate up-regulation of PGP 9.5 (+) Langerhans cells in the epidermis of CCI animals. Langerhans cells are antigen-presenting cells in the epidermis. They have close contact with neural elements (1, 21, 46–48). This is consistent with the observation that epidermal Langerhans cells are a potential marker of nerve injury to the skin (23). Because Langerhans cells are an important source of cytokines or pro-inflammatory molecules, such phenotypic changes provide another dimension to explore the complex mechanisms of neuropathic pain (13, 40).

Conclusion

We demonstrate a partial depletion of epidermal nerves in chronic constriction injury of the sciatic nerves in rats. Rats with CCI show thermal hyperalgesia. In contrast, rats with tight ligation of the sciatic nerves exhibit thermal anesthesia and a total loss of cutaneous nerves. These findings suggest that a partial injury to the nerves and the resultant partial epidermal denervation are related to neuropathic pain.
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REFERENCES


