Generalized depletion of free nerve endings and decrease of cutaneous nervous innervation in streptozotocin-induced painful and painless diabetic rats

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Abstract

Background: The loss of peripheral nerve fiber is evident in chronic painful diabetic neuropathy. However, the correlation between peripheral fiber loss and the genesis of pain is unclear. Using the streptozotocin-induced diabetic rat model and focusing on free nerve endings, we attempted to investigate the peripheral changes that elicit pain syndromes in diabetes.

Methods: Diabetes was induced in rats using 75 mg/kg streptozotocin, while controls received saline solution. “Painful” rats with thermal or mechanical hypersensitivity and “painless” rats (without significant threshold changes) were enrolled. The peripheral nerve endings were immunostained using protein gene product 9.5 in footpad skin sections. The peripheral nerve densities in each behavior group were calculated and averaged.

Results: A progressive loss of protein gene product 9.5-blotted nerve fibers was noted after diabetes was induced and as the duration of hyperglycemia proceeded. Painful and painless diabetic rats have similar histological nerve fiber loss including depleted epidermal free nerve endings.

Conclusion: The results indicated that there are undiscovered pathological changes that are sensitizing the injured nerve fiber in periphery.

Keywords: free nerve endings; innervation density; painful diabetic neuropathy; protein gene product 9.5; streptozotocin

1. Introduction

Painful diabetic neuropathy (PDN) is a common complication of both type 1 and type 2 chronic diabetes. Although its pathophysiology is not clear, most authors believe PDN to be neuropathic in origin.1-3 However, previous investigations focusing on certain classes of peripheral nerve fibers or morphological differences failed to find a correlation between pain generation and peripheral nerve dysfunction in diabetic patients.4-6

In recent years, the cutaneous free nerve ending (FNE) has been increasingly studied in peripheral neuropathic disorders, including diabetic neuropathy. These FNEs are unmyelinated sensory nerve fibers terminating in the epidermal—dermal junction.7 These nerve terminals may play pain-perceptive roles which involve responses to thermal, mechanical, chemical, and nociceptive stimuli.8,9 The cutaneous FNEs have
been demonstrated to be responsible for the majority of neurotransmission induced by nociceptive stimuli, and are putatively able to induce nociception by sensory sensitization. Although it is highly likely that these FNEs play a role in triggering PDN, the correlation between cutaneous FNEs and the pathogenesis of PDN remains unclear.

In order to examine the mechanisms contributing to pain genesis, we investigated the peripheral changes in PDN. First, by using the pan-neural marker protein gene product 9.5 (PGP 9.5) and the widely used streptozotocin (STZ)-induced PDN model, we estimated the changes in PGP 9.5 innervation density using immunohistochemical analysis through the diabetic course. Second, we recorded behavior changes including thermal hyperalgesia, mechanical allodynia, and painless (those without significant threshold changes) in diabetic rats. Last, we examined the density of PGP 9.5 in the footpad skin from different behavioral phenotypes in diabetic rats to elucidate the underlying changes.

2. Methods

2.1. Diabetic animal model

This study was approved by the Animal Care Ethics Committee of the National Defense Medical Center (IACUC-05-031) and was conducted according to the guidelines of the Ethics Committee of the International Association for the Study of Pain. Experimental diabetes was induced in male Sprague–Dawley rats weighing 250–300 g by a single intraperitoneal injection of 75 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA) to achieve a maximal induced diabetic ratio and to maintain a stable, chronic hyperglycemic state. The rats in the control group were of the same gender and weight, and received intraperitoneal saline. Blood glucose levels were checked for 7 consecutive days following STZ injection and at every interval after behavior testing. Glucose was measured with a glucose meter (ADVANTAGE, Roche, Nutley, NJ, USA) using whole blood taken from the ventral tail vein under isoflurane anesthesia. Diabetes was confirmed by a persistently elevated blood glucose level greater than 270 mg/dL (15 mM). All rats were maintained in plastic-bottomed cages covered with sawdust bedding and given free access to sufficient food and water. Colony cages were cleaned daily and were kept on a 12-hour light–dark cycle at 22°C, with an independent air supply. Diabetic rats with body weight that fell below 200 g, ceased significant exploratory activities, or had obvious evidence of infection were excluded from the study and were euthanized.

2.2. Behavioral tests

Behavior tests were conducted according to our previous experiments examining nociception and the analgesic effects of drugs in rodents. Baseline thermal and mechanical thresholds were determined before STZ injection and rechecked every week after the induction of diabetes. Body weight and blood glucose levels were also measured after the behavior tests to ensure a persistent hyperglycemic state.

2.3. Thermal thresholds

Paw withdrawal latency to radiant heat was used to assess thermal responses (using a paw stimulator analgesia meter, model 390; IITC Life Science, Woodland Hills, CA, USA). Rats were placed in dark plastic cages on a glass platform and were kept silent and dry for at least 30 minutes. The heat source was positioned directly beneath the plantar surface (between the first and second pair of footpads) of the hind paws and was adjusted to a mean baseline value about 10 seconds with an activated intensity of 25%, and a cutoff time of 15 seconds to prevent tissue damage. Room temperature was kept between 22°C and 24°C. The hind paws were tested alternately, with at least 8-minute intervals between consecutive tests. A minimum of five tests were performed, and withdrawal latencies were recorded and averaged.

2.4. Mechanical thresholds

Mechanical thresholds were measured directly by paw withdrawal using an electronic von Frey anesthesiometer (model 2290; IITC Life Science). Rats were transferred to dark plastic cages with metal mesh bottoms and were allowed to acclimate for 30 minutes. Gently and gradually increased pressure was applied with no. 14–16 von Frey filaments from underneath the metal mesh to the plantar surface of the hind paws until withdrawal (cutoff = 60 g to prevent tissue damage). The hind paws were tested alternately with at least 8-minute intervals between consecutive tests. A minimum of five tests were performed, and the withdrawal pressures were recorded and averaged.

2.5. Animal groups and preparations

In the first portion of our study, the PGP 9.5 immunoblotted nerve fiber density in footpad skin sections of Sprague–Dawley rats before STZ treatment (baseline) and at 6, 8, 12, 16, and 24 weeks after diabetes was induced, were measured (n = 5 in baseline and at each time point of diabetes). Body weight and blood glucose level at the time point of study (sacrifice) were also recorded. In the study’s second portion, STZ-induced diabetic rats were assigned for observation of the characteristics of painful as well as painless behaviors as the diabetic duration proceeded until 24 weeks.

Rats with a significant decrease in thermal or mechanical thresholds compared with baseline values were considered to have thermal hyperalgesia or mechanical allodynia, respectively. The rats without significant change in either the thermal or mechanical thresholds were considered to have no presence of painful behaviors (painless). The rats with significant increase of thresholds were hypoalgesic.

In the final portion of our study, rats (n ≥ 6 in each group) at 6–8 weeks after the induction of diabetes from different behavioral phenotypes (thermal hyperalgesia, group T; mechanical allodynia, group M; and painless, group N) were investigated. Controls (C) were body weight matched rats that received saline.
2.6. Immunohistochemistry

Immunohistochemical analysis was performed as described in our previous experiments. In brief, rats were deeply anesthetized and euthanized with intraperitoneal thiopental at 10 mg/kg, and then a sternotomy was performed to allow needle cannulation into the left ventricle. Perfusion of 200–250 mL normal saline was followed by 500 mL 4% paraformaldehyde through a perfusion pump (MASTERFLEX, Cole-Parmer, model 7553-70, Vernon Hills, IL, USA). The footpad skin of the bilateral hind paws was harvested and cryoprotected in sucrose overnight. Samples were then washed, fixed, and embedded. Frozen sections were sliced to a thickness of 18–20 μm, with a minimum separation of 80 μm, using an ultramicrotome (Leica CM3000; Leica Microsystems, Wetzlar, Germany) and were stored in phosphate buffer.

Sixteen to 20 sections were selected randomly from each rat and were mounted on a gelatin-coated slide. Samples were washed with Tris-buffered solution (pH 7.4), and then 1% H₂O₂ was added to remove endogenous peroxidase activity. Goat serum with 0.5% Triton X-100 was used to block nonspecific binding. Sections were incubated with the primary antibody (rabbit anti-PGP 9.5, 1:4000; UltraClone, Yarmouth, Isle of Wight, UK) at 4°C overnight. Controls were incubated with buffer only (not shown in figures). After rinsing in Tris-buffered solution, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (1:200; Vector, Peterborough, UK) at room temperature for 30 minutes, followed by incubation of the avidin-biotin complex (1:300; Vector) for another 30 minutes. Subsequently, the reaction products were visualized with 3',3-diamino-benzidine. Thereafter, the 3',3-diamino-benzidine-stained sections were dehydrated, counterstained with methyl green, and then coverslipped.

2.7. Image analysis

Each section was examined under light microscopy by a trained observer who was blinded to the status of the sample’s treatment. The region of interest (ROI) was designated as the papillary dermal and epidermal areas (Fig. 1A). Five to six representative fields containing one or more ROI at 200× magnification were chosen from each section, and the digital images were obtained by using a microscopy-based digital camera system (Microphoto-Fxa; Nikon, Tokyo, Japan). Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD, USA) was used to examine the distribution of PGP 9.5-stained fibers. The PGP 9.5-stained epidermal nerve fiber density (ENFD) was derived and expressed as fibers/mm. Briefly, the length of the epidermal layer along the upper margin of the stratum corneum in each section was measured, and the number of epidermal FNEs was counted to calculate the ENFD. Additionally, PGP 9.5-stained fiber innervation density in dermal and epidermal area was measured. Because of the extensive decrease of blotted intensity in diabetic samples, quantitative analysis was performed by placing a transparent counting grid (modified from Yam et al20) arranged in a regular square array (16 × 12, each square 15 × 15 μm²), over each image. The number of squares with nerve fibers was counted and averaged. The resulting value directly represented the degree of nerve fiber innervation in the ROI of each skin section (Fig. 1B).

2.8. Statistical analysis

Results were presented as mean ± standard deviation (SD). For comparing differences, Mann–Whitney U-test was used.

3. Results

3.1. STZ-induced diabetic rat model and the progressive loss of skin innervation

In our study, a single injection of 75 mg/kg STZ produced a persistent hyperglycemic state in more than 90% of subject rats. The diabetic rats maintained a continuous growth pattern of body weight until the 8th week, and after that body weight
decreased significantly (Fig. 2A). In contrast, those rats that received saline kept growing, and the body weight rapidly exceeded 500 g. Hyperglycemia occurred within 1 week (mean 4.7 ± 1.2 days) after STZ injection, and the uncontrolled blood glucose level increased progressively until the 24th week (Fig. 2B). Meanwhile, the blood glucose level of control rats remained below 270 mg/dL.

In the first portion, the cutaneous nerve density in the footpad skin sections of diabetic rats decreased significantly at the 6th week, and then was maintained at the same level for the following 6 weeks. After this, the density depleted profoundly until the 24th week (Fig. 2C). The progressive decrease in cutaneous nerve innervation reflects the background of extensive nerve degeneration in our animal model.

3.2. Painful and painless behavioral phenotypes

In the second portion, the onset of painful behaviors in diabetic rats varied from 2 to 10 weeks after induction of diabetes, and the duration of the painful behavior was at least 3 weeks. About half of the rats developed painful behaviors as early as 6–8 weeks after the induction of diabetes, at which time the loss of peripheral nerve fibers became prominent. These included: thermal hyperalgesia (Fig. 3A) and mechanical allodynia (Fig. 3B). Moreover, a portion of the animals did not exhibit significant thermal or mechanical threshold change (painless, Fig. 3C) in the period. As the diabetic duration extended past 16 weeks, most of the rats became hypoaalgic (significant increased in threshold) accompanied by decreased body weight and activity that implied a decompensated state. There were no significant differences in body weight and blood glucose levels among the different behavioral types of the diabetic animals.

3.3. Depleted FNEs and skin denervation in diabetic rats

In the skin sections of control rats, there were moderate to heavily PGP 9.5-stained fibers that were abundant around sweat glands, which extended continuously into the dermal–epidermal layers. The epidermal nerve fibers typically ascended vertically from the dermal papillae into the epidermal layer, and some radiating branches were also observed. The mean calculated ENFD was 10.8 ± 2.1 fibers/mm. The PGP 9.5-stained fibers usually terminated at the distal end of the dermal papillae, with or without branches. Some of these terminal fibers form a specialized nervous
plexus with other horizontal dermal fibers present below the dermal papillae.

In the diabetic groups T (behavior phenotype of thermal hyperalgesia), M (mechanical allodynia), and N (painless = no significant thermal or mechanical threshold change compared with baseline value), there was a generalized depletion of epidermal nerve fibers in skin sections (Fig. 4A–D). Occasionally, a single pale, thin stream or dot-like epidermal nerve fiber could be found under magnification (200×). However, no more than two epidermal fibers could be identified in a section, and the calculated ENFD was less than 0.1 fiber/mm. Likewise, the staining intensity of PGP 9.5 in the diabetic rats was obviously decreased, and the lightly stained fibers varied in their morphologies as thin or thick, wriggly, and winding, and most fibers were fragmented. A remarkable reduction of dermal innervation was also noted, although some small fibers radiated from a remnant fiber trunk. A near-total depletion of epidermal and dermal fibers was observed in some sections in the ROI.

The PGP 9.5 immunodensity score was calculated to represent the extent of nerve fiber innervation in the skin. This score was 35.3 ± 2.0 in control samples, but decreased in diabetic animals to 16.6 ± 0.9 in group T, 22.9 ± 2.6 in group M, and 20.5 ± 2.2 in group N. Quantitative analysis revealed a significant decrease in PGP 9.5 immunodensity in all diabetic groups. However, there was no statistically significant difference among the diabetic groups T, M, and N (Fig. 4E). These results demonstrate the generalized suppression of nerve fiber innervation in painful and painless diabetic rats.

4. Discussion

The STZ-induced diabetic rat model is widely used in the study of PDN. The early decreases in thermal and mechanical thresholds combined with pronounced nerve degeneration in diabetic rats correspond to the frequently observed painful symptoms and peripheral nerve fiber depletion in patients with chronic diabetes.21 It is accepted that STZ at a dosage of 50–60 mg/kg is able to induce a hyperglycemic state by destroying the pancreatic islet cells.22 However, Pabbidi et al22 used a different, higher STZ dose (200 mg/kg) with a mouse model.
and they failed to estimate a background of extensive fiber loss that is recognized in chronic diabetics. The onset of painful behavior varied from 2 to 10 weeks after STZ treatment and induced diabetes in our study. During this pain-genesis period, the rats exhibited a moderate extent of nerve fiber loss but maintained a stable body weight and blood glucose level, which implied an underlying regeneration/repair activity in the periphery. After this period, the rats revealed depleted peripheral nervous innervation and experienced profound hypoalgesia to external stimuli. The above nervous regeneration period is reasonably critical to the development of PDN. The painful diabetic rats collected at 6th to 8th weeks demonstrated hypersensitivity to peripheral thermal and/or mechanical stimuli. On the other hand, a normal “painless” state was also found in some diabetic rats. Some of these “painless” rats eventually developed PDN, but in some cases not for 16 weeks. This finding indicates that there are corresponding pathological changes in the peripheral

Fig. 4. Microscopic findings in the region of interest (ROI) in skin sections immunostained with PGP 9.5 to reveal the innervation of intraepidermal and dermal nerve fibers at 200× magnification. (A) Sections in control rats, which revealed abundant nerve fibers extending from dermal to epidermal layers with abundant branches. (B) Sections in rats with mechanical allodynia (M); (C) thermal hyperalgesia (T); and (D) painless group (N). Sections B–D revealed an obvious diminution in nerve fiber innervation. The interrupted, lightly stained residual fibers appeared mainly in the dermal layer with little branching. (Black arrows: epidermal-free nerve endings; red arrows: residual dermal fibers. Scale bar = 50 μm. PGP 9.5 = protein gene product 9.5.) (E) PGP 9.5 immunoreactivities in different groups of rats at diabetic weeks 6–8. Significant decreases in PGP 9.5 expression (*) was noted in all diabetic rats (T, M, and N). C = body weight-matched controls; M = mechanical allodynia; N = painless group; PGP 9.5 = protein gene product 9.5; T = thermal hyperalgesia. (Data = mean ± SD, n = 5 in controls and n = 6–8 in each diabetic group).
nerve degeneration and regeneration, which lead to subsequent pain genesis. As the duration of the hyperglycemic state persists, the nerve fiber density depletes progressively, to the extent that signal transduction is terminated, or nerve conduction is significantly delayed such that the animals eventually become hypalgesic.

It is believed that changes in cutaneous innervation are more sensitive and may reflect early peripheral neuropathologies in diabetes.23 One hypothesis of the pain genesis in diabetic rats is the over-regenerated nerve endings in the peripheral areas after injury. It could cause a focal increase in nerve density and lead to peripheral sensitization. Our study results show the early, progressive depletion of FNEs as well as a decrease in epidermal and dermal innervation in diabetic rats, irrespective of the presence of painful behaviors, thus subverting the above hypothesis. The peripheral sensitization occurred in a background of generalized cutaneous denervation. Although these findings are largely compatible with the recent report that the extent of skin denervation in diabetic patients increased with diabetic disease duration,19 it revealed the fact that nerve degeneration alone cannot produce hyperalgesic or allodynic behavior, as evident by the phenotype of diabetic rats with no pain symptoms (group N). The depletion of peripheral-free nerve terminals caused by hyperglycemic-induced demyelination could be the first neuropathic event in diabetes. Subsequent neuroplasticity, including activation of a certain type of neuropeptides, growth factors, or cellular mediators,24—26 should be the stepwise triggers that induce the genesis of PDN.

Conventional wisdom dictates that decreased innervation and depleted FNEs would lead to diminished peripheral nociceptive input to the spinal cord and have an impact on proposed underlying spinal or central mechanisms.3 In fact, complementary evidence that peripheral nociceceptor activation takes place during PDN has been reported.27,28 In our study, the diabetic rats in the painful groups evidently experienced hypersensitivity to peripheral thermal or mechanical stimuli, such that even the painless rats could have transient normal nociceptive responses. This suggests that the residual dermal nerve fibers are sufficient to receive and transmit nociception throughout the epidermal and dermal layers in peripheral neuropathy. If the residual nociceptive pathway is replaced by a subset of sensitized fibers and/or neuropeptides, hypersensitivity occurs. A report noting preserved conduction velocity with C-fiber hyper-responsiveness to mechanical stimuli in diabetic rats demonstrated by Chen and Levine29 also confirmed our point of view. Accordingly, sensitization of the residual fibers produced a hyperalgesic phenotype similar to animal groups T and M. The other animals without the sensitization of residual fibers would be normoperceptive, as in the case of rats in group N.

In conclusion, our study results revealed a generalized depletion in peripheral nerve innervation in diabetic rats with or without PDN. The most feasible explanation for this apparent discrepancy is that peripheral sensitization was activated by concomitant neuropeptides, growth factors, or cellular mediators in the residual nerve terminals. Further experiments and observations focus on above activators are necessary to disclose the pathogenesis of PDN.

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References


