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Summary

Background: Skin-scratching behavior, a common response observed in patients with pruritus, is supposed to promote the sprouting of cutaneous sensory nerve fibers. Thus, it sometimes exacerbates the original lesions. However, the precise changes that develop in cutaneous sensory nerve fibers after skin-scratching have not yet been elucidated.

Objective: To investigate how and what kinds of cutaneous sensory nerve fibers increase and how nerve growth factor (NGF) and its receptors change after skin-scratching.

Methods: After scratching the dorsal skin of anesthetized ICR mice, change in cutaneous nerve fibers was detected by immunofluorescence for protein gene product 9.5 (PGP9.5), substance P (SP) and/or calcitonin gene-related peptide (CGRP). To investigate the involvement of NGF signaling, the production of NGF and the expression of its receptors were examined using ELISA and/or immunofluorescence, respectively.

Results: Skin-scratching dramatically induced the sprouting of cutaneous nerve fibers. Both dermal and epidermal nerve fibers began to increase and reached a peak at days 3–7. At the same time, nerve fibers containing SP or CGRP increased significantly. NGF in the scratched skin increased immediately and reached a peak at days 1–3. The expression of NGF receptors, such as phosphorylated trk A and p75, on nerve fibers was remarkably upregulated within 2 days.

KEYWORDS
Murine model; Skin-scratching; Cutaneous sensory nerve fiber; Neuropeptide; Nerve growth factor

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Abbreviations: CGRP, calcitonin gene-related peptide; NGF, nerve growth factor; PGP9.5, protein gene product 9.5; SP, substance P

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1. Introduction

Patients with pruritic skin diseases sometimes scratch their skin repeatedly, which may aggravate the skin lesions and in turn further increase itching sensations. Thus, vicious itch-scratch circles are easily formed and maintained [1]. In itchy skin conditions, such as atopic dermatitis or prurigo, PGP9.5-immunoreactive nerve fibers are increased [2–5]. Further, in lesional skin of allergic contact dermatitis, PGP9.5-immunoreactive nerve fibers and the content of nerve growth factor (NGF) were significantly increased [6]. Judging from these clinical observations, skin-scratching is supposed to play crucial roles in the pathogenesis of cutaneous nervous changes, although evidence of how skin-scratching may induce the growth of nerve fibers has not been presented.

Animal models have been used to analyze the effect of skin-scratching on the cutaneous nervous system. For example, strong correlations among scratching (or itching), growth of cutaneous nerve fibers and severity of dermatitis have been demonstrated [7–9]. In addition, the scratching behavior of NC/Nga mice caused increases in transepidermal water loss and in the severity of skin lesions, leading to the development of dermatitis [10]. Skin-scratching stimulation comprises several components such as pressing, extension of cells and stripping of the cornified layer and the epidermis. Each component may affect the cutaneous nervous system and lead to hypersensitivity and/or to enhanced neurogenic inflammation. For example, barrier disruption with tape stripping and/or acetone treatment led to an increase in epidermal NGF mRNA [11] and induced growth of cutaneous nerve fibers [12]. Taken together, skin-scratching stimulation is supposed to have effects altering the cutaneous nervous system.

Sensory nerve fibers derived from dorsal root ganglia (DRG) are distributed throughout the skin. In the epidermis, a three-dimensional network of unmyelinated fine nerves is innervated and converged into the dermis [13]. These sensory nerves, especially C-polymodal nociceptors, which respond to noxious, mechanical, thermal and/or chemical stimuli, predominantly synthesize and release neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and α-melanocyte stimulating hormone (α-MSH) [13–15]. Among those neuropeptides, the roles of SP and CGRP in neurogenic inflammation have been well-elucidated. SP and CGRP in sensory neurons are synthesized under the regulation of NGF [16] and then play important roles through proinflammatory responses such as sensory neurotransmission, mast cell degranulation, vasodilation and cytokine release, thus inducing neurogenic inflammation [14].

NGF, a representative neurotrophic factor, is synthesized in keratinocytes and in other cutaneous cells in response to conditions such as UVB irradiation and cutaneous wounds [17]. In addition to its multiple roles in cutaneous cells, NGF has a variety of functions for neurons such as neuronal survival, axonal regeneration after injury and collateral reinnervation in wounded skin [18]. For these neuronal functions, NGF binds to two NGF receptors (trk A and p75) at the axon terminus, is taken up by sensory neurons and is transported in retrograde fashion to the cell body in DRG [18,19]. Thus, the sprouting of nerve fibers observed in the skin after skin-scratching stimulation and/or in patients with pruritus may be attributable to the innervating function of NGF. However, this possibility has not been elucidated so far.

In the present study, we first examined the time course and the distribution of nerve sprouting in scratched skin to address the question of whether and how skin-scratching induces increases in cutaneous sensory nerve fibers. Second, we examined immunoreactivity to SP and CGRP in order to characterize the sprouting of nerve fibers with respect to enhanced neurogenic inflammation. Third, we investigated changes in the expression of NGF and its receptors, since NGF is the most probable factor that may induce the sprouting of nerve fibers after skin-scratching.

2. Materials and methods

2.1. Mice

Male ICR mice (7 weeks old, body weight: 31–36 g) were used in this study and were purchased from Nisseizai (Tokyo, Japan). They were housed under...
conditions of controlled temperature (24°C), humidity (40–60%) and illumination (8:00–20:00) and were allowed free access to food and water.

2.2. Skin-scratching

Skin-scratching stimulations were given on the dorsal skin of mice using two methods. The first method (designated as shaker scratching) was performed using a horizontal shaker (Labo Shaker BC-7300, Biocraft, Tokyo, Japan) and a porcine hair brush fixed on the holder. Mice were anesthetized with pentobarbital (Nembutal, Dainippon Sumitomo Pharma, Osaka, Japan) and were put on the shaker. By shaking the mice, fixed brushes scratched their dorsal skin periodically and repeatedly. Scratching conditions were as follows: scratching pressure; 100 gf/cm², scratching frequency; 90 times/min, duration time; 30 min. To give mice stable skin-scratching stimulations, adjustments of the scratching position and the pressure were conducted during the scratching periods as necessary. The second method was manual using brushes made of horse tail (designated as manual scratching). Scratching conditions were as follows: scratching pressure; 100 gf/cm², scratching frequency; 90 times/min, duration time; 10 min. To keep the manual scratching stable and constant, the scratching pressure was checked frequently using plate pressure gauges. In addition, the scratching time was divided into three parts and each scratching time was conducted by a different person. All procedures involving animals were conducted in accordance with the guidelines of the Animal Care and Use Committee, Nippon Medical School.

2.3. Immunofluorescent analysis

At designated times after scratching, skin tissues were biopsied from mice and were immediately fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C, overnight. The next day, specimens were washed in sucrose solution with several changes (10% sucrose in 0.01 M PBS for 4 h, 15% sucrose in 0.01 M PBS for 4 h, and 20% sucrose in 0.01 M PBS overnight). On the third day, the specimens were frozen in OCT compound and kept −45°C. For immunofluorescent staining for PGP9.5, SP or CGRP, the specimens were cryo-sectioned, air-dried and rinsed with PBS. The sections were then blocked with 10% normal serum in PBS for 30 min, and were preincubated with the primary antibody at room temperature overnight. The next day, specimens were washed with PBS and incubated with an FITC- or rhodamine-labeled secondary antibody at room temperature for 60 min. After washing, each specimen was mounted on a slide using glycerol mounting medium (Dako, Carpenteria, CA, USA). Primary antibodies used in this study were rabbit polyclonal anti-PGP antibody (Ultraclone, Cambridge, UK), rat anti-SP antibody

After washing, each specimen was mounted on a slide using glycerol mounting medium (Dako, Carpenteria, CA, USA). Primary antibodies used in this study were rabbit polyclonal anti-PGP antibody (Ultraclone, Cambridge, UK), rat anti-SP antibody
(Oxford Biotechnology, Oxfordshire, UK), goat anti-CGRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-phosphorylated trk A (Santa Cruz Biotechnology) and anti-p75 (Santa Cruz Biotechnology). For the amplification of signals, an ABC Kit (Vector Laboratories, Burlingame, CA, USA) and TSA fluorescent tyramide reagent (Perkin-Elmer, Yokohama, Japan) were used according to the manufacturer's instructions. Cell nuclei were counterstained with DAPI (Molecular Probes, Eugene, OR, USA). After double staining with these antibodies in pairs, images were captured using a digital camera system DP70 (Olympus, Tokyo, Japan).

2.4. Immunohistochemical analysis

For immunohistochemical staining of NGF, fixed skin tissue sections frozen in OCT compound (described above) were used. First, sections were air-dried and endogeneous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in methanol. After washing with PBS, sections were blocked with 10% goat normal serum and a biotin-blocking reagent (Dako Cytomation, Kyoto, Japan). After double staining with these antibodies in pairs, images were captured using a digital camera system DP70 (Olympus, Tokyo, Japan).

2.5. Evaluation of changes in nerve fibers

Nerve fibers in each section were visualized by immunofluorescent staining for PGP 9.5, SP and/or CGRP. To evaluate increase in nerve fibers, images were taken from at least six fields per section with a digital camera DP70 (Olympus, Tokyo, Japan). PGP 9.5-immunoreactive nerve fibers were evaluated with image analysis software Lumina Vision ver 2.2.0 (Mitani Corporation, Tokyo, Japan). To evaluate changes in nerve fibers in the dermis or in the epidermis, areas or lengths of PGP 9.5-immunoreactive nerve fibers were calculated with this software.

Fig. 2 Changes in epidermal nerve fibers and epidermal thickness after skin-scratching. Cutaneous nerve fibers were stained with anti-PGP9.5 antibody and images were taken with a DP70 digital camera. Scale bar: 50 μm. (a) Epidermal nerve fibers before skin-scratching at day 0. (b) Epidermal nerve fibers at day 7. (c) Changes in epidermal nerve fibers after skin-scratching. Lengths of epidermal nerve fibers were evaluated with Lumina Vision ver 2.2.0. Each value represents the percentage compared with day 0. (d) Changes in epidermal thickness. Epidermal thickness was measured in images. Mean ± S.D. (n = 4), *p < 0.05; **p < 0.01 (Tukey’s method).
respectively. Lengths of SP- or CGRP-immunoreactive nerve fibers were measured with scales. To obtain objective results, three independent persons counted the nerve fibers in stained preparations. The lengths of nerve fibers were compared with those at day 0 and rates were calculated.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Skin tissues taken from mice at designated times were immediately frozen and kept at −80°C. All samples were processed at the same time and NGF concentrations were determined with an ELISA using the NGF E Max Immunoassay System (Promega, Madison, WI, USA). All procedures were performed according to the manufacturer’s instructions. Briefly, collected skin tissues were put in lysis buffer including a protease inhibitor cocktail (Sigma–Aldrich, Saint Louis, MO, USA), cut into small pieces with scissors and homogenized using a sonicator (Astron Ultrasonic Processor, Misonic Incorporated, Tokyo, Japan). During these procedures, samples were kept on ice to prevent proteinase activity. Homogenized samples were then centrifuged at 8000 × g 10 min at 4°C and supernatants were recovered. Protein concentrations were determined by the Bradford method with using Protein assay CBB solution (Nalalai Tesque, Kyoto, Japan). Each sample (0.5 mg, 100 µl for each well) was applied to a well of a flat bottom 96-well plate coated with anti-NGF polyclonal antibody and protein concentrations of NGF were determined by the sandwich ELISA system.

2.7. Statistical analysis

Data were analyzed by Tukey’s method or by Student’s t-test for independent samples using SPSS.
11.5 for Windows. All results are expressed as means ± S.D. (n = 4). **p < 0.01 and *p < 0.05.

3. Results

3.1. Increase in nerve fibers in the dermis and in the epidermis

First, we evaluated the sprouting of cutaneous nerve fibers after skin-scratching. To visualize nerve fibers, cryosections were stained using an anti-PGP antibody and were detected by immunofluorescence. After images were taken with a digital camera, areas of dermal nerve fibers were evaluated with image analysis software. After scratching, dermal nerve fibers began to sprout immediately. Three days after skin-scratching, they were significantly increased, which lasted until about day 7 (Fig. 1). There were no significant differences in the increasing rate of dermal nerve fibers between mice subjected to shaker scratching and to manual scratching.

The epidermal nerve fibers also became longer after scratching, went through epidermal cells and were densely distributed in the epidermis like a network (Fig. 2a and b). The increased pattern of epidermal nerve fibers was similar to that of dermal nerve fibers. Namely, 3 days after skin-scratching, they were significantly increased, which lasted until about day 7 (Fig. 2c). Epidermal thickness also showed a similar increased pattern (Fig. 2d).

After reaching a peak at about day 7, both dermal and epidermal nerve fibers began to decrease gradually and returned to the basal level after about 2–3 weeks (Figs. 1 and 2). This means that the
increase in cutaneous nerve fibers induced by skin-scratching is a transient response and that it returns to the basal level after about 2–3 weeks, if scratching stimulation does not continue. In other words, at least 2 weeks are necessary for the increased nerve fibers to return to the basal state, when there is no further scratching.

3.2. Increase in nerve fibers which are immunoreactive to SP or CGRP

As shown in Fig. 3c, double staining was carried out using anti-PGP9.5 and anti-SP antibodies. Thus, SP-immunoreactive nerve fibers co-localized with PGP9.5-immunoreactive nerve fibers. Next, to

![Fig. 5](image)

**Fig. 5** Changes in NGF after skin-scratching. Immunohistochemical staining of NGF was carried out as described in Section 2. (a) Day 0; (b) day 1; (c) day 3; (d) day 7. Scale bar: 100 μm. (e) Changes in NGF protein content. NGF protein contents in samples taken from scratched sites were examined with ELISA as described in Section 2. Mean ± S.D. (n = 4), *p < 0.05, **p < 0.01 (Tukey’s method).
evaluate the increase in nerve fibers that were immunoreactive to SP, sections were stained with the anti-SP antibody and lengths of SP-immunoreactive nerve fibers were measured from images taken with the digital camera. As shown in Fig. 3b, SP-immunoreactive nerve fibers increased significantly at day 7 and returned to the basal level by day 14 (Fig. 3d). Similarly, as shown in Fig. 4c, CGRP-immunoreactive nerve fibers co-localized with PGP9.5-immunoreactive nerve fibers. To evaluate the increase in nerve fibers that were immunoreactive to CGRP, lengths of CGRP-immunoreactive nerve fibers were measured from images taken with the digital camera (Fig. 4b). Similar to SP-immunoreactive nerve fibers increased significantly at day 7 and returned to the basal level at day 14 (Fig. 4d). These results indicate that not only total nerve fibers increased significantly at day 7 and returned to the basal level by day 14 (Fig. 5 e). The two methods of skin-scratching stimulation used did not make a significant difference in the pattern except for a slight shift of peak time.

3.3. Evaluation of NGF synthesized in scratched skin

Since NGF is supposed to be responsible for the sprouting of cutaneous sensory nerves, we examined changes in NGF in the scratched skin. Immunohistochemical analysis revealed that the expression of NGF began to increase significantly in epidermal keratinocytes, in hair follicles, and in dermal fibroblasts at day 1 and the increases continued until day 3 (Fig. 5a–d). ELISA showed that NGF protein started to increase significantly at 6–8 h, reached a peak at days 1–3 and then decreased to the basal level at day 7 (Fig. 5e). The two methods of skin-scratching stimulation used did not make a significant difference in the pattern except for a slight shift of peak time.

3.4. Expression of NGF receptors on cutaneous sensory nerve fibers

Next, the expression of two NGF receptors on cutaneous sensory nerve fibers was examined, to investigate the effect of increasing NGF on the cutaneous sensory nervous system. First, the expression of phosphorylated trk A on nerve fibers was examined by double staining using anti-phosphorylated trk A and anti-PGP9.5. Phosphorylated trk A is a marker of activated trk A by NGF signal transduction [19]. As shown in Fig. 6a–d, the expression of phosphorylated trk A on cutaneous sensory nerve fibers at day 2 was significantly upregulated compared to that at day 0. The tendency persisted until days 2–3, after which it gradually returned to the basal level at days 5–7 (data not shown). Next, the expression of p75 on nerve fibers was examined by double staining using anti-p75 and anti-PGP9.5 antibodies. As shown in Fig. 6e and f, the expression of p75 on cutaneous sensory nerve fibers at day 1 was significantly upregulated compared with that at day 0. The tendency persisted until days 2–3, after which it gradually returned to the basal level at days 5–7 (data not shown).

4. Discussion

To resolve whether the stimulation of skin-scratching really induces the sprouting of cutaneous nerve fibers, we used a mouse model of skin-scratching. In the clinical situation, increases in cutaneous nerve fibers have been observed in patients with pruritic skin diseases such as atopic dermatitis [2–4] and/or prurigo [5]. Further, in skin lesions of NC/Nga mouse, a model for atopic dermatitis, strong correlations among skin-scratching behavior, severity of skin lesions and increases in cutaneous nerve fibers were found [7–9]. From those observations, skin-scratching is supposed to be a major causative factor for increases in cutaneous nerve fibers, which may then lead to the exacerbation of lesions. However, there has been no evidence to verify this hypothesis. In the present study, we present, for the first time, evidences that the stimulation of skin-scratching really increases cutaneous nerve fibers in mice. These results suggest that skin-scratching behavior in patients with pruritus may be, at least in part, responsible for increases in nerve fibers observed in the clinical situations.

In addition, we examined the time course and the distribution of sprouting nerve fibers after skin-scratching. After a single scratching, cutaneous nerve fibers increase and then decrease, exhibiting a transient peak. Cutaneous nerve fibers increased significantly within 3 days after a single scratching both in the dermis and in the epidermis. Nerve fibers continued to increase until about day 7, and thereafter gradually decreased and eventually returned to the basal level after about 2 or 3 weeks. In the epidermis, nerve fibers were distributed in a dense network between epidermal cells at the peak time. Getting a similar knowledge from clinical observations is quite difficult, for patients are sometimes exposed to multiple environmental stimulations and they usually scratch themselves repeatedly with irregular durations and intensities. Since the present study was focused on changes in nerves induced by a single scratching, we did not investigate the effect of repeated scratchings. Nevertheless, our findings suggest useful information about how and
when symptoms of patients may really develop (exacerbate or recover) in clinical situations.

Next, to evaluate the effects of skin-scratching on cutaneous hypersensitive responses and/or on susceptibility to neurogenic inflammation, we examined SP- or CGRP-immunoreactivity on cutaneous nerve fibers after a single skin-scratching. SP- or CGRP-immunoreactive nerve fibers increase in patients with pruritic skin diseases such as atopic dermatitis and nodular prurigo [5,20—23]. Our findings revealed that significant increases in SP- or CGRP-immunoreactive nerve fibers developed at about day 7, when the number of PGP9.5-immunoreactive nerve fibers was at its peak. Taken together, these results suggest that scratching behavior in patients with pruritus may be, at least in part, responsible for the increases in SP- or CGRP-immunoreactive nerve fibers. In our mouse model of skin-scratching, after the number of nerve fibers reached a peak, it returned to the basal level by day 14, thus exhibiting a transient changing pattern. The immunoreactivity of SP or CGRP in cutaneous nerve fibers in human skin lesions may well be variable, since the stimulations in the clinical situations are usually irregular and the timing of sample taking is different in various stages of the diseases.

SP and CGRP are well-known neuropeptides that are involved in the hypersensitivity of itchy sensations [24—26] and/or neurogenic inflammation [14]. Therefore, it is suggested that skin-scratching may lead to hypersensitive responses through increases in SP or CGRP in the cutaneous environment. We will examine, in future studies, whether scratching-induced increases in SP- or CGRP-immunoreactive nerves may be responsible for the hypersensitivity of itchy sensations and/or the susceptibility to neurogenic inflammation.

To explain why SP- or CGRP-immunoreactive nerve fibers increased, we hypothesized two potential mechanisms. First, SP- or CGRP-immunoreactive nerve fibers themselves might increase after skin-scratching. Second, the synthesis and/or transport of these neuropeptides from DRG neurons might be enhanced by skin-scratching, thus leading to increased immunoreactivity for SP or CGRP on nerve fibers. Judging from the fact that changes in nerve fibers immunoreactive to PGP9.5, SP or CGRP have similar patterns, we suppose that increases in nerve fibers themselves might be, at least in part, responsible for increases in SP- and/or CGRP-immunoreactive nerve fibers.

Since NGF is supposed to be the molecule most likely to be involved in the induction of nerve sprouting both in skin-scratched mice and in patients with pruritus, we examined changes in NGF protein synthesis and expression of its receptors after skin-scratching. Immunohistochemical analysis revealed that expression of NGF was significantly enhanced at days 1—3 in epidermal keratinocytes, fibroblasts and hair follicles. In accordance with that result, synthesis of NGF protein detected by ELISA increased remarkably at days 1—3 and then returned to the basal level at around day 7. In addition, immunofluorescent analysis by double staining with anti-phosphorylated trk A, anti-p75 and anti-PGP9.5 antibodies revealed that the expression of both receptors is significantly upregulated on cutaneous nerve fibers within 2 days after skin-scratching. For the binding and uptake of NGF, both trk A and p75 are necessary, and NGF transported in a retrograde manner remains associated with p75 and activated trkA receptors [16,19,27].

These results demonstrate that after scratching, NGF protein is immediately synthesized in cutaneous cells and that it is soon bound to receptors at the axonal terminus. These results suggest that NGF signaling is effectively working on nerve fibers. In addition, the peak time of NGF synthesis is at days 1—3 after skin-scratching while the peak time of increase in nerve fibers is at days 3—5, indicating that the sprouting of nerve fibers begins several days after synthesis and uptake of NGF by neurons. The finding that SP- and/or CGRP-immunoreactive nerves increases further supports the assumption that sprouting of nerve fibers is induced, at least in part, via NGF signaling, since SP or CGRP in sensory neurons is regulated by NGF and they are released from NGF-dependent nerves [16,28]. In general, cutaneous sensory nerves are divided into two groups: an NGF-dependent group and a GDNF-dependent group [29]. Thus, we plan to elucidate in future studies whether GDNF-dependent nerve fibers increase after skin-scratching.

In conclusion, we show in this study that skin-scratching induces a transient increase in PGP9.5-, SP- and/or CGRP-immunoreactive nerve fibers and that NGF and its receptor system are significantly

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**Fig. 6** Expression of NGF receptors on cutaneous sensory nerve fibers was examined by immunofluorescence. Sections were stained with anti-phosphorylated trk A, anti-p75, anti-PGP 9.5 and/or DAPI. (a) Expression of phosphorylated trk A on cutaneous sensory nerve fibers at day 0. Merges of images stained with anti-phosphorylated trk A (FITC), anti-PGP 9.5 (TRITC) or DAPI. (b) Expression of phosphorylated trk A (FITC) at day 2. Nuclei were stained with DAPI. (c) Expression of PGP9.5 (TRITC) at day 2. (d) Merged image of b and c. This represents the expression of phosphorylated trk A on cutaneous sensory nerve fibers (arrow) at day 2. (e and f) Expression of p75 on cutaneous sensory nerve fibers. Images stained with anti-p75 (FITC), anti-PGP 9.5 (TRITC) or DAPI are merged. (e) Day 0; (f) day 1. Scale bar: 100 μm.
upregulated soon after the stimulation. These findings suggest that skin-scratching behavior in patients with pruritus might be responsible for enhanced neurogenic inflammation in relation to a transient increase in cutaneous sensory nerve fibers, in which NGF signaling may play crucial roles. This understanding about cutaneous sensory nerve fibers may be useful for treating patients with pruritic skin diseases.

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**References**