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Development of Numerous Nerve Fibers in the Epidermis of Hairless Mice With Atopic Dermatitis-Like Pruritic Skin Inflammation

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Abstract. Itching is the most important symptom in atopic dermatitis because the persistent scratching in response to itching aggravates the disease. However, the etiologic mechanisms of itching in atopic dermatitis remain uncertain. HR-1 hairless mice fed a special diet, HR-AD, develop atopic dermatitis-like symptoms with prolonged scratching episodes. The purpose of this study was to examine whether skin nerve fiber changes were involved in the prolonged scratching seen in this mouse model. On day 56 after the start of feeding, prolonged scratching, as well as atopic dermatitis-like skin changes, were clearly observed in HR-AD-fed mice, while no abnormal changes were observed in mice fed a normal diet. Immunohistochemical analyses of the skin using antibody to protein gene product 9.5 showed the development of numerous immunoreactive nerve fibers in the epidermis of HR-AD-fed mice. Furthermore, after cessation of HR-AD feeding, the reduction in intraepidermal nerve fibers coincided with decreased scratching. Neither the prolongation of scratching nor the increase in intraepidermal nerve fibers was affected by dexamethasone treatment. Thus, the increased number of intraepidermal nerve fibers could be involved in the aggravation of itch-related scratching observed in this model.

Keywords: itch, atopic dermatitis, nerve fiber, hairless mice, protein gene product 9.5

Introduction

Itching is defined as an uncomfortable sensation that elicits the desire to scratch (1); it appears to be transmitted through the free nerve endings of C-fibers, which are mainly located in the epidermis and at the dermo-epidermal junction (2, 3). Atopic dermatitis is a common dermatologic disease that is accompanied by severe itching, which is the most important symptom in the disease (4, 5). Furthermore, in patients with atopic dermatitis, the severe itching not only influences the patient's quality of life, but also elicits intense and persistent scratching, which aggravates the lesions (6). Thus, when treating atopic dermatitis, one has to develop an efficient strategy for controlling itching and scratching. However, the precise etiologic mechanisms of itching in atopic dermatitis remain unclear.

Protein gene product (PGP) 9.5, also known as ubiquitin C-terminal hydrolase (7), is widely used as a neuronal marker in studies of the peripheral and central nervous systems. Immunohistological analyses of skin biopsies using antibodies to PGP 9.5 (8, 9) have allowed the visualization of cutaneous innervation and the investigation of the changes that occur in cutaneous innervation with different clinical conditions. An altered distribution of skin nerve fibers has been found in atopic dermatitis; numerous nerve fibers extend into the epidermis of atopic skin lesions (10, 11). In addition, there is a higher density of PGP 9.5-positive peripheral nerves in the lesions of patients with atopic dermatitis than in uninvolved skin (12). These findings raise the possibility that the changes in the distribution of skin nerve fibers are related to the development of itching in patients with atopic dermatitis; however, this relationship has not been fully documented.

Appropriate animal models are required to understand the mechanisms of disease development. We previously reported that hairless mice fed a special diet, HR-AD, but not a normal diet, developed dermatological changes, including severe dry skin, erythema, epidermal hyperplasia, and massive infiltration of inflammatory cells into the dermis, as well as systemic changes,

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including an increased number of blood T cells and an elevated serum immunoglobulin E level (13). These features, which are caused by feeding hairless mice with HR-AD, appear to be similar to those seen in patients with atopic dermatitis. In addition, HR-AD-fed mice were found to have prolonged episodes of scratching that were attenuated by the μ-opioid receptor antagonist naloxone, but not by the H_1-receptor antagonist mepyramine (14). Similar observations have been reported clinically in atopic dermatitis patients (15 – 18). Therefore, the mechanisms underlying the scratching behavior in HR-AD-fed mice partially reflect those of patients with atopic dermatitis.

In the present study, the role of peripheral skin nerve fibers in the itching sensation in experimental atopic dermatitis was examined. Nerve fibers in the skin of hairless mice fed a normal diet or HR-AD were detected and quantified using immunohistological techniques involving the anti-PGP 9.5 antibody. The changes in the distribution of skin nerve fibers, as well as changes in the scratching response, during and after HR-AD feeding were investigated. Furthermore, the effects on these parameters of treatment with a corticosteroid, dexamethasone, were assessed.

Materials and Methods

Animals and diets

Female HR-1 hairless mice (4-week-old) weighing 10 – 12 g were obtained from Hoshino Experimental Animal Center (Yashio). The mice were housed in a conventional animal room under a controlled temperature of 23 ± 1°C and a humidity of 60 ± 10% with lights on from 08:00 to 20:00. They were fed a normal diet (F-2; Funabashi Farm, Chiba) or a special diet developed for HR-1 mice (HR-AD manufactured diet; Nosan Corp., Yokohama), and given tap water ad libitum. The detailed ingredients of these diets have been previously described (13).

The Experimental Animal Research Committee at Kyoto Pharmaceutical University approved this animal study.

Reagents

The following reagents were purchased from the indicated sources: normal goat serum and biotin-conjugated goat anti-rabbit IgG antibody (Dako, Glostrup, Denmark), rabbit anti-PGP 9.5 polyclonal antibody (UltraClone Limited, Isle of Wight, UK), normal rabbit serum (Cederlane Laboratories, Ltd., Burlington, Canada), streptavidin-horseradish peroxidase and the 3,3'-diaminobenzidine (DAB) substrate kit (BD Pharmingen, San Diego, CA, USA), Entellan® (Merck, Darmstadt, Germany), and bovine serum albumin (BSA) and Mayer's hematoxylin (Wako Pure Chem., Osaka).

Histology

Mice were sacrificed by bleeding under diethyl ether anesthesia. Then, the cervical dorsal skin was isolated, fixed in 10% neutral formalin, and embedded in paraffin. The paraffin-embedded tissues were sliced into 4-μm sections and then dried on a slide at 37°C. Following deparaffinization, the thin section was stained with hematoxylin and eosin (H&E).

Analysis of spontaneous scratching behavior

The spontaneous scratching behavior of the mice was analyzed as described previously (13). In brief, the mice were acclimatized for 10 min in a measuring cage, and then their scratching behavior was videotaped for 1 h. The scratching was analyzed by playing back the videotape. Over a period of 1 h, the frequency and cumulative duration of the scratching behavior were determined using a specifically developed instrument that allows analysis of two aspects of the behavior: 1) scratch frequency, which is determined by an observer who touches a switch each time scratching is observed while watching the video; and 2) cumulative duration of scratching behavior, which is determined by having the observer touch the switch for as long as scratching behavior is observed. The duration of one scratching bout was calculated by dividing the cumulative duration by the frequency of scratching episodes.

Immunohistochemistry

The skin sample was collected and prepared as described above. A 4-μm section was deparaffinized and then washed with several changes in 0.1% Tween 20-containing tris-buffered saline (T-TBS). To quench endogenous peroxidase in the tissue, the sections were preincubated with 3% H_2O_2 methanol solution for 10 min at room temperature (RT). Following washing with T-TBS, the sections were incubated with T-TBS containing 5% normal goat serum for 30 min at RT to block the non-specific coloring reaction. They were then washed and incubated overnight at 4°C with rabbit anti-PGP 9.5 antibody (1:200, 0.3 mg protein/ml) diluted in 1% BSA-containing T-TBS. After washing, the sections were incubated with biotin-conjugated goat anti-rabbit IgG antibody in 1% BSA-containing T-TBS at a dilution of 1:3,000 for 30 min at RT, followed by streptavidin horseradish peroxidase treatment for 30 min at RT. PGP 9.5-immunoreactive nerve fibers were visualized under an optical microscope using the DAB substrate kit. Finally, the sections were thoroughly washed with distilled water, counterstained with

hematoxylin, and mounted in Entellan®.

As a negative control, in each sample, normal rabbit serum (1:323, 0.3 mg protein/ml) was also used as a substitute for rabbit anti-PGP 9.5 antibody.

Quantification of PGP 9.5-immunoreactive nerve fibers in the skin (Fig. 1)

The PGP 9.5-immunoreactive nerve fibers in the epidermis and at the dermoepidermal junction were quantified as follows: Ten representative areas, randomly selected from the PGP 9.5-immunostained sections, were photographed using a digital camera (DP12; Olympus Optical, Tokyo) equipped with an optical microscope at ×1,000 magnification. Subsequently, curved lines were drawn on the upper and lower margins of the epidermis, and a line parallel to the lower line was drawn 50 μm below the lower line (Fig. 1A). Then, the stained section was separated into epidermal (Fig. 1B) and dermoepidermal (Fig. 1D) regions. PGP 9.5-immunoreactivities (brown area in Figs. 1: B and D) were extracted, and the extracted area (black area in Figs. 1: C and E) was quantified, using NIH image software (version 1.62; National Institutes of Health, Bethesda, MD, USA). The area of PGP 9.5-immunoreactive nerve fibers in the epidermis and at the dermoepidermal junction was expressed as $\times 10^2$ μm^2 per mm of the length of the upper epidermal margin and as $\times 10^3$ μm^2 per mm^2 of the dermoepidermal area, respectively.

In preliminary experiments, a non-specific coloring reaction was faintly seen at the dermoepidermal junction, but not in the epidermis. Therefore, at the dermoepidermal region, the net area of PGP 9.5-immunoreactive nerve fibers was determined by subtracting the value of the respective negative control.

Drug administration

From the start of HR-AD feeding, dexamethasone (Wako Pure Chem.), suspended in distilled water, was given orally once daily at a dose of 1 mg/kg. The control group was only given the vehicle. On day 63 after the start of treatment, histological changes, scratching behavior, and PGP 9.5-immunoreactive nerve fiber changes were assessed as described above.

Statistical analyses

The data are shown as the mean ± S.E.M. Statistical differences between the two groups were determined using Student's unpaired *t*-test. To compare the three groups, Bonferroni's multiple test was used after doing one-way analysis of variance. Values of $P<0.05$ were considered significant.

Results

Development of atopic dermatitis-like symptoms and prolongation of scratching in HR-AD-fed mice

Figure 2A shows the appearance of a mouse fed a normal diet and a mouse fed a special diet (HR-AD) on day 56 after the start of feeding. The normal diet-fed mouse shows no apparent skin changes, whereas the HR-AD-fed mouse shows systemic erythema and wrinkled skin. From results of histology of the skin of these mice on day 56 (Fig. 2: B and C), the skin of HR-AD-fed mice shows inflammatory changes such as epidermal hyperplasia and abundant infiltration of some inflammatory cells. These changes observed in HR-AD-fed mice seem to be similar to those of patients with atopic dermatitis (19, 20).

Figure 2, D – F, shows the changes in spontaneous scratching behavior in normal diet- and HR-AD-fed mice. There was no difference in the frequency of scratching behavior between both groups of mice on days 28 and 56 (Fig. 2D). Although the cumulative duration of HR-AD-fed mice tended to increase compared with that of normal diet-fed-mice, the difference on day 56 was not statistically significant (Fig. 2E). In contrast, compared to normal diet-fed mice, the duration of one scratching bout in HR-AD-fed mice was markedly prolonged, and the duration depended on the number of days they had been fed the HR-AD diet (Fig. 2F). Thus, the duration of one scratching bout was used as an index of scratching in response to itching in the following studies.

Changes in PGP 9.5-immunoreactive nerve fibers in the skin during HR-AD feeding

To evaluate the distribution of nerve fibers in the skin of the mice, immunohistochemical analysis with antibody to PGP 9.5 was done. Figure 3 shows representative examples of PGP 9.5-immunostained sections obtained from normal diet- and HR-AD-fed mice. On days 28 and 56, no obvious morphological changes were observed in the normal diet-fed mice (Fig. 3: A – C), while marked epidermal hyperplasia was seen in the HR-AD-fed mice (Fig. 3: D – F). A slight degree of PGP 9.5-immunoreactivity was found in the epidermis and at the dermoepidermal junction of normal diet-fed mice; however, there was no significant difference in the reactivity levels between days 28 and 56 (Fig. 3: A and B). In HR-AD-fed mice, on day 28, the PGP 9.5-positive reaction was similar to that seen in normal diet-fed mice (Fig. 3D). In contrast, on day 56, the HR-AD-fed mice showed strong PGP 9.5-immunoreactivity in the skin, especially in the epidermis (Fig. 3E). Furthermore, the extension of PGP 9.5-

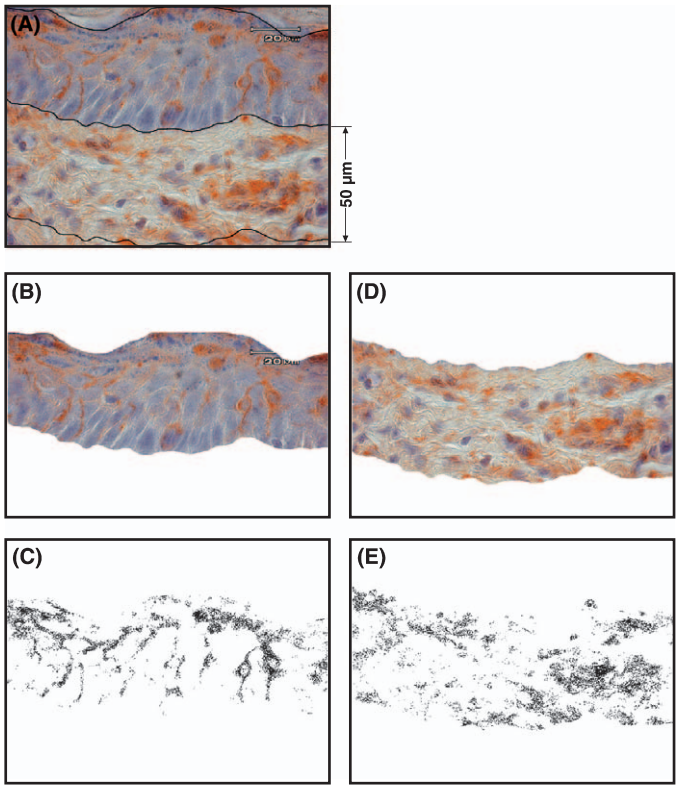

Fig. 1. Quantification of protein gene product (PGP) 9.5-immunoreactive nerve fibers in the epidermis and at the dermoepidermal junction of the skin collected from hairless mice. On the photograph, randomly selected from the PGP 9.5-immunostained section (A), black curved lines were drawn on the upper and lower margins of the epidermis, and an identical line was drawn 50-μm below the lower line. Then, the stained section was separated into the epidermal (B) and the dermoepidermal (D) regions. The PGP 9.5-immunoreactivites (brown area in panels B and D) were extracted, and the extracted area (black area in panels C and E) was quantified, using NIH image software.

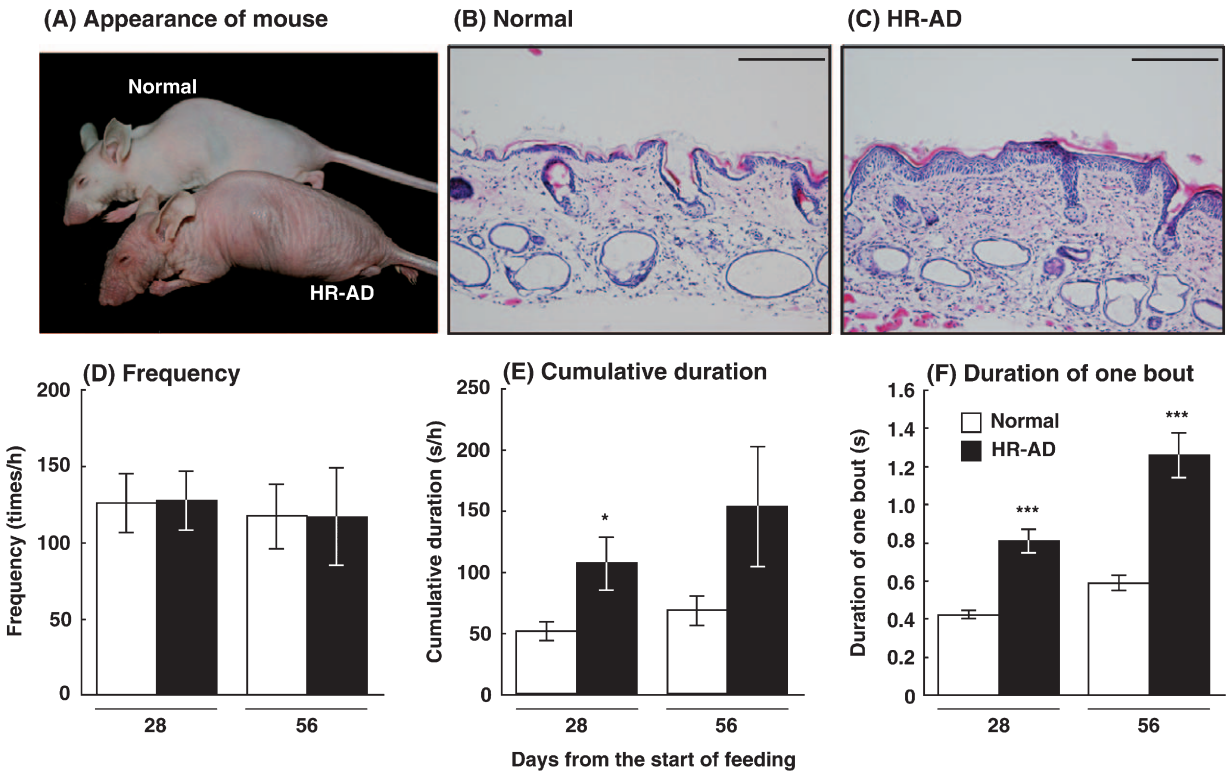

Fig. 2. The appearance (A) and the histology (B and C) of hairless mouse fed a normal diet and the special diet (HR-AD) on day 56. The collected skin was stained with H&E. Scale bar in panels B and C represent 200 μm. Time-course changes in frequency (D), cumulative duration (E), and duration of one bout (F) of spontaneous scratching behavior in normal diet- and HR-AD-fed mice. Each column represents the mean ± S.E.M. of 9 – 12 animals. *P<0.05 and ***P<0.001.

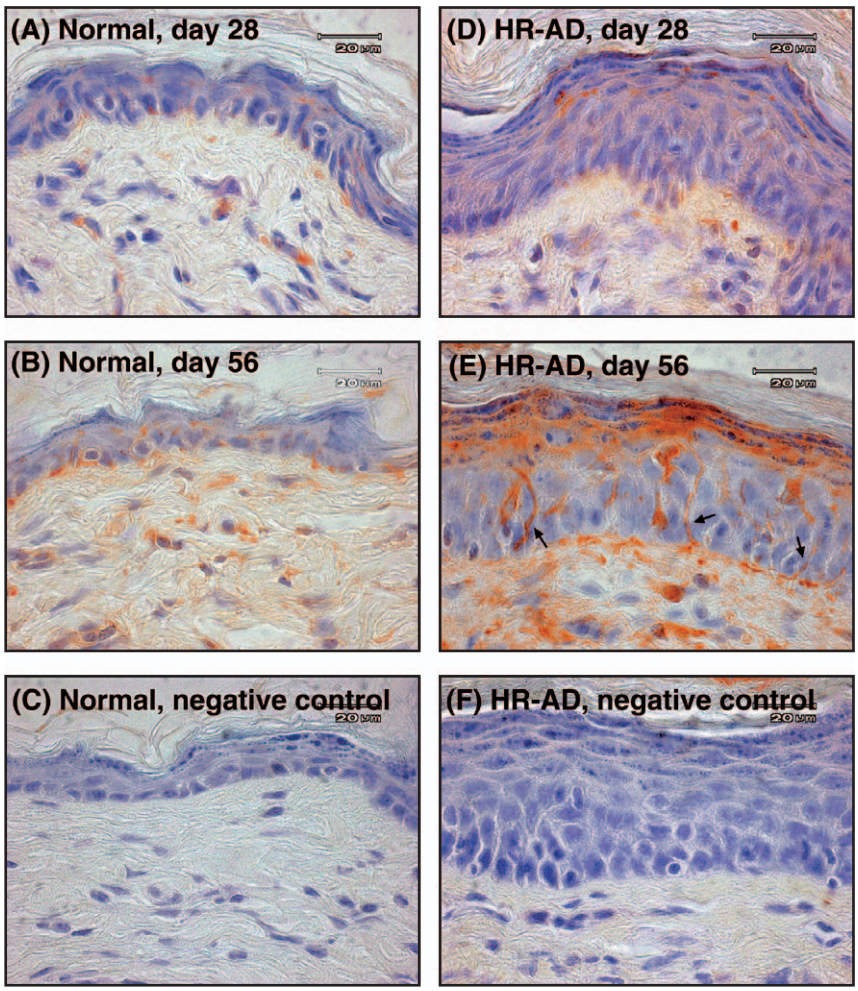

Fig. 3. Protein gene product (PGP) 9.5-immunostained sections of the skin collected from hairless mice fed a normal diet (A – C) and the special diet (HR-AD, D – F). The respective PGP 9.5-immunostained sections represent the skin of a normal diet-fed mouse on day 28 (A) and day 56 (B), and that of an HR-AD-fed mouse on day 28 (D) and day 56 (E). The skin of normal diet-fed (C) and HR-AD-fed (F) mice on day 56 was treated with normal rabbit serum as a substitute for anti-PGP 9.5 antibody. Arrows in panel E indicate the extension of PGP 9.5-immunoreactive nerve fibers into the epidermis.

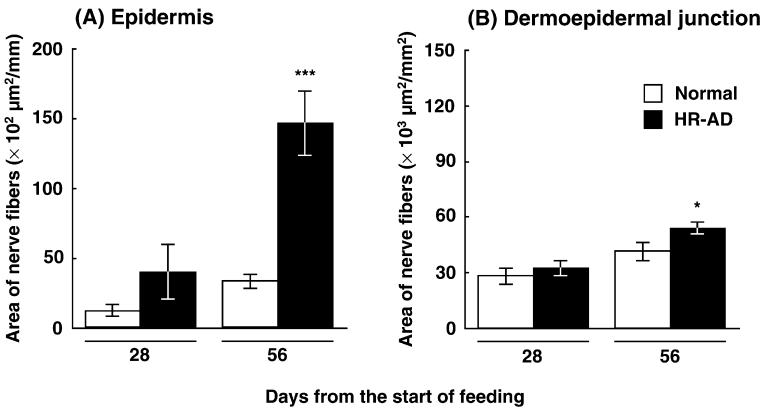

Fig. 4. Time-course changes in the area of the protein gene product (PGP) 9.5-immunoreactive nerve fibers in the epidermis (A) and at the dermoepidermal junction (B) of hairless mice fed a normal diet or the special diet (HR-AD). Each column represents the mean ± S.E.M. of 8 – 12 animals. **P*<0.05 and ****P*<0.001.

immunoreactive nerve fibers from the dermoepidermal junction to the granular layer of the epidermis was noted (arrows in Fig. 3E). On the other hand, in the skin treated with normal rabbit serum as a substitute for the anti-PGP 9.5 antibody, no non-specific coloring reaction was detected in the epidermis, although a few dermal cells showed a weak coloring reaction (Fig. 3: C and F).

The results of the quantitative evaluation of stained nerve fibers in the epidermis and at the dermoepidermal junction are shown in Fig. 4. On day 28, the area of PGP 9.5-immunoreactivity in the epidermis of HR-AD-fed

mice was slightly increased compared to that of normal diet-fed mice, but the increase was not statistically significant (Fig. 4A). On the other hand, on day 56, the area of PGP 9.5 immunoreactivity was obviously enlarged in HR-AD-fed mice compared to that in normal diet-fed mice (Fig. 4A). On day 28, there was no difference in the area of PGP 9.5-immunoreactivity at the dermoepidermal junction between normal diet- and HR-AD-fed mice, while on day 56, the area of PGP 9.5 immunoreactivity at the dermoepidermal junction was marginally but significantly increased in HR-AD-fed mice compared to normal diet-fed mice (Fig. 4B).

Changes in the prolongation of scratching and in the number of PGP 9.5-immunoreactive nerve fibers in the skin after cessation of HR-AD feeding

We examined the changes in prolonged scratching and in the increase of PGP 9.5-immunoreactivity after cessation of HR-AD feeding. On day 64 after the start of feeding with HR-AD, HR-AD feeding was changed to a normal diet in the H – N group. In the H – N group, 1 week after the feeding change, the duration of scratching and the PGP 9.5-immunoreactive area were clearly reduced compared to mice continuously fed with HR-AD (H – H group), but the levels of these parameters remained high compared to mice continuously fed a normal diet (N – N group) (Fig. 5).

On the other hand, there was no significant difference in the PGP 9.5-positive areas at the dermoepidermal junction among the three groups of mice (data not shown).

Effects of dexamethasone on changes in the prolongation of scratching and the increase in the epidermal PGP 9.5-immunoreactive nerve fibers in HR-AD-fed mice

The effects of dexamethasone on the skin inflammation, prolonged scratching, and increased PGP 9.5 immunoreactivity caused by HR-AD feeding are shown in Fig. 6. Consistent with a previous study (13), the skin of dexamethasone-treated mice shows the reduction of epidermal hyperplasia and inflammatory cellular infiltrates (Figs 6: A – C). On the other hand, the prolonged scratching was not affected by dexamethasone (Fig. 6D). Similarly, the PGP 9.5-immunoreactive area of dexamethasone-treated mice did not significantly differ from that of the control mice (Fig. 6E).

Discussion

The present study investigated whether changes in skin nerve fibers were associated with the itch-related scratching seen in HR-AD-fed mice. The immunohistochemistry of skin treated with anti-PGP 9.5 antibody showed that on day 56, the immunoreactive nerve fibers were markedly increased in the epidermis of HR-AD-fed mice, which at that time also had atopic dermatitis-like symptoms and prolonged scratching. In contrast, in normal diet-fed mice, neither the distribution of intraepidermal nerve fibers nor the duration of scratching changed during the observation period. Thus, these results appear to indicate that the increase in nerve fibers is related to prolongation of scratching in HR-AD-fed mice. However, on day 28, the increase in the intraepidermal nerve fibers expressing PGP 9.5 was not statistically significant, even though prolonged scratching was already present at that time. Nevertheless, changing the HR-AD to a normal diet reduced the number of intraepidermal nerve fibers and the prolonged scratching. On the other hand, compared to the increase in PGP 9.5-immunoreactive fibers in the epidermis seen with HR-AD feeding, there was not a marked increase in PGP 9.5-immunoreactive nerve fibers at the dermoepidermal junction. Taken together, our results suggest that an increase in intraepidermal nerve fibers could be involved in the aggravation rather than the onset of itch-related scratching in HR-AD-fed mice.

In patients with atopic dermatitis, a weak exogenous

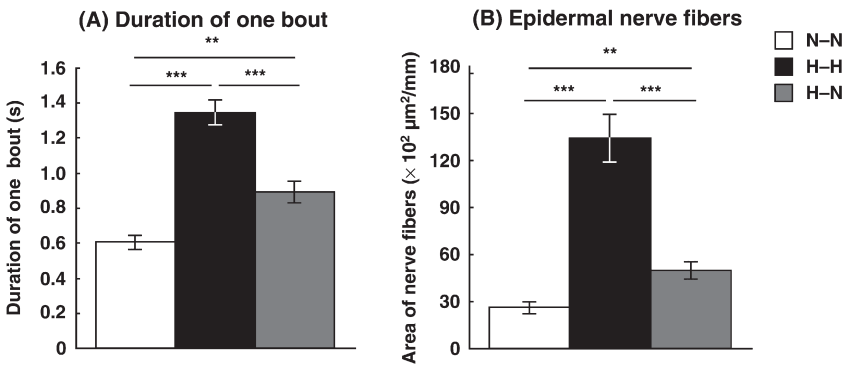

Fig. 5. Changes in the prolonged duration of one scratching bout (A) and the increase in the area of protein gene product (PGP) 9.5-immunoreactive nerve fibers in the epidermis (B) after cessation of HR-AD feeding. On day 64 after the start of feeding with HR-AD, feeding with HR-AD was changed to feeding a normal diet. The parameters were assessed 7 days after the feeding change. N – N group: mice continuously fed a normal diet. H – H group: mice continuously fed HR-AD. H – N group: mice fed a normal diet after being fed with HR-AD for 64 days. Each column represents the mean ± S.E.M. of 8 – 10 animals. $^{**}P<0.01$ and $^{***}P<0.001$.

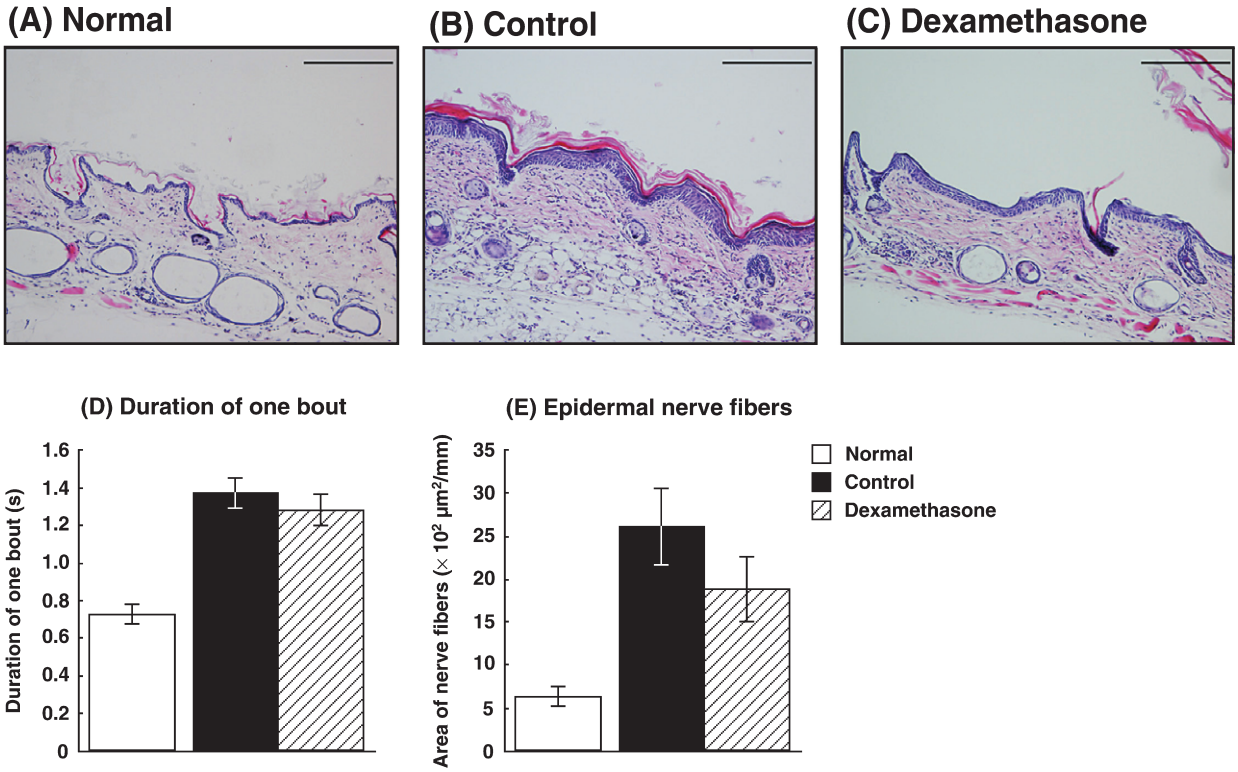

Fig. 6. Effects of dexamethasone on the skin inflammation (A, B, and C), the prolonged duration of one scratching bout (D) and the increase in the area of protein gene product (PGP) 9.5-immunoreactive nerve fibers in the epidermis (E) of HR-AD-fed mice. Dexamethasone (1 mg/kg) was given daily from the start of HR-AD feeding. The mice were assessed on day 63. Scale bar in panels A, B, and C represent 200 μm. Normal: mice fed normal diet. Each column represents the mean ± S.E.M. of 9 or 10 animals.

irritant, such as contact with wool fibers, has been shown to produce itching (21). This indicates that atopic skin has a lower threshold of itching in response to exogenous irritants than the skin of healthy individuals; however, the main reason for this lower itching threshold is uncertain. On day 56, the nerve fibers in the skin of HR-AD-fed mice were obviously extended to the sub-layer of the stratum corneum, which is the main barrier of the skin. This observation raises the possibility that HR-AD-fed mice are highly susceptible to exogenous irritants, which increases the itching sensation. On the other hand, patients with atopic dermatitis have skin barrier dysfunction (22). Similarly, in HR-AD-fed mice, skin barrier dysfunction coincides with prolonged scratching on and after day 28, as reported previously (13, 14). Since prolonged scratching is inhibited by the application of petrolatum ointment, which helps recover the skin barrier (14), this barrier defect could be involved in the itching sensation in HR-AD-fed mice. Together, the nerve fiber changes and the skin barrier dysfunction may be the essential changes that are responsible for the itching in HR-AD-fed mice and probably in patients with atopic dermatitis.

Topical corticosteroids are most frequently used to treat atopic dermatitis and are effective in reducing skin inflammation through their anti-inflammatory properties. On the other hand, they do not seem to have a direct antipruritic action, as there is little direct evidence of their antipruritic properties. It has been reported that in NC/Nga mouse, which has been used as an atopic dermatitis model, spontaneous long duration scratching has been attenuated by dexamethasone treatment (23). However, in some animal allergic dermatitis models (24, 25), corticosteroid administration aggravated or did not influence the scratching behavior associated with the dermatitis. Thus, the effects of corticosteroid on the itching in atopic dermatitis-like skin conditions are controversial. Consistent with previous report (13), HR-AD-fed mice show atopic dermatitis-like skin inflammation, including epidermal hyperplasia and inflammatory cellular infiltrates; these inflammatory changes are significantly suppressed by dexamethasone treatment. Despite the anti-inflammatory actions of dexamethasone, prolonged scratching was not at all affected. Therefore, this suggests that the inflammatory changes are not directly involved in

the prolonged scratching seen in HR-AD-fed mice. On the other hand, dexamethasone did not affect the increase in PGP 9.5-immunoreactive intraepidermal nerve fibers in HR-AD-fed mice. As well, the skin barrier dysfunction in HR-AD-fed mice was not affected by dexamethasone treatment, as previously shown (13). Thus, these findings support the view that the increase in intraepidermal nerve fibers, as well as the skin barrier dysfunction, plays a crucial role in the increased itching sensation in HR-AD-fed mice.

The mechanisms by which the nerve fibers extend in HR-AD-fed mice remain unclear. Various studies provide evidence that some neurotrophins can contribute to neural extension. Nerve growth factor (NGF) is well known to play a critical role in cutaneous biology (26). In patients with atopic dermatitis, plasma NGF levels are well correlated with disease severity (27). It has also been reported that NGF is abundantly released from human keratinocytes in culture (28) and is highly expressed in the epidermis of the NC/Nga mouse (29, 30). Furthermore, in NC/Nga mice, treatment with an anti-NGF antibody abolished not only nerve fiber extension but also the skin lesions (30). On the other hand, recent studies have shown that in atopic dermatitis patients, circulating levels of brain-derived neurotrophic factor were correlated with disease severity (31). These findings suggest that elevated neurotrophin levels contribute to neural extension in HR-AD-fed mice.

Taken together, the present results suggest that the extension of nerve fibers into the epidermis could be involved in the itch-related scratching of HR-AD-fed mice. Further investigations on the relationship between itching and the neural changes in this model may shed light on the etiology of itching in human atopic dermatitis.

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