Original Article

Effects of neuropeptides in the development of the atopic dermatitis of mouse models

Masako Kojima,1 Michiko Aihara,1 Masako Yamada,1 Setsuko Matsukura,1 Tsutomu Hirasawa2 and Zenro Ikezawa1

1Department of Environmental Immuno-Dermatology, Yokohama City University Graduate School of Medicine, Yokohama and 2Discovery Research Laboratories, Shionogi and Co. Ltd, Osaka, Japan

ABSTRACT

Background: Neuropeptides are considered to be factors that trigger, exacerbate or modulate allergic diseases and inflammatory skin reactions. In addition, it has been indicated that allergic diseases are highly correlated with stress.

Methods: 2,4,6-Trinitrochlorobenzene (TNCB) and crowding stress were applied to mouse atopic dermatitis (AD) models for the purpose of provoking chronic dermatitis. In addition, TNCB-sensitized mice were given olopatadine hydrochloride (10 or 3 mg/kg), an anti-allergic agent, orally. The neuropeptide content in the skin tissues and serum IgE levels were measured in these mice.

Results: Repeated local application of TNCB solution induced itching skin lesions, together with an increase in levels of substance P, a decrease in calcitonin gene-related peptide levels and early augmentation of IgE production. Treatment with crowding stress brought about a transient increase in substance P. Furthermore, we observed significant decreases in substance P and serum IgE levels when the administration of olopatadine hydrochloride was started before the elicitation of chronic dermatitis caused by repeated application of TNCB solution. These changes were more definite in the group administered a higher dose of the drug (10 mg/kg).

Conclusions: It is suggested that repeated contact allergic dermatitis or mental stress may promote the development and exacerbation of AD and that substance P has a role in this response. In addition, it seems that an anti-allergic drug, such as olopatadine hydrochloride, possibly downregulates substance P, thereby suppressing the development of AD. In the future, the development and clinical application of a drug that strongly influences the release of neuropeptides, such as substance P, and the expression of neuropeptide receptors would be expected for the treatment of AD.

Key words: atopic dermatitis, calcitonin gene-related peptide, neuropeptides, olopatadine hydrochloride, substance P.

INTRODUCTION

Sensory and autonomic nerves are widely distributed in the skin, together with more than 20 types of neuropeptides. Neuropeptides do not simply act as neurotransmitters or neuromodulators, but also exhibit diverse physiological activities in the skin. They are considered to be factors that trigger, exacerbate or modulate allergic diseases. In addition, it is indicated that allergic diseases are highly correlated with stress.1–3

In the present study, we measured the neuropeptide contents of skin tissues and serum IgE levels in mouse atopic dermatitis (AD) models, with the aim of investigating the involvement of neuropeptides in the development and exacerbation of AD, as well as the factors affecting neuropeptide content.
METHODS

Experimental animals

Female NC/Nga mice aged 7–11 weeks and raised in specific pathogen-free (SPF) conditions were purchased from Japan SLC (Shizuoka, Japan). Five week-old female DS-Nh mice raised in SPF conditions (Shionogi, Pharmaceutical Research and Development Division, Osaka, Japan) were acclimatized under conventional conditions before being used in the experiments.

The animal room was controlled at a temperature of 23 ± 3°C, 55 ± 10% humidity and a 12 h light–dark cycle. Animals were kept in cages lined with sterilized sawdust and were given sterilized solid feed (CE-2; CLEA Japan, Tokyo, Japan) and water ad libitum.

Drugs and reagents

Olopatadine hydrochloride (Kyowa Hakko Kogyo, Tokyo, Japan), which was used as the test drug, was dissolved in distilled water. A 1% solution of the sensitizing antigen 2,4,6-trinitrochlorobenzene (TNCB) was made in a solvent of acetone : olive oil (4 : 1) and used in the experiments.

Sensitization and elicitation of chronic dermatitis

2,4,6-Trinitrochlorobenzene (100 µL of a 1% solution) was applied to the dorsal skin of NC/Nga and DS-Nh mice for sensitization. Seven days after sensitization, 50 µL of the same solution was applied to the skin of the same area to induce skin lesions. Thereafter, 50 µL of the same solution was applied repeatedly to the same skin area every other day (48 h intervals) for the purpose of provoking chronic dermatitis.

Stress treatment

Female NC/Nga mice raised under SPF conditions were purchased at 7 weeks of age and housed 6 per cage in cages intended for 10 mice until 12 weeks of age. After reaching 12 weeks of age, mice were housed 12 per cage in cages intended for five mice to produce crowding stress. Skin biopsies and blood collections were performed before stress treatment and after 2, 4, 7 and 13 days under stress.

Olopatadine administration

The TNCB-sensitized DS-Nh mice were administered olopatadine hydrochloride, an anti-allergic agent, orally using a stomach tube 5 days a week for 2 weeks. Mice were allocated to either a 10 or 3 mg/kg group. Olopatadine hydrochloride was dissolved in 150 µL distilled water and administered to the TNCB-treated groups. Control mice were administered the same volume of distilled water. The effect of olopatadine hydrochloride in mice was compared between groups in which drug administration was started either 1 day before or 1 week after the elicitation of chronic dermatitis.

Evaluation of the severity of skin symptoms

Erythema, edema, scratch marks or erosion and exfoliation were scored on a four-point scale (from 0 to 3, where 0 was defined as no symptoms, 1 = mild, 2 = moderate and 3 = severe).

Measurement of neuropeptide content in skin tissues

Dorsal mouse skin was placed in 10 mL of 0.5 mol/L acetate buffer (including 1 µg/mL aprotinin and 1 µg/mL leupeptine), heated at 95°C for 10 min and homogenized. The homogenate was centrifuged at 40 000 g for 30 min. Then, 2 mL diethyl ether was added to approximately 10 mL supernatant. After shaking vigorously, the tube was left to stand and the ether layer was removed. This procedure was repeated five times and the aqueous layer was then freeze-dried. The freeze-dried sample was reconstituted in enzyme immunoassay (EIA) buffer for the measurement of neuropeptides. Substance P was measured using the Substance P Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI, USA) and calcitonin gene-related peptide (CGRP) was determined using the RAT CGRP Enzyme Immunoassay Kit (SPIbio, Messy, France). Measurements were made using a microplate reader (model 3550; Bio-Rad, Hercules, CA, USA) equipped with Microplate Manager III Macintosh data-analysis software (ver. 1.57; Bio-Rad).

Serum IgE measurements

After blood collection, serum was separated and stored at −80°C. The serum was thawed before IgE determination. A mouse IgE measurement kit (Yamasa EIA;
Yamasa Shoyu, Chiba, Japan) was used with the microplate reader (model 3550; Bio-Rad).

**Histopathological analysis**

Mice were shaved the day before skin tissue collection. Under anesthesia with Nembutal (sodium pentobarbital; Dainippon Pharmaceutical, Osaka, Japan), the dorsal skin was collected, fixed in 10% formalin and paraffin embedded. Sections (2 μm) were cut and stained with hematoxylin–eosin (HE) or Giemsa stain. In addition, rabbit anti-protein gene product (PGP) 9.5 polyclonal antibodies (Chemicon International, Temecula, CA, USA) were used as the anti-PGP9.5 antibodies to stain nerve fibers.

**Statistical analysis**

Results are expressed as the mean ± SEM. Student’s t-test was used to compare two groups. In all analyses, P < 0.05 was considered significant. Statistical analyses were performed using StatView (SAS Institute, Cary, NC, USA).

**RESULTS**

**Factors promoting AD development in individuals with atopic disposition**

*Induction of a contact hypersensitivity reaction to TNCB*

The NC/Nga and DS-Nh mice were sensitized with TNCB and chronic skin lesions were induced by repeated local application of TNCB.

Repeated local application of TNCB solution induced itching skin lesions, together with an increase in levels of substance P (Fig. 1a) and a decrease in levels of CGRP (Fig. 1b), changes that were significant in NC/Nga mice and mild (i.e. not significant) in DS-Nh mice. Serum IgE levels were not elevated in NC/Nga mice that had not been treated with TNCB, but were slightly, but not significantly, elevated in TNCB-treated NC/Nga mice. In the case of TNCB-treated DS-Nh mice, serum IgE levels were augmented considerably more and earlier than untreated DS-Nh mice (Fig. 2). These serum IgE levels showed no significant differences. Although the development of skin lesions was observed at the site of application of TNCB application on the dorsal area (Fig. 3), lesional skin was

![Fig. 1](image_url)

**Fig. 1** Effects of sensitization with 2,4,6-trinitrochlorobenzene (TNCB) on (a) substance P and (b) calcitonin gene-related peptide (CGRP) levels in NC/Nga and DS-Nh mice. Repeated local application of TNCB solution induced skin lesions, together with an increase in levels of substance P and a decrease in levels of CGRP. (□), untreated (control); (■), TNCB treated (+ skin lesions). Data are the mean ± SEM. *P < 0.05 compared with control.
NC/Nga mouse

DS-Nh mouse

Fig. 2  Effects of sensitization with 2,4,6-trinitrochlorobenzene (TNCB) on serum IgE levels in NC/Nga and DS-Nh mice. (■), DS-Nh mice sensitized with TNCB; (□), untreated DS-Nh mice; (▲), NC/Nga mice sensitized with TNCB; (△), untreated NC/Nga mice. In DS-Nh mice, early augmentation of IgE production was observed compared with untreated mice. Data are the mean ± SEM.

Fig. 3  Clinical features of skin lesions induced by repeated local application of 2,4,6-trinitrochlorobenzene in NC/Nga and DS-Nh mice. Symptoms commonly observed in spontaneously developing skin lesions are shown.

Fig. 4  Effects of mental stress on (a) neuropeptides (■, substance P; ▲, calcitonin gene-related peptide) in the dorsal skin and (b) bodyweight of NC/Nga mice (■, stressed mice; ▲, control mice) under specific pathogen-free conditions. Twelve mice were housed in one cage (17 cm × 15 cm) for 2 weeks. † Two of six mice developed skin lesions. Data are the mean ± SEM. *P < 0.05 compared with day 0.
also found on the face and auricles, which did not receive any direct application of antigen.

**Treatment with crowding stress**

In experiments using NC/Nga mice, substance P and CGRP levels decreased mildly without significant differences on day 2 under stress conditions. On day 4, substance P concentrations increased to levels higher than before the stress had been induced, whereas levels of CGRP did not show any change. On day 7, concentrations of both substance P and CGRP recovered to levels seen before the stress had been induced (Fig. 4a). Serum IgE levels showed no apparent increase under stress.

However, a loss of bodyweight was observed immediately after animals were placed under stress (Fig. 4b) and lesional skin developed in the neck region on day 4 under stress conditions. Skin lesions developed in two of six mice. No development of new lesional skin was observed thereafter.

**Effect of an anti-allergic agent on neuropeptide content**

In DS-Nh mice given the anti-allergic drug olopatadine hydrochloride on consecutive days starting from the day before TNCB application, there was a significant decrease in substance P (Fig. 5a), although no changes
in CGRP levels (Fig. 5b) were observed and serum IgE levels showed no significant difference, but decreased obviously (Fig. 5c) was observed. Skin lesions were clearly milder (Fig. 5d). These changes were more definite in the group given the higher dose of olopatadine hydrochloride (Fig. 5).

When the administration of olopatadine hydrochloride was started 1 week after the induction of chronic dermatitis caused by the repeated application of TNCB solution, although the severity of the skin lesions was diminished (Fig. 5d), there were no definite changes on the levels of substance P (Fig. 5a), CGRP (Fig. 5b) and serum IgE (Fig. 5c).

**Histopathological observations**

When samples of lesional skin tissues, induced by the repeated application of TNCB, were stained with HE, marked acanthosis, hyperkeratosis and cellular infiltration were observed (Fig. 6). When tissues were stained with Giemsa, an increased number of mast cells and active degranulation were seen in lesional skin tissues compared with non-lesional skin tissues (Fig. 7). Using the anti-PGP9.5 stain, a thickening of cutaneous nerve fibers in the upper layers of the dermis and elongation of some nerve fibers to the epidermis were observed (Fig. 8).
DISCUSSION

The NC/Nga mouse strain was developed in 1957 and reported as a murine AD model in 1997. These mice, kept under conventional conditions, are known to develop skin lesions accompanied by elevated serum IgE levels from 8 weeks of age, showing intense scratching behavior resulting in bleeding or even partial loss of tissues.

The DS/Nh mice were introduced as mice in which dermatitis occurred spontaneously associated with Staphylococcus aureus. When DS-Nh mice are transferred from SPF to conventional conditions, S. aureus is isolated from the skin and strongly itching AD-like dermatitis develops spontaneously, mainly on the face, from 5 weeks of age. These mice also show elevated IgE levels after 20 weeks of age. Because the pathology observed in DS-Nh mice resembles AD, the usefulness of these mice as a model for AD is attracting attention, as in the case of NC/Nga mice.

Neuropeptides are small amino acid compounds contained in the neurons of the brain, spinal cord and peripheral nerves, where they act as both neurotransmitters and neuromodulators. Neuropeptides are present throughout the body and may be produced by and localized in cells other than those of the central and peripheral nervous systems. Neuroendocrine cells in the respiratory tract and skin contain several peptides. In addition, they have been identified in various inflammatory cells, including eosinophils, mast cells and mononuclear and polymorphonuclear leukocytes. Once released, they act on target cells through specific receptors and are finally degraded into biologically inactive fragments by several enzymes, namely peptidases.

In the skin, peripheral nerves are abundantly distributed and more than 20 types of neuropeptides are found. Substance P and CGRP, released antidromically from C-fibers of sensory nerves by epicutaneous stimulation, lead to vasodilation and the leakage of plasma proteins and produce erythema and edema. Substance P promotes degranulation of mast cells and the release of histamine, leukotriene B4 or tumor necrosis factor (TNF) as an inflammatory cytokine. In addition, these neuropeptides, directly or indirectly, lead to augmentation of the expression of adhesion molecules in vascular endothelial cells and the infiltration of inflammatory cells.

Although abnormal expression of neuropeptides, especially substance P and CGRP, in the skin of AD patients has been reported, there are almost no reports of quantitative determinations of skin neuropeptides levels. The present study provides interesting data on murine AD, especially results concerning CGRP levels.

We have compared the neuropeptide content of skin tissues from NC/Nga, DS-Nh, BALB/c and C57BL/6 mice, aiming to elucidate the relationship between AD and neuropeptides. We have reported that there is an increase in substance P levels and a decrease in CGRP levels in skin lesions from NC/Nga mice kept under conventional conditions compared with levels seen in non-lesional skin in the same mouse strain. Markedly higher skin levels of substance P were observed in non-lesional skin of NC/Nga mice kept under conventional conditions compared with non-lesional skin of the same mouse strain and the other strains under SPF conditions.
Some reports have shown an increase in substance P- and CGRP-containing nerve fibers in the skin of AD patients. In contrast, the results of the present study revealed a paradoxical decrease in CGRP levels, despite an increase in levels of substance P. The imbalance between substance P and CGRP, which has immunosuppressive effects, may play an important role in the development and maintenance of AD-like dermatitis.

In the present study, chronic contact allergy was induced by repeated application of TNCB to the dorsal skin of mice kept under SPF conditions. Histopathologically, acanthosis, hyperkeratosis, cellular infiltration, increases in the number of mast cells with degranulation and nerve fibers elongating to the epidermis were observed in the skin lesions, together with an increase in IgE, as observed in human AD. It was thought that chronic contact allergic dermatitis induced by repeated application of TNCB triggers AD-like skin lesions, accompanied by augmentation of IgE production.

In skin lesions on the dorsal areas of NC/Nga mice with chronic dermatitis induced by repeated application of TNCB, a significant increase in levels of substance P was observed compared with non-lesional dorsal skin in untreated mice. In contrast, CGRP levels were significantly lower in the skin lesions of TNCB-sensitized NC/Nga mice compared with non-lesional skin in untreated mice. Although DS-Nh mice also showed the same tendency of changes in neuropeptide content, the degree of change observed in DS-Nh mice was milder than that seen in NC/Nga mice and not statistically significant. In DS-Nh mice with chronic dermatitis induced by repeated application of TNCB, an early and marked increase in serum IgE levels was observed. However, in untreated NC/Nga mice, a slight, albeit not significant, increase in serum IgE levels was observed.

Matsukura et al. have found that the development of skin eruptions and increases in serum total IgE in DS-Nh mice are not caused by Th2-predominant mechanisms but, rather, by Th1-predominant mechanisms through an increase in the production of interleukin (IL)-13 (S Matsukura et al., unpubl. obs., 2003), whereas NC/Nga mice have been reported as being a Th2-predominant AD mouse model in previous studies. Therefore, differences in effects on serum IgE levels between the two models may reflect differences in the predominance of Th1 and Th2 mechanisms in these mice. These findings suggest that the difference in the mechanisms responsible for the development of skin lesions and the production of IgE between the two strains is related to changes in neuropeptides.

We focused on stress, which is known to have important direct and indirect effects on various diseases, such as exacerbation of inflammatory diseases. The word 'stress' advocated by Selye has taken root as a daily word with strong psychological implications; however, in the scientific field, the scope of application of this word is extremely ambiguous. In stress experiments conducted in animals, physical stress is commonly used as the stressor, such as restraint, abnormal environmental temperature and forced exercises including swimming. However, in the present study, we tried to eliminate the direct effects of physical treatment and, thus, used crowding stress, which is defined as a sociopsychological stress. According to former studies, crowding induces weight loss, increased adrenal gland weight and increased production of monoamine metabolites (neurotransmitters), all of which are characteristic of depression and stress.

Accompanying recent advances in immunology, it is now known that the nervous, endocrine and immune systems are closely interrelated and regulate body functions via neurotransmitters, hormones and cytokines, and that this balance is greatly impaired and the immune system is changed markedly during stress. Hosoi et al. reported a correlation between serum corticosterone levels and immobilization stress. That study also suggested that stress affects the cutaneous immune response. Furthermore, another report has also indicated that the psychological state is related, to some extent, to the immunological state.

In the study of factors that affect AD, mental stress induced a transient increase in substance P levels and development of skin lesions in NC/Nga mice. Because acute immobilization stress has been reported to trigger skin mast cell degranulation via corticotropin-releasing hormone and substance P, these results strongly suggest a relationship between an increase in substance P induced by stress and the induction of skin lesions.

In contrast, CGRP levels showed no definite changes at the time when substance P levels were increased. Tsuchiya et al. reported that immobilization stress did not influence the cutaneous CGRP content determined by radioimmunoassay or the number of CGRP-immunoreactive nerve fibers in the skin. These facts suggest that crowding and immobilization-induced stress do not produce the antidromic mechanism of CGRP in the
primary afferent nerves of the skin and that the effects of such stress, if any, are less than that induced by cutaneous stimuli, such as aggravating mechanical stimuli or irritating cold.

The transient increase in substance P levels observed in the present study supports the hypothesis that the adaptive phenomenon under chronic stress recovers the immune function to the prestress state.25

We conducted another study on the effects of an anti-allergic drug, namely olopatadine hydrochloride, on skin symptoms and the expression of neuropeptides. Olopatadine is a dibenzo-oxepin derivative possessing carboxyl and amino groups in its molecule and it is a novel anti-allergic agent developed by Kyowa Hakko Kogyo Co. Ltd. Olopatadine is a selective histamine H\textsubscript{1} receptor antagonist possessing inhibitory effects on the release of inflammatory lipid mediators, such as leukotrienes and thromboxanes, from human polymorphonuclear leukocytes and eosinophils. Olopatadine also inhibits the tachykininergic contraction in guinea pig bronchi by prejunctional inhibition of peripheral sensory nerves.26 In addition, further pharmacological actions,26,27 including inhibition of eosinophilic infiltration, have been reported for olopatadine.

In the present study, the development of skin lesions was clearly inhibited only when the administration of olopatadine was started before the elicitation of chronic dermatitis caused by the repeated application of TNCB. Substance P levels in skin tissues were markedly suppressed in mice given the high dose of olopatadine. However, when the drug was administered after skin lesions had developed, although the skin lesions diminished, there was no clear effect on levels of substance P in the skin tissues. These results suggest significant involvement of substance P in the pathogenesis of AD. Furthermore, some of the effect of olopatadine on the development of AD may be due to the suppression of substance P.

In conclusion, it is suggested that repeated contact allergic dermatitis or mental stress may promote the development and exacerbation of AD and that substance P has a role in the response. In addition, it seems that an anti-allergic drug, such as olopatadine hydrochloride, possibly downregulates substance P and thereby suppresses the development of AD. In the future, the development and clinical application of drugs strongly influencing the release of neuropeptides like substance P and the expression of neuropeptide receptors would be expected for the treatment of AD.

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REFERENCES