

Effects of high-affinity nerve growth factor receptor inhibitors on symptoms in the NC/Nga mouse atopic dermatitis model

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Summary

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Key words

AG879, atopic dermatitis, K252a, NC/Nga mouse, nerve growth factor, tropomyosin-related kinase A

Conflicts of interest

None declared.

Background Nerve growth factor (NGF) is an important substance in the skin, where it modulates nerve maintenance and repair. However, the direct link between NGF and pruritic diseases such as atopic dermatitis is not yet fully understood. Our previous study showed that NGF plays an important role in the pathogenesis of atopic dermatitis-like skin lesions in NC/Nga mice. NGF mediates its effects by binding to two classes of transmembrane receptors, a high-affinity receptor (tropomyosin-related kinase A, TrkA) and a low-affinity receptor (p75).

Objectives To determine the significance of NGF receptors in the pathogenesis of atopic dermatitis, the effects of TrkA inhibitors AG879 and K252a on the symptoms of NC/Nga mice were evaluated.

Methods Male NC/Nga mice with severe skin lesions were used. AG879 or K252a was applied to the rostral part of the back of mice five times a week. The dermatitis score for the rostral back was assessed once a week. The scratching behaviour was measured using an apparatus, MicroAct (Neuroscience, Tokyo, Japan). Immunofluorescence examinations were made in the rostral back skin for nerve fibres, NGF and TrkA receptor.

Results Repeated applications of AG879 or K252a significantly improved the established dermatitis and scratching behaviour, and decreased nerve fibres in the epidermis. NGF was observed more weakly in keratinocytes, and a lower expression of TrkA was observed in stratum germinativum of the epidermis of mice treated with AG879 or K252a compared with those treated with vehicle.

Conclusions We suggest that NGF plays an important role in the pathogenesis of atopic dermatitis-like skin lesions via the high-affinity NGF receptor. These findings provide a new potential therapeutic approach for the amelioration of symptoms of atopic dermatitis.

Itching is a characteristic symptom in various forms of dermatosis, especially atopic dermatitis; it consequently constitutes a major diagnostic criterion.^{1,2} The itch-scratch cycle – a strong scratching action facilitating susceptibility to more itching and the aggravation of skin lesions in patients with atopic dermatitis^{3,4} – is a well-known phenomenon. The most effective strategy would be to prevent this aggravation of skin lesions and to improve quality of life for patients with atopic dermatitis by reducing itching and scratching.⁵

Cutaneous nerve fibres are present at higher densities in the epidermis of the lesional skin found in atopic derma-

titis.^{6,7} This increase in nerve fibres in the epidermis is partly responsible for the intense itching sensations in the lesional skin of patients with atopic dermatitis. It is now generally accepted that nerve growth factor (NGF), which is released from keratinocytes in the skin, is one of the major mediators determining skin innervation density.^{8–10} NGF is an important substance in the skin environment, where it modulates nerve maintenance and repair.^{11,12} However, the nature of the relationship between NGF and pruritic conditions such as atopic dermatitis is still not fully understood. A recent study has reported that NGF is overexpressed in

prurigo nodularis, hypothesizing that NGF and its receptors contribute to the neurohyperplasia of the disease.¹³ NGF protein levels are higher in psoriatic skin than in nonlesional and normal skin,¹⁴ and psoriatic keratinocytes express higher amounts of NGF than normal keratinocytes.¹⁵ Patients with atopic dermatitis also showed significantly higher plasma NGF levels compared with controls, and a strong correlation was noted between plasma NGF and the severity of symptoms.¹⁶ We conducted our investigation using NC/Nga mice, considered a suitable model for atopic dermatitis.^{17–19} We previously observed that the epidermis of lesional skin expresses high levels of NGF compared with nonlesional skin, and that repeated administration of NGF-neutralizing antibody significantly inhibited skin lesions and scratching behaviour in NC/Nga mice.²⁰ These findings suggest that NGF may play an important role in the pathogenesis of atopic dermatitis, possibly regulating the development of atopic dermatitis lesions.

NGF mediates its effects by binding to two classes of transmembrane receptors, a high-affinity receptor (tropomyosin-related kinase A, TrkA), for which NGF is the primary ligand, and the nonspecific neurotrophin receptor p75, which binds all known neurotrophins including NGF, brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5 with low affinity.^{21–23} The binding of NGF to the TrkA receptor is responsible for the transmission of NGF signals for mitogenesis and differentiation.^{24,25} The role of p75 in these processes is less clear, but it may function as an accessory protein that modifies ligand-binding affinity,²⁶ and recent evidence has implicated it as having a role in the apoptotic cell death cascade.^{27–29} In atopic dermatitis, the roles played in pathogenesis by the two types of NGF receptors remain unclear. To determine the significance of NGF receptors in the pathogenesis of atopic dermatitis, we evaluated the efficacies of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (AG879) and an alkaloid-like material isolated from the culture broth of *Nocardopsis* sp. (K252a), high-affinity NGF receptor inhibitors,^{30–33} in NC/Nga mice.

Materials and methods

Animals

Male NC/Nga mice purchased from SLC Japan (Shizuoka, Japan) were all housed under conditions of controlled temperature (23 ± 3 °C), humidity ($55 \pm 15\%$) and lighting (lights on from 07.00 to 19.00 hours). To increase the incidence of dermatitis-related scratching behaviour, they were kept together with mice of the same strain with severe skin lesions (males, 25–30 weeks of age),³⁴ and then used at 25 weeks of age. Eight mice in each group were used. Food and tap water were provided *ad libitum*. All studies reported here have been reviewed by the Taisho Pharmaceutical Animal Care Committee and have met the Japanese Experimental Animal Research Association Standards as defined in the Guidelines for Animal Experiments (1987).

Materials

AG879 ($0.1 \mu\text{mol L}^{-1}$; Calbiochem, San Diego, CA, U.S.A.) and K252a ($2.0 \mu\text{mol L}^{-1}$; Alomone Labs, Jerusalem, Israel) were dissolved in dimethyl sulphoxide (Wako, Osaka, Japan). Concentrations for these compounds were set at 10 times the dose necessary to inhibit neurite generation initiated by NGF *in vitro*.^{30,33}

Evaluation of inhibitory effects of AG879 or K252a on established dermatitis and scratching behaviour

One hundred microlitres of AG879 or K252a was applied to the rostral part of the back of mice five times a week. The dermatitis score for the rostral back was assessed once a week (0, none; 1, slight; 2, moderate; 3, severe). This scoring was based on the severity of erythema/haemorrhage and pilosis. The total weekly score (minimum 0, maximum 6) for each mouse was obtained by summation. The scratching behaviour of NC/Nga mice was measured after at least 24 h had passed following the last application of each compound. The scratching behaviour was measured using an apparatus, MicroAct (Neuroscience, Tokyo, Japan). After mice were acclimatized for 30 min in an observation chamber (11 cm in diameter, 18 cm high), the number of scratching movements directed at the rostral back was measured for 30 min as an index for the itch sensation.^{35,36}

Immunofluorescence for nerve fibres, nerve growth factor and tropomyosin-related kinase A receptor in the skin

The rostral back skin of each mouse was extracted and fixed in 10% neutral buffered formalin. The fixed samples were embedded in paraffin and 3- μm sections prepared. After deparaffinization with xylene and graded ethanol solutions, these sections were preincubated in 1% bovine serum albumin-phosphate-buffered saline (PBS) for 30 min at room temperature and rinsed with PBS, then incubated with antibodies as described below.

The sections were incubated with polyclonal rabbit antibody against protein gene product 9.5 (UltraClone Limited, Welling, Isle of Wight, U.K.; dilution 1:400) overnight at 4 °C and washed with PBS, then incubated with Alexa Fluor goat 488 antirabbit IgG ($10 \mu\text{g mL}^{-1}$; Molecular Probes, Eugene, OR, U.S.A.) for 1 h at room temperature away from light.

The sections were treated with avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, U.S.A.). After washing with PBS, the sections were incubated overnight with rabbit polyclonal antibody against mouse NGF (Alomone Labs; dilution 1:60) or anti-TrkA receptor (sc-188; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.; dilution 1:200) at 4 °C. After washing with PBS, these sections were incubated with $10 \mu\text{g mL}^{-1}$ biotinylated goat antirabbit IgG (Vector Laboratories) for 1 h at room temperature. After that, the sections were incubated with $10 \mu\text{g mL}^{-1}$ Alexa Fluor 568-conjugated streptavidin (Molecular Probes) for 1 h at room temperature away from light.

Finally, all these sections were observed via confocal laser scanning microscopy (TCS-SP5; Leica Microsystems, Heidelberg, Germany).

Data analysis

Experimental values for dermatitis scores are given as the median and interquartile range, and for scratching counts are given as mean and SEM. Statistical comparisons were made using Dunnett's multiple comparison; $P < 0.05$ was considered as having statistical significance.

Results

Effects of AG879 and K252a on established dermatitis and scratching behaviour in NC/Nga mice

In both the AG879 and K252a groups, dermatitis scores improved significantly from week 1 compared with the mice treated with vehicle (Fig. 1a). At week 4, scratching behaviour had decreased for mice in both AG879 and K252a groups following repeated applications (Fig. 1b).

Immunohistochemical staining of nerve fibres, nerve growth factor and tropomyosin-related kinase A in the skin of NC/Nga mice

In the mice treated with vehicle, nerve fibres were observed at many sites in the epidermis, and epidermal thickening was observed (Fig. 2a). In contrast, few nerve fibres were observed in the epidermis of mice treated with AG879 (Fig. 2b) and K252a (Fig. 2c). NGF immunoreactivity was observed strongly in keratinocytes and mast cells of mice treated with vehicle (Fig. 3a), and moderately in mast cells but weakly in keratinocytes of mice treated with AG879 (Fig. 3b) and K252a (Fig. 3c). High expression of TrkA was observed in stratum germinativum of the epidermis of mice treated with vehicle

(Fig. 4a), compared with that of mice treated with AG879 (Fig. 4b) and K252a (Fig. 4c).

Discussion

NC/Nga mice were originally established as an inbred strain of Japanese fancy mice. Under conventional conditions, NC/Nga mice develop spontaneous skin lesions with diagnostic characteristics of high concentrations of total IgE in plasma and invasion of inflammatory cells into skin lesions.^{17,19} NC/Nga mice with severe skin lesions frequently scratch their face, ears and the rostral part of the back with their hind paws.¹⁸ As all of these traits are similar to those observed in patients with atopic dermatitis, NC/Nga mice are considered a suitable model for human atopic dermatitis. Our previous study showed that nerve fibres were significantly increased in the epidermis of lesional skin, the NGF content of the serum and skin was significantly elevated, and repeated administration of an NGF-neutralizing antibody significantly inhibited skin lesions and scratching behaviour in NC/Nga mice.²⁰

NGF mediates its effects by binding to two classes of transmembrane receptors, TrkA and p75.^{21–23} TrkA and p75 are expressed not only in neurones, but also in keratinocytes.^{37,38} AG879 and K252a were characterized as protein kinase inhibitors.^{30–33} In the present study, the effects of these TrkA inhibitors on the symptoms of NC/Nga mice were evaluated. Dimethyl sulphoxide, used as a vehicle in this study, has anti-inflammatory effects when applied topically.³⁹ Dermatitis scores were slightly reduced by topical application of dimethyl sulphoxide. However, repeated applications of AG879 or K252a significantly improved the established dermatitis, scratching behaviour, and innervation in the epidermis in NC/Nga mice compared with the vehicle group. The effects observed in this study were similar to results of the effects of NGF-neutralizing antibody reported previously.²⁰ These results strongly suggest that TrkA is responsible for the effects exerted by NGF in the pathogenesis of atopic dermatitis-like

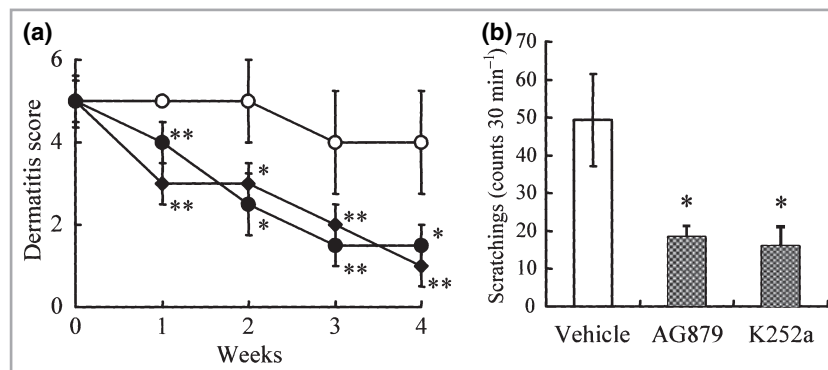


Fig 1. Effects of AG879 or K252a on established dermatitis and increased scratching behaviour of NC/Nga mice. Vehicle (open circles), AG879 (closed circles) or K252a (closed lozenges) was applied to the rostral part of the back of mice five times a week. The dermatitis score for the rostral back was assessed once a week (a). Values are the median and interquartile range for eight mice. The number of scratching movements directed at the rostral back was measured for 30 min (b). Values are the mean and SEM for eight mice. * $P < 0.05$, ** $P < 0.01$.

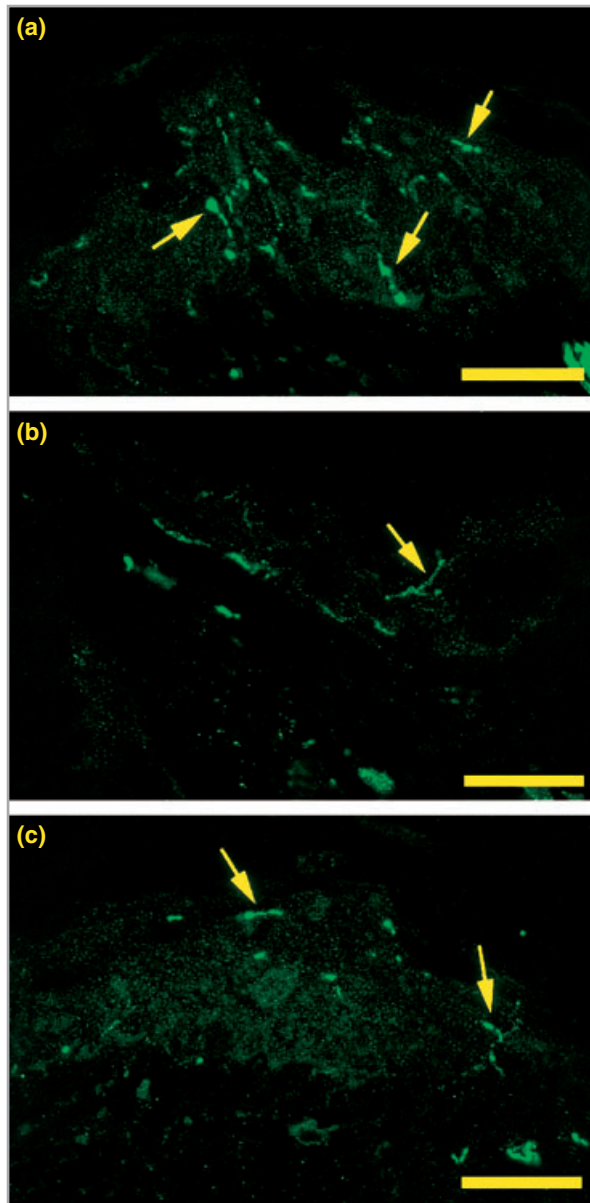


Fig 2. Protein gene product (PGP) 9.5 immunoreactivity of skin of NC/Nga mice following vehicle (a), AG879 (b) and K252a treatment (c). PGP9.5 immunoreactivity was seen in the epidermis (arrows). Scale bars = 25 μ m.

symptoms in NC/Nga mice. NGF stimulates TrkA phosphorylation in human keratinocytes, and functions as a survival factor for keratinocytes through TrkA.^{38,40,41} Although levels of p75 mRNA and protein in human keratinocytes increase during their exponential growth phase,³⁷ K252a, but not anti-p75, inhibits NGF-induced keratinocyte proliferation,³⁸ strongly suggesting that TrkA is the functional NGF receptor in neurones and keratinocytes. NGF is released from keratinocytes in the skin.

So far, no studies have reported on NGF receptor expression in the skin in atopic dermatitis. In NC/Nga mice, higher expression of TrkA was observed in the epidermis of lesional

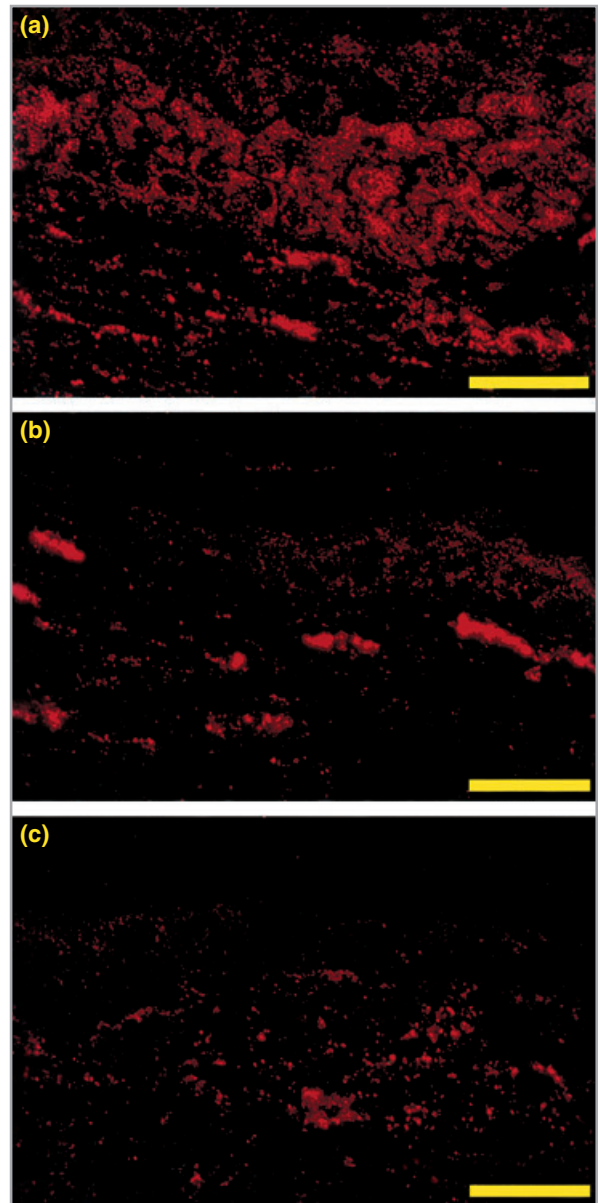


Fig 3. Immunofluorescence for nerve growth factor (NGF) in the skin of NC/Nga mice. NGF immunoreactivity was observed strongly in keratinocytes and mast cells of mice treated with vehicle (a), and moderately in mast cells but weakly in keratinocytes of mice treated with AG879 (b) or K252a (c). Scale bars = 25 μ m.

skin than in nonlesional skin (data not shown). The elevated expression of TrkA in lesional skin declined to the same level as that in nonlesional skin via repeated application of AG879 or K252a, as is also the case with NGF levels. NGF is released from keratinocytes in the skin, and TrkA is expressed in keratinocytes. A TrkA inhibitor can induce apoptosis in keratinocytes.⁴² There is some concern that the apoptosis induced by TrkA inhibitors may result in lower NGF levels and TrkA expression in the epidermis. On the other hand, there was no significant difference between the expression of p75 in lesional and in nonlesional skin, and no change was observed

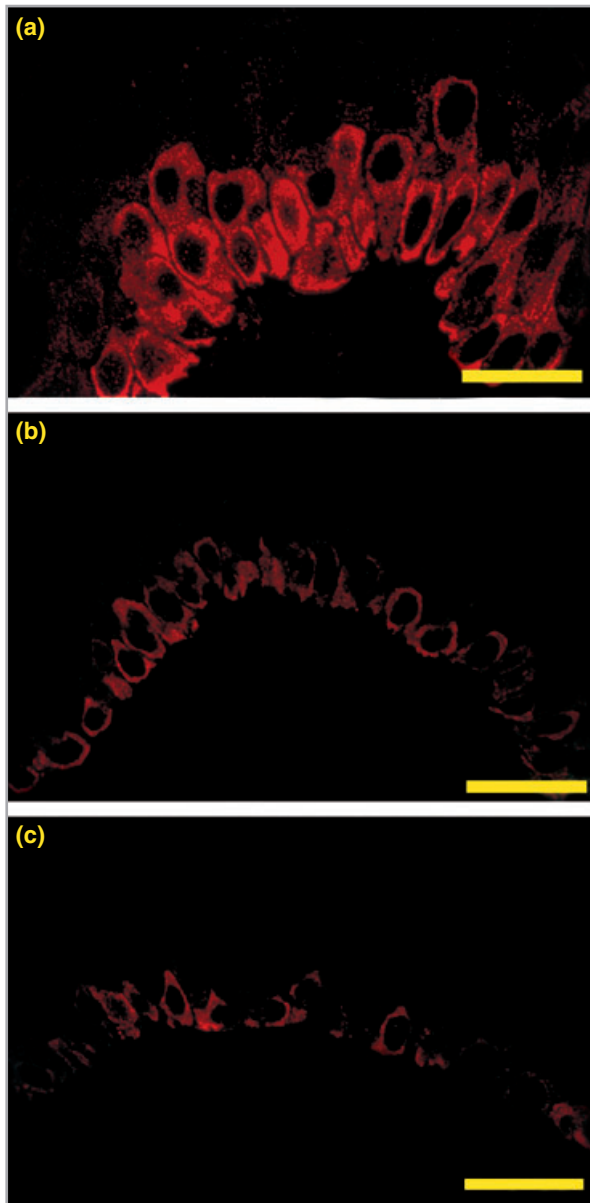


Fig 4. Immunofluorescence for tropomyosin-related kinase A (TrkA) in the skin of NC/Nga mice. High expression of TrkA was observed in stratum germinativum of the epidermis of mice treated with vehicle (a), compared with those treated with AG879 (b) or K252a (c). Scale bars = 25 µm.

following the application of AG879 or K252a (data not shown). Reports regarding the action of p75 indicate that this receptor is a TrkA coreceptor that can enhance or suppress neurotrophin-mediated TrkA activity, and autonomously activates signalling cascades that result in the induction of apoptosis or in the promotion of survival.^{43–46} Further study is needed to determine the action of NGF via p75 in the pathogenesis of atopic dermatitis.

In summary, our study demonstrated that repeated application of TrkA inhibitors can suppress skin lesions, scratching behaviour, and innervation into the epidermis in an atopic

dermatitis animal model, NC/Nga mice. We suggest that NGF plays an important role in the pathogenesis of atopic dermatitis-like skin lesions via the high-affinity NGF receptor. These findings provide a new potential therapeutic approach for ameliorating the symptoms of atopic dermatitis.

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