

Semaphorin3A Alleviates Skin Lesions and Scratching Behavior in NC/Nga Mice, an Atopic Dermatitis Model

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Topical steroids and antihistamines are commonly used for the treatment of atopic dermatitis (AD). However, in a substantial number of patients with AD, these treatments are not sufficiently effective. In AD patients, C-fibers in the epidermis increase and sprout, inducing hypersensitivity, which is considered to aggravate the disease. Semaphorin3A (Sema3A), an axon guidance molecule, is a potent inhibitor of neurite outgrowth of sensory neurons. To investigate the effect of Sema3A on AD, we administered recombinant Sema3A intracutaneously into the skin lesions of NC/Nga mice, an animal model of AD. Sema3A dose-dependently improved skin lesions and attenuated the scratching behavior in NC/Nga mice. Histological examinations revealed a decrease in: (a) epidermal thickness; (b) the density of invasive nerve fibers in the epidermis; (c) inflammatory infiltrates, including mast cells and CD4 + T cells; and (d) the production of IL-4 in the Sema3A-treated lesions. Because the interruption of the itch-scratch cycle likely contributes to the improvement of the AD-like skin lesions, Sema3A is promising in the treatment of patients with refractory AD, as well as overall itching dermatosis.

Journal of Investigative Dermatology advance online publication, 10 July 2008; doi:10.1038/jid.2008.150

INTRODUCTION

Atopic dermatitis (AD) is defined as “an itchy, chronic, or chronically relapsing, inflammatory skin condition.” Because itch is an unavoidable symptom and markedly reduces quality of life in AD patients, its control is an important issue in the treatment of AD. For the treatment of AD, topical corticosteroids, topical immunosuppressants, and antihistamines are commonly used (Friedmann and Holden, 2004). However, there are a substantial number of AD patients in whom such medications cannot control the itch sufficiently.

The itch-scratch cycle has attracted attention as a factor in the aggravation and chronicity of AD (Ikoma *et al.*, 2006; Paus *et al.*, 2006). Itch is conducted by afferent C-fibers, which are unmyelinated nerve fibers originating from dorsal root ganglia neurons. Normally, C-fibers possess free nerve

endings at the boundary between the epidermis and the dermis, and some nerve fibers invade the epidermis. In AD, however, C-fibers in the epidermis sprout. This is probably caused by nerve growth factor (NGF), which is released from keratinocytes or fibroblasts due to stimulations including scratching. Semaphorins, a large family of guidance cues for axonal/dendritic projections, are composed of both secreting and membrane-bound proteins sharing a sema domain (Luo *et al.*, 1993; Semaphorin Nomenclature Committee, 1999). The class 3 subfamily, including semaphorin3A (Sema3A), are secreted molecules that function as chemorepulsive/attractive cues in the central and peripheral nervous systems (Luo *et al.*, 1993; Kolodkin, 1998; Raper, 2000). Neuropilin-1 (NRP-1) binds to Sema3A with high affinity and is necessary for Sema3A-mediated repulsive guidance events (Nakamura *et al.*, 1998, 2000; Takahashi *et al.*, 1999; Raper, 2000).

Sema3A potentially induces retraction of NGF-sensitive neurons among dorsal root ganglia neurons (Dontchev and Letourneau, 2002). The expression levels of NRP-1 are increased by NGF (Pond *et al.*, 2002). Therefore, it is likely that Sema3A can induce retraction of invasive nerve fibers in the epidermis of AD. If it is possible to interrupt the itch-scratch cycle by use of Sema3A, this may provide the basis for a novel strategy for the treatment for AD. To investigate the effects of Sema3A, we locally administered Sema3A to skin lesions of NC/Nga mouse, which is commonly used as an AD model. The results showed a marked effect of Sema3A to reduce the scratch behavior and improve AD-like lesions both macroscopically and histologically in the NC/Nga mice. Histological examination revealed a reduction of the invasive

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Abbreviations: AD, atopic dermatitis; NGF, nerve growth factor; NRP-1, neuropilin-1; Sema3A, semaphorin3A

Received 22 November 2007; revised 7 April 2008; accepted 7 April 2008

nerve fibers in the epidermis and a decrease in the infiltration of inflammatory cells and IL-4 production in the dermis. Thus, it is likely that Sema3A exerts the effect through both neurological and immunological mechanisms.

RESULTS

Sema3A macroscopically improved AD

To investigate the effect of Sema3A on AD, 1.0 ml solution of Sema3A (750 U) or vehicle control was intracutaneously injected into the dorsal skin lesion of 12- to 14-week-old NC/Nga mice. Sema3A was administered to each mouse twice a day for 5 consecutive days. The mice were observed until 11 days after the first injection, and then skin biopsy was performed. Lesions were evaluated with clinical skin scores as described previously (Suto *et al.*, 1999; Takakura *et al.*, 2005). Macroscopically, the intracutaneous administration of Sema3A markedly improved the lesions in a time-dependent

fashion, leading to a significant reduction in the clinical skin score. Sema3A tended to decrease the clinical skin score at day 2, and the score was lowest at day 8 (Figure 1a and b). The vehicle-control treatment produced no effect on the skin lesions (Figure 1a and b). The improvement of skin lesions was only observed in those lesions to which Sema3A was administered and not elsewhere, for instance, the face or ear, demonstrating the local action of Sema3A. The effect of Sema3A continued for more than 6 days after the discontinuation of the treatment (Figure 1b), and then skin lesions gradually deteriorated after that. At day 16, the Sema3A-treated group had the same clinical skin score as the no-treatment and control groups (data not shown). To investigate whether the effect of Sema3A depended on the injected dose, we administered different doses of Sema3A and found that Sema3A reduced the clinical skin score in a dose-dependent manner (Figure 2).

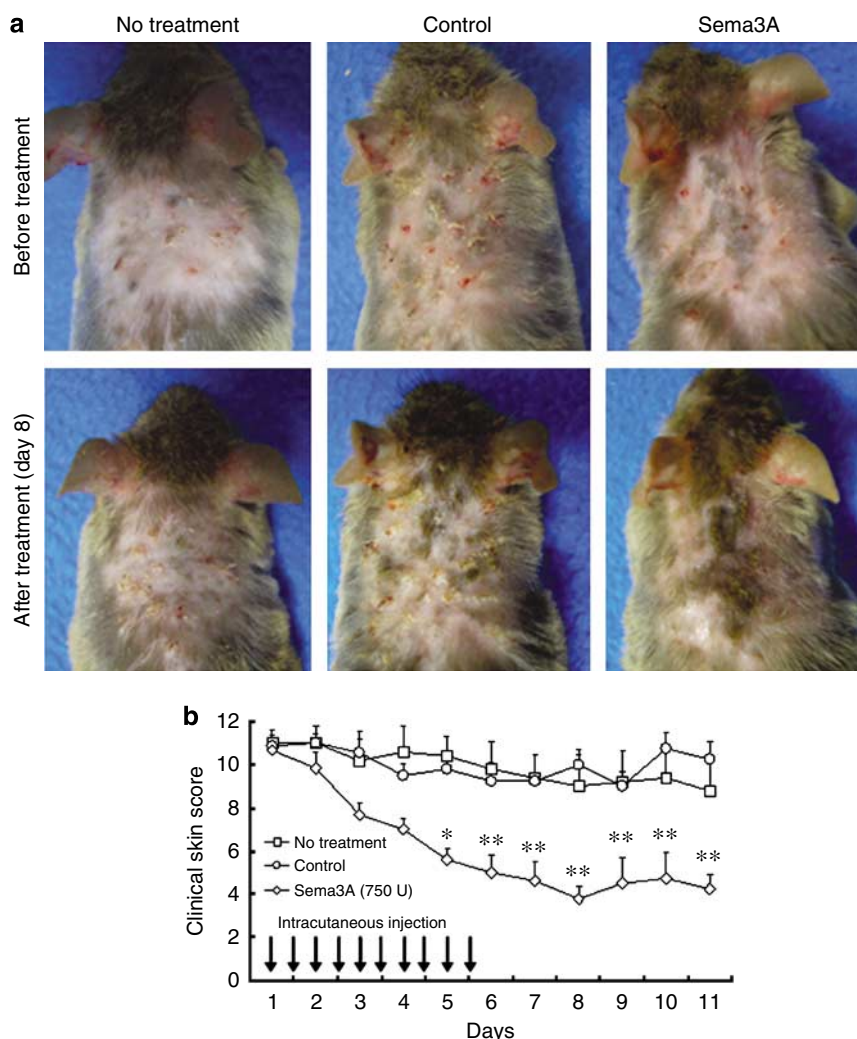


Figure 1. Sema3A improves AD-like skin lesions of NC/Nga mice. (a) Sema3A markedly suppressed erythema, scaling, erosion, and crust formation in the skin 8 days after the start of the Sema3A treatment. Sema3A (750 U) was intracutaneously injected into the dorsal skin lesions of NC/Nga mice aged 12–14 weeks. Mice were administered no treatment, vehicle control, or Sema3A twice a day for 5 consecutive days and then observed for 11 days. (b) The improvement of lesion was evaluated by the clinical skin score. A decrease in the clinical skin score was observed only in the Sema3A-treated group. The effect of Sema3A was observed only at the site of Sema3A injection and not elsewhere. The improvement of the skin lesion continued for several days after discontinuation of Sema3A. Values are mean $\pm$ SEM ($n=6$). * $P<0.05$, ** $P<0.01$, Mann-Whitney U -test, compared with no-treatment and control groups.

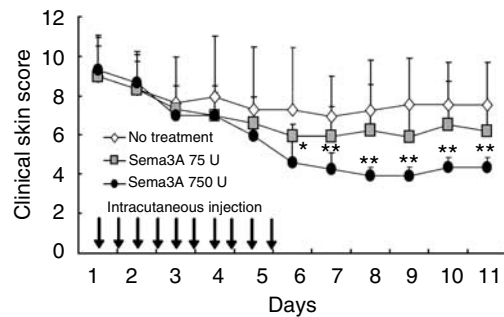


Figure 2. Sema3A improves the skin lesions in a dose-dependent manner. Sema3A (75 or 750 U) dose-dependently improved skin lesion in NC/Nga mice. The administration protocol and lesion evaluation were as described in Figure 1. No improvement of the skin lesion was observed in the no-treatment group. Values are mean $\pm$ SEM ($n=6$). * $P<0.05$, ** $P<0.01$, Mann-Whitney U -test, compared with no-treatment and control groups. Other details are described in the legend of Figure 1.

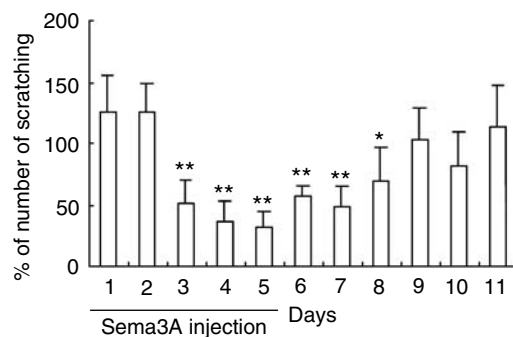


Figure 3. Sema3A interrupts the scratching behavior of NC/Nga mice. Sema3A was administered as described in the Materials and methods. Simultaneously, we counted the number of times the mice scratched on their dorsal skin lesions for 15 min on 11 consecutive days. Sema3A caused a decrease in the scratching behavior, which was accompanied by improvement of skin eruptions. The scratching behavior resumed immediately after discontinuation of Sema3A treatment, followed by recurrence of the eruptions. * $P<0.05$, ** $P<0.01$, Mann-Whitney U -test, when compared with the value on day 1. The scratching behavior was estimated as a percentage of the control calculated from the mean value of the corresponding no-treatment group; for each day, the average values of the no-treatment group were normalized to 100%. Values are mean $\pm$ SEM ($n=6$). Other details are described in the legend of Figure 1.

Sema3A treatment suppressed the scratching behavior of NC/Nga mice

We next investigated the effects of intracutaneous injection of Sema3A on the scratching behavior and the severity of AD-like lesions in NC/Nga mice. Twice a day for 5 consecutive days, we administered Sema3A to their dorsal lesions. On the same experimental groups, we observed the mouse once a day and counted the number of times the mouse scratched its dorsal skin lesion for 11 consecutive days. Sema3A caused a reduction in the number of scratches at day 3 after the start of administration (Figure 3), which was accompanied by an improvement of the eruptions (data not shown). An increase in the scratching behavior of the mice was again observed immediately after the discontinuation of the Sema3A injection, followed by a recurrence of eruptions.

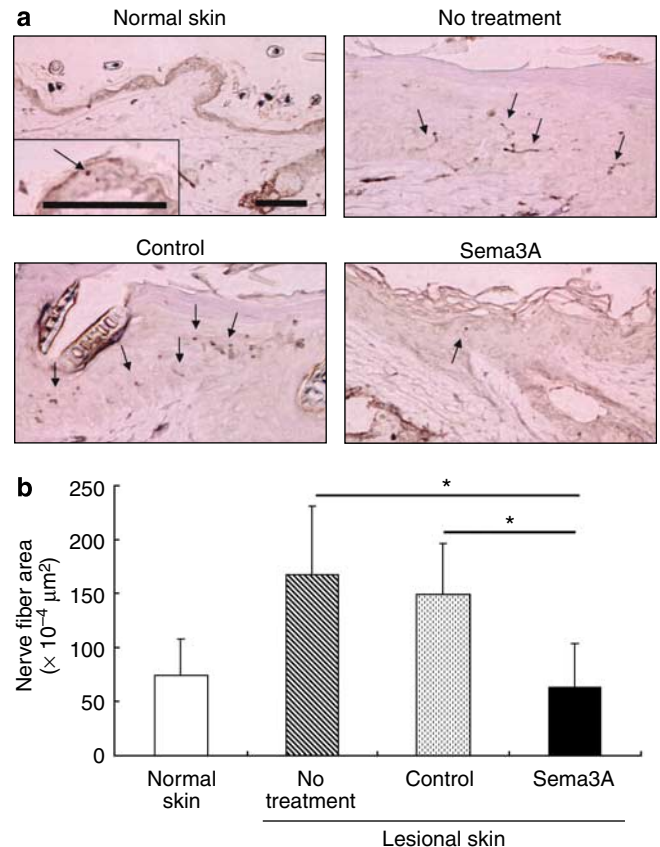


Figure 4. Sema3A decreases the density of invasive nerve fibers in the epidermis. After the administration of no treatment, vehicle control, or Sema3A, the skin with and without lesion was subjected to biopsy, fixed, and stained with PGP9.5 antibody. (a) In the Sema3A group, the density of invasive nerve fibers in the epidermis decreased to a level similar to that in the normal skin. In contrast, the densities of the nerve fibers remained high in the epidermis of the no-treatment and vehicle-control groups. Arrows indicate immunoreactive nerve fibers. Original magnification $\times 400$; Bar = 100 μm . (b) The nerve fiber area was quantified by the imaging software (Image J). Values are mean $\pm$ SEM ($n=6$). * $P<0.01$, Mann-Whitney U -test, compared with no-treatment and vehicle control.

The suppression of the scratching behavior in NC/Nga mice by Sema3A was almost completely reversed to the basal levels on day 4 after the discontinuation of Sema3A (Figure 3).

Sema3A decreased the invasive nerve fiber count in the epidermis

To investigate the effect of Sema3A on the density of the invasive nerve fibers into the skin, we performed immuno-histochemical examination of the sensory neurons using anti-PGP9.5 antibody, which recognizes a ubiquitin hydrolase (McCarthy *et al.*, 1995; Horiuchi *et al.*, 2005), a marker protein of peripheral nerves. At biopsy on day 11, in NC/Nga mice, an extensive invasion of the PGP9.5-positive nerve fibers was observed in the epidermis of the skin lesion. Sema3A (750 U) treatment decreased the PGP9.5-positive nerve fiber count per area in the epidermis to a similar level of the normal skin (Figure 4a). In contrast, the density of

invasive nerve fibers in the epidermis remained high in the no-treatment and vehicle-control groups (Figure 4b).

Sema3A histologically improved AD

Acanthosis and the infiltration of inflammatory cells including lymphocytes, mast cells, and eosinophils are histopathological changes that occur in the skin lesion of AD patients and NC/Nga mice (Matsuda *et al.*, 1997; Friedmann and Holden, 2004). After completion of an 11-day observation period, lesions of each group were subjected to biopsy, fixed, and stained with hematoxylin and eosin. A decrease in the epidermal thickness and in the number of infiltrating cells in the dermis were observed only in the Sema3A-treated skin

areas (Figure 5a and c). Using toluidine blue staining, we also observed a significant decrease in the number of infiltrating mast cells in the dermis (Cerio and Calonje, 2004). Again, the decrease in the number of infiltrating cells was observed only in the dermis of the Sema3A-treated skin areas (Figure 5b and d). These results indicate that histological as well as macroscopic improvement of AD was achieved by the intracutaneous administration of Sema3A.

Sema3A suppresses CD4 + T cells infiltration and IL-4 production

Several semaphorins play critical roles in the immune system (Kikutani and Kumanogoh, 2003; Lepelletier *et al.*, 2007). In

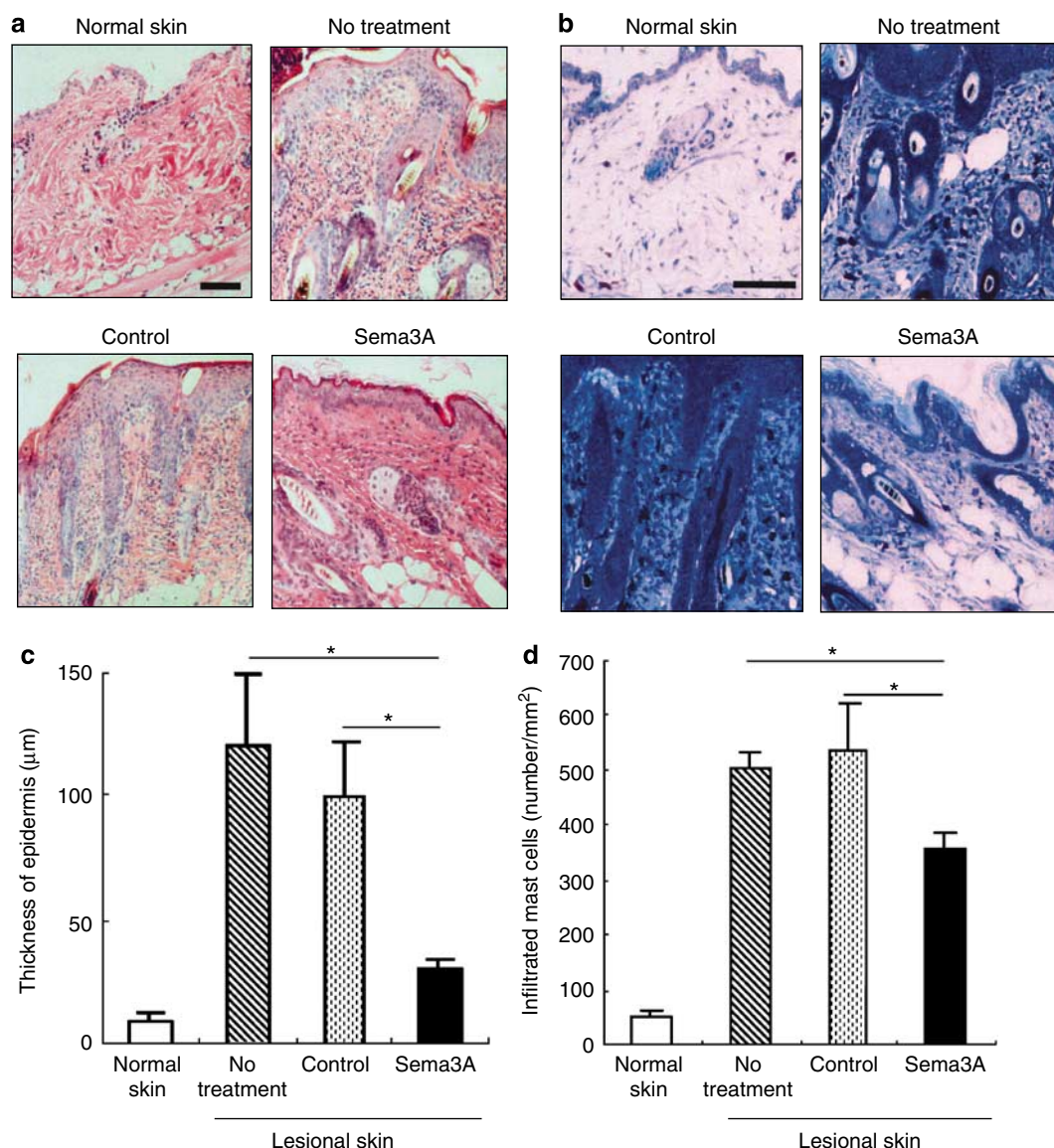


Figure 5. Sema3A suppresses acanthosis, and lymphocyte and mast cell infiltration in AD-like skin lesions. Mice were administered vehicle control or Sema3A and were observed for 11 days. The lesion sites of the skin were then subjected to biopsy in each group. (a, c) Hematoxylin-and-eosin staining showed an improvement of acanthosis and a decrease in the number of invasive lymphocytes in the dermis only in the Sema3A-treated group. (b, d) Toluidine blue staining showed a significant decrease in the mast cell count in the dermis only in the Sema3A-treated group. The number of infiltrated mast cells in the dermis was quantified by the imaging software (Image J). Values are mean $\pm$ SEM ($n = 6$). * $P < 0.01$, Mann-Whitney U -test, compared with no-treatment and control groups. Original magnification $\times 200$; Bar = 100 μ m.

particular, *Sema3A* and *NRP-1* are expressed in dendritic cells, which were generated from monocytes, and T cells (Tordjman *et al.*, 2002; Lepelletier *et al.*, 2006). To investigate the effects of *Sema3A* on skin T cells, we immunostained CD4⁺ T cells and IL-4 in the skin. As a result, a decrease in the number of infiltrating CD4⁺ T cells and IL-4 production were observed in *Sema3A*-treated skin area (Figure 6a-d).

Discussion

We demonstrate for the first time that *Sema3A* markedly improves AD in NC/Nga mice, an animal model of AD. *Sema3A* acts locally and exerts a continuous effect for a while even after discontinuation. Histopathological examinations revealed an improvement of acanthosis and a decrease of inflammatory infiltrate, including mast cells, and CD4⁺

T cells in the dermis in the *Sema3A*-treated skin area. The treatment of *Sema3A* markedly decreased the density of invasive nerve fibers in the *Sema3A*-treated area but not elsewhere on each mouse. Moreover, the physical alteration was associated with the suppression of scratching behavior in *Sema3A*-treated animals. This effect of *Sema3A* was reversible, because the decrease in scratching behavior returned to the basal levels immediately after the discontinuation of the *Sema3A*, followed by recurrence of lesions. These results suggest that scratching behavior aggravates AD, and *Sema3A* can ameliorate the symptoms of AD by interrupting the itch-scratch cycle, one cause of aggravation and chronicity of AD. Quite recently, there has been an interesting report that epidermal *Sema3A* levels were decreased in patients with AD compared with healthy volunteers, concomitant with the

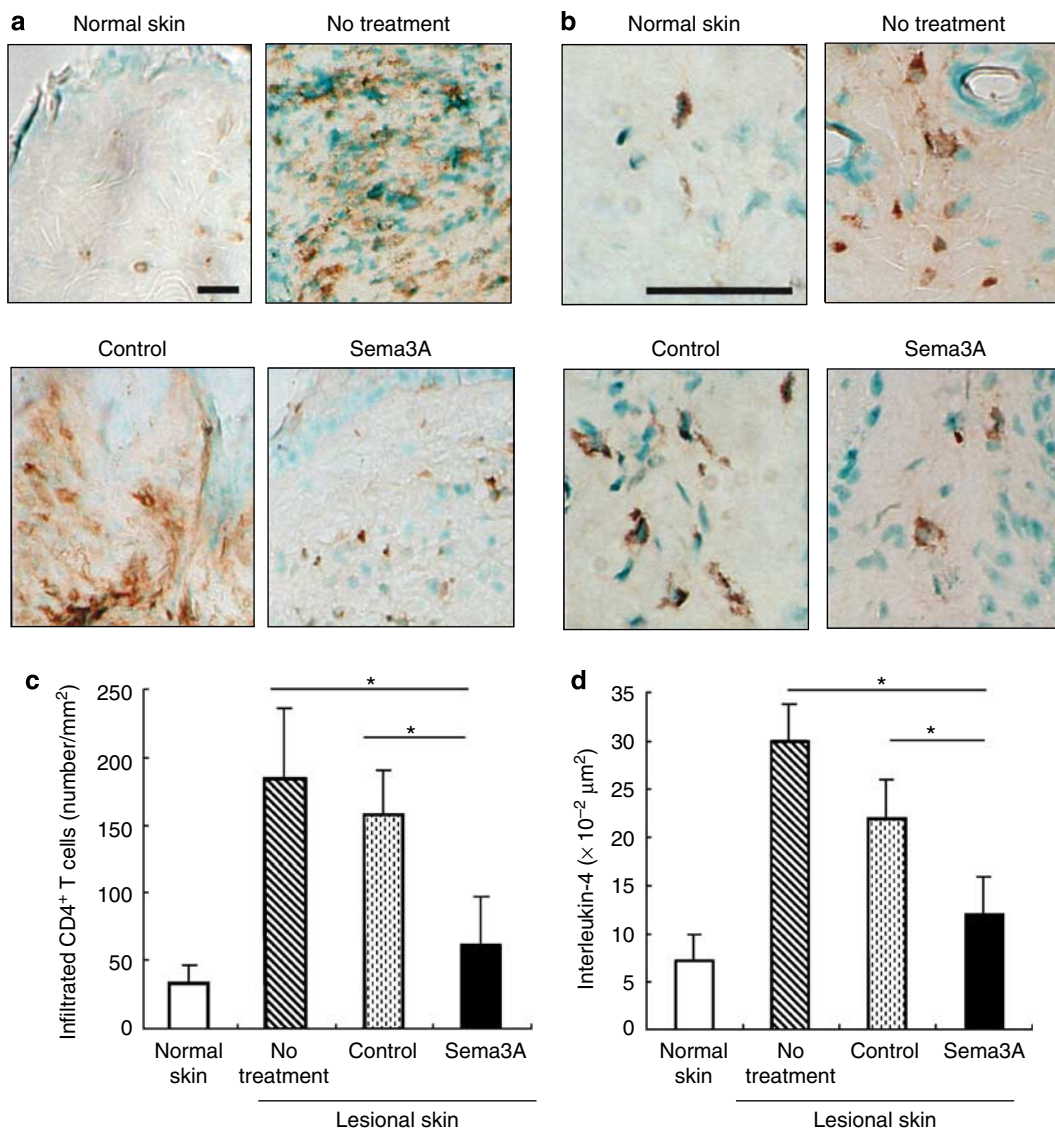


Figure 6. Sema3A suppresses CD4⁺ T cells and IL-4 production. To investigate the effects of *Sema3A* on skin inflammatory infiltrate, we immunostained CD4⁺ T cells and IL-4 production. A decrease in the number of infiltrating CD4⁺ T cells and the area of IL-4 were observed in *Sema3A*-treated skin area (**a**, **b**, **c**, **d**). The areas of the dermis and the IL-4 production were quantified by the imaging software (Image J). Values are mean $\pm$ SEM ($n = 6$). * $P < 0.01$, Mann-Whitney *U*-test, compared with no-treatment and control groups. Original magnification $\times 200$; Bar = 100 μm .

increase of epidermal nerve densities (Tominaga *et al.*, 2008). This result shows a good correlation between epidermal innervation and Sema3A levels and provides a supportive evidence for our therapeutic claim. Many drugs have been developed for the treatment of AD. However, there is no treatment for AD other than Sema3A that targets the invasive nerve fibers in the epidermis. Therefore, Sema3A is expected to be effective even for patients with AD, which is resistant to such drugs that have other pharmacological actions. Our finding provides the first example for a potential clinical application of semaphorins.

Recently, attention has been attracted to the transient receptor potential vanilloid receptor subtype 1 and capsaicin. The generally accepted basis for the therapeutic application of capsaicin to mitigate itch is the well-appreciated desensitizing effect of this vanilloid. However, the most notorious clinical limitation of capsaicin application might be the transient receptor potential vanilloid receptor subtype 1-coupled acute excitation of the sensory C-afferents, which results in a marked "hot painful burning" sensation (Bíró *et al.*, 2007). Does the local application of Sema3A cause such a sensation? Does reduction in innervation density of thin fibers by Sema3A cause sensory deficits? In our experiments, no apparent abnormalities in antinociceptive response as well as in general symptoms were observed upon Sema3A treatment (data not shown). Clearly, answering the above questions will be required before therapeutic attempts in humans.

Sema3A, when intracutaneously administered repeatedly, improved AD lesions on day 2 or 3 after the start of the injections. The effects of Sema3A may involve other mechanisms such as regulation of the immune and vascular systems, in addition to the suppression of the invasive sensory neurons in the epidermis. In addition, NRP-1 and Sema3A are expressed in human keratinocytes (Kurschat *et al.*, 2006). The keratinocyte NRP-1 and Sema3A may be relevant to the suppressive effect of Sema3A on acanthosis observed in our present study. Recent evidence indicates that semaphorins play diverse roles unrelated to axon guidance, including organogenesis, vascularization, and angiogenesis (Kitsukawa *et al.*, 1995; Behar *et al.*, 1996; Soker *et al.*, 1998). In particular, attention has been given to the fact that several semaphorins play critical roles in the immune system (Kikutani and Kumanogoh, 2003; Lepelletier *et al.*, 2007). Studies have demonstrated that Sema3A (Delaire *et al.*, 2001; Lepelletier *et al.*, 2007), Sema3C (Mangasser-Stephan *et al.*, 1997), Sema4A (Kumanogoh *et al.*, 2002), Sema4D (Bougeret *et al.*, 1992; Hérolde *et al.*, 1995; Hall *et al.*, 1996; Wu *et al.*, 1998; Kumanogoh *et al.*, 2000, 2002; Delaire *et al.*, 2001; Watanabe *et al.*, 2001), and Sema7A (Holmes *et al.*, 2002) function in various phases of immune system. Sema3A and NRP-1 are expressed in dendritic cells, which were generated from monocytes, and T cells (Tordjman *et al.*, 2002; Lepelletier *et al.*, 2006). Consistently, our present study clearly demonstrated that Sema3A suppressed CD4⁺ T cells infiltration and IL-4 production in the dermis of NC/Nga mice, in which dermatitis is closely associated with excessive IL-4 production as well as inflammatory cell infiltration in the

dermis (Matsuda *et al.*, 1997). In 2005, Takano *et al.* reported that anti-NGF antibodies inhibited the development and proliferation of skin lesions and epidermal innervation, and any growth in scratching in the NC/Nga mouse. They also speculate that anti-NGF antibodies may suppress symptoms of the mice by blocking response to immune cells. Thus, Sema3A might be similar to anti-NGF antibodies in that both the agents have immunological as well as neurological mechanisms. Anti-NGF antibody, however, may affect the responsiveness of dorsal root ganglia neurons to Sema3A. NGF is known to increase the expression levels of NRP-1 (Pond *et al.*, 2002) and to augment the effect of Sema3A to induce axon repulsion (or growth cone collapse) of dorsal root ganglia neurons (Messersmith *et al.*, 1995). Therefore, the combination therapy of Sema3A with anti-NGF antibody may not be justified for AD. Further studies are required to elucidate the mechanism of action of Sema3A on AD.

In summary, we demonstrated that Sema3A macroscopically and histologically improved AD model mice. Sema3A may be effective for the treatment of patients with refractory AD, who are resistant to existing drugs. In addition, there are several skin diseases other than AD in which the itch-scratch cycle is involved. Sema3A may also prove to be widely effective against these refractory itching skin diseases.

MATERIALS AND METHODS

Animals

Male NC/Nga mice purchased from SLC (Shizuoka, Japan) were housed in the standard mouse facility and fed with autoclaved diet and water. Under conventional conditions for 7 weeks, NC/Nga mice developed spontaneous skin lesions with diagnostic characteristics of high concentrations of total immunoglobulin E in plasma and invasion of inflammatory cells into the skin lesions. NC/Nga mice with severe skin lesions frequently scratched their face, ears, and dorsal skin with their hind paws. Their long-duration scratching behavior is one index for evaluating the itching in cases of AD (Takano *et al.*, 2003). NC/Nga mice with similar levels of skin lesion were used for each experiment. Each experimental group contained six mice, and more than three experiments were performed for each experiment. Throughout the experimental procedures, all efforts were made to minimize the number of animals used and their suffering, and the experimental procedures have been approved by ethical committee of Yokohama City University.

Administration of recombinant Sema3A

Recombinant Sema3A was prepared as described previously (Goshima *et al.*, 1997; Nakamura *et al.*, 1998). In brief, conditioned medium containing Sema3A was collected 3–5 days after transfection and was applied to Talon (Clontech, Palo Alto, CA) column according to the manufacturer's instructions. The column was washed with 20 mM Tris-HCl (pH 7.6) buffer containing 0.5 M NaCl. The recombinant Sema3A was eluted primarily in the 50 mM imidazole fraction. Conditioned medium of human embryonic kidney 293T cells transfected with the empty vector DNA was processed in parallel, and the column fraction with 50 mM imidazole was used as a control. The activity of Sema3A was assessed by growth cone collapse assay using chick dorsal root ganglia neurons, as described previously (Goshima *et al.*, 1995, 1997). One unit was

defined as the amount of Sema3A per milliliter that induced growth cone collapse by 50%. The purified Sema3A was diluted with saline (1:40 v/v) and 1.0 ml of the solution was administered to the dorsal skin lesion of the mice twice a day for 5 consecutive days. We could not find any behavioral changes after the injection of Sema3A as compared with vehicle control.

Clinical skin score

The dorsal skin of each mouse was photographed before, during, and after Sema3A treatment. The severity of AD-like dorsal skin lesion was assessed once a day with the following clinical skin score. Dorsal lesions were evaluated for four symptoms: erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. Each symptom was graded from 0 to 3 (none, 0; mild, 1; moderate, 2; severe, 3). Clinical skin score was defined as the sum of the individual scores, ranging from 0 to 12 (Suto *et al.*, 1999; Takakura *et al.*, 2005).

Histology

After treatment with or without Sema3A, the NC/Nga mice were anaesthetized and tissue samples of the skin from the dorsal surface were fixed in 10% formalin solution. The samples were embedded in paraffin by the conventional method, cut into 2- μ m-thick sections, and stained with either hematoxylin and eosin or toluidine blue (Cerio and Calonje, 2004). Thickness of the epidermis in hematoxylin and eosin and cell numbers in toluidine blue (number of mast cells) were expressed as the mean in five fields per mouse. The dermal area infiltrated mast cells, and the nerve fiber area in epidermis were quantified by the imaging software (Image J, National Institutes of Health, MD).

Immunohistochemistry

Formalin-fixed, paraffin-embedded skin sections were deparaffinized with xylene and graded ethanol. Endogenous peroxidases were blocked using peroxide for 30 min at room temperature. After washing with PBS, sections were incubated in normal goat serum for 15 min at 37°C. Sections were then incubated at 4°C overnight with polyclonal rabbit antibodies against protein gene-product 9.5 (PGP9.5), a pan-neuronal marker (UltraClone Ltd., Isle of Wight, UK). PGP9.5 is a member of the ubiquitin C-terminal hydrolase family and presents as a cytoplasmic protein in peripheral nerves (McCarthy *et al.*, 1995). PGP9.5 is generally used to visualize nerve fibers in the skin (Horiuchi *et al.*, 2005). The sections were washed with PBS and then incubated with biotinylated goat anti-rabbit IgG antibody for 30 min at 37°C. Slides were washed with PBS and then incubated with avidin-biotinylated peroxidase complex. Peroxidase activity was visualized using stable diaminobenzidine. Staining was monitored under a bright-field microscope, and sections were washed with distilled water to stop the reaction. Sections were then counterstained with methyl green, washed, and mounted. We analyzed nerve fiber amount in the epidermis according to the method employed by Takano *et al.* (2005), in which the NC/Nga mice were used. They randomly quantified an area of epidermis and the area of immunoreactive nerve fibers in the epidermis into five fields per mouse by imaging software (Image J). The ratio of the nerve fiber area in the epidermis to the epidermal area was then calculated (μm^2 per 1 μm^2 epidermis). For staining of CD4+ T cells and IL-4, frozen section was used. Briefly, optimal cutting temperature

compound-embedded skin slices were fixed in acetone for 10 min, incubated with rat monoclonal antibodies against mouse CD4 and IL-4 (Pharmingen, San Diego, CA), followed by goat anti-rat IgG conjugated with peroxidase (Jackson, West Grove, PA). Visualization of the reaction products was performed with diaminobenzidine. Sections were then counterstained with methyl green. The number of infiltrated CD4+ T cells and the area of IL-4 production were expressed as the mean in five fields per mouse. The areas of the dermis and the IL-4 production were quantified by the imaging software (Image J).

Evaluation of the effects of Sema3A on established lesions and scratching behavior

The scratching behavior was recorded on video once a day for 11 consecutive days. We counted the number of times a mouse scratched on the dorsal skin lesion within a period of 15 min. Because the average number of scratches in each mouse varied daily, the scratching behavior was estimated as the percentage of the control calculated from the mean value of the no-treatment group.

Statistics

Experimental values are given as means $\pm$ SEM. The statistical difference was determined by two-sided Mann-Whitney *U*-test. Differences with $P < 0.05$ were considered to be significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank T Okada, A Morita, and S Takeshita for their technical advice. This work was partly supported by a grant for 2006–7 Strategic Research Project (no. K19032) of Yokohama City University, Japan (J.Y.), a grant of Yokohama Foundation for Advancement of Medical Science (J.Y.) and Grants-in-Aid for Scientific Research in a Priority Area and Targeted Proteins Research Program from the Ministry of Education, Science, Sports and Culture (Y.G.).

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