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Modulation of semaphorin 3A expression by calcium concentration and histamine in human keratinocytes and fibroblasts

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ABSTRACT

Background: Both neurotrophins and chemorepellents are involved in the elongation and sprouting of itch-associated C-fibers in the skin. Nerve growth factor (NGF) and semaphorin 3A (Sema3A) are representatives of these two types of axon-guidance factors, respectively.
Objective: We investigated the effects of calcium concentration and histamine on the expression of NGF and Sema3A in normal human epidermal keratinocytes (NHEK) and normal human fibroblasts (NHFb).
Methods: NHEK and NHFb were cultured under different calcium concentrations (0.15–0.9 mM) with or without histamine, and the expression of mRNA for *NGF* and *SEMA3A* was assessed by real-time PCR analysis. An immunohistochemical study was performed for Sema3A using normal skin and skin cancer specimens.
Results: In NHEK, *SEMA3A* expression was elevated by high calcium concentration and reduced by low calcium condition, while *NGF* expression was not dependent on calcium. Their expressions were unchanged by calcium in NHFb. Immunohistochemically, keratinocytes in the prickle layer of normal epidermis and squamous cell carcinoma cells were positive for Sema3A, sparing basal cells and suprabasal cells. The addition of histamine to NHEK at 10 μg/ml enhanced *SEMA3A* expression but depressed *NGF* expression. In NHFb, however, histamine decreased both *NGF* and *SEMA3A* levels.
Conclusions: Sema3A inhibits C-fiber elongation/sprouting in the upper layers of the epidermis, where calcium concentration is high, thereby determining the nerve endings. Histamine reduces Sema3A production by fibroblasts, allowing C-fibers to elongate in the dermis. In contrast, the histamine-augmented keratinocyte production of Sema3A might suppress C-fiber elongation and exaggerated pruritus.

1. Introduction

C-fiber is known as a primary afferent nerve to deliver itch to the central nerve system [1]. Numerical increment and elevated sprouting of C-fiber in the epidermis easily produce itch and accelerate the itch–scratch cycle [2]. In addition, C-fiber plays pluripotential roles for itch-related events. For example, neuropeptides released from C-fibers stimulate keratinocytes and mast cells to produce pruritogenic factors that again stimulate C-fiber [3–5].

Both neurotrophins and chemorepellents regulate the elongation and sprouting of itch-associated C-fibers in the skin. Nerve growth factor (NGF) [6,7] and semaphorin 3A (Sema3A) [8] are representatives of these two types of nerve axon-guidance factors, respectively. NGF and Sema3A have opposite effects on C-fiber elongation, which is related to promotion of itch in the skin. In atopic dermatitis, the number of peripheral nerve endings is increased and may amplify itch [2]. NGF is known to initiate the sprouting of epidermal nerve fibers in atopic dermatitis and its model NC/Nga mice [9]. Changes in Sema3A expression/production has also been reported in the lesional skin of atopic dermatitis [10,11]. A reduction in Sema3A expression allows nerve fibers to sprout into the upper epidermis, and photochemotherapy may exert a therapeutic effect by increasing epidermal cell production of Sema3A [12]. On the other hands, in normal skin, the extracellular calcium concentration critically determines a variety of biological activities of keratinocytes [13]. The calcium concentration increases towards the outer epidermis, forming a "calcium gradient" within the epidermis [14]. It is possible that this physiological condition influences on the production of the nerve guidance factors. In addition, histamine released from mast cells in certain allergic conditions possibly modulates their production.

To address the regulatory mechanisms underlying C-fiber elongation, we investigated the effects of calcium concentration on the expression of *NGF* and *SEMA3A* in normal human epidermal keratinocytes (NHEK). Upon allergic stimulation, histamine and

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tryptase are produced by mast cells, and keratinocytes express H1 receptors for histamine and protease-activated receptor-2 (PAR-2) for tryptase [15,16]. Therefore, it is an interesting issue to investigate the effects of histamine and a PAR-2 agonist, SLIGRL-NH_2 (SLIGRL) on the expression of these guidance factors in NHEK and normal human fibroblasts (NHFb). Results suggest that the physiological and pathological conditions of the skin modulate the production of NGF and Sema3A by keratinocytes and fibroblasts.

2. Material and methods

2.1. Chemicals

Histamine and a PAR-2 agonist SLIGRL were purchased from Wako (Osaka, Japan) and Tocris (Ellisville, MO), respectively. These substances were freshly diluted by distilled water before use.

2.2. Cell and cell culture

Primary culture of NHEK was obtained from Lonza (Basel, Switzerland). NHEK were grown in Keratinocyte Growth Medium-2 (KGM-2) for expansion and Keratinocyte Basal Medium-2 (KBM-2; Lonza) for experiments at 37 °C in a 5% CO_2 atmosphere. Primary NHFb were obtained from Takara (Osaka, Japan) and cultured in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine.

2.3. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

NHEK were grown to subconfluence in KGM-2, unstimulated or stimulated with histamine or SLIGRL under various calcium concentration in KBM-2, and then harvested. cDNA was synthesized directly from cell lysate solution using TaqMan Gene Expression Cells-to-CTTM Kit (Austin, TX) according to manufacturer's instructions. The conditions of reverse transcription were as follows: 60 min at 37 °C, 5 min at 95 °C, and then hold at 4 °C. Amplification reaction was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems; Carlsbad, CA) with TaqMan Gene Expression Assays (Applied Biosystems). Primers and TaqMan probe for human *SEMA3A* and human *NGF* were purchased from Applied Biosystems. Amplification of human β-actin, *ACTB* (TaqMan β-actin Control-Reagent Kit) was used as an endogenous control for quantification. The conditions of the real-time PCR were as follows: 2.0 min at 50 °C (reverse transcription), 10 min at 95 °C (RT inactivation and initial activation), and then 40 cycles of amplification consisting of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing and extension). All heating and cooling steps were performed with a slope of 20 °C/s. Samples were analyzed in triplicates and averages were calculated for analysis of the expression ratios.

2.4. Western blotting

NHEK were cultured at 0.15 mM or 0.6 mM of calcium concentration in 6-well plates for 12 h, harvested with rubber policeman, and subjected to extraction by RIPA buffer (50 mM Tris–HCl [pH8.0], 150 mM sodium chloride, 0.5 w/v% sodium deoxycholate, 0.1 w/v% sodium dodecyl sulfate, and 1.0 w/v% NP-40 substitute; Wako Chemical Co., Tokyo, Japan). Ten μg protein samples were electrophoresed by 8% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes for 2 h at 180 mA. After blocking with 5% skim milk solution, the membranes were incubated with rabbit anti-human Sema3A (sc-28867; 1:1000, Santa Cruz Co., Santa Cruz, CA), NGF (sc-548; 1:1000), or Loricrin (PRB-145P; 1:1000, Covance, NJ) polyclonal antibodies, and the reaction was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000, Bio-Rad, Hercules, CA). Immunoblots were visualized using the ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, England) according to the manufacturer's protocol.

2.5. Cytometric beads array

IL-8 and GM-CSF concentration in the culture supernatants were determined with Cytometric Bead Array (CBA) Flex Set System purchased from Becton Dickinson and Company (Franklin Lakes, NJ) using FACSCanto (Becton Dickinson).

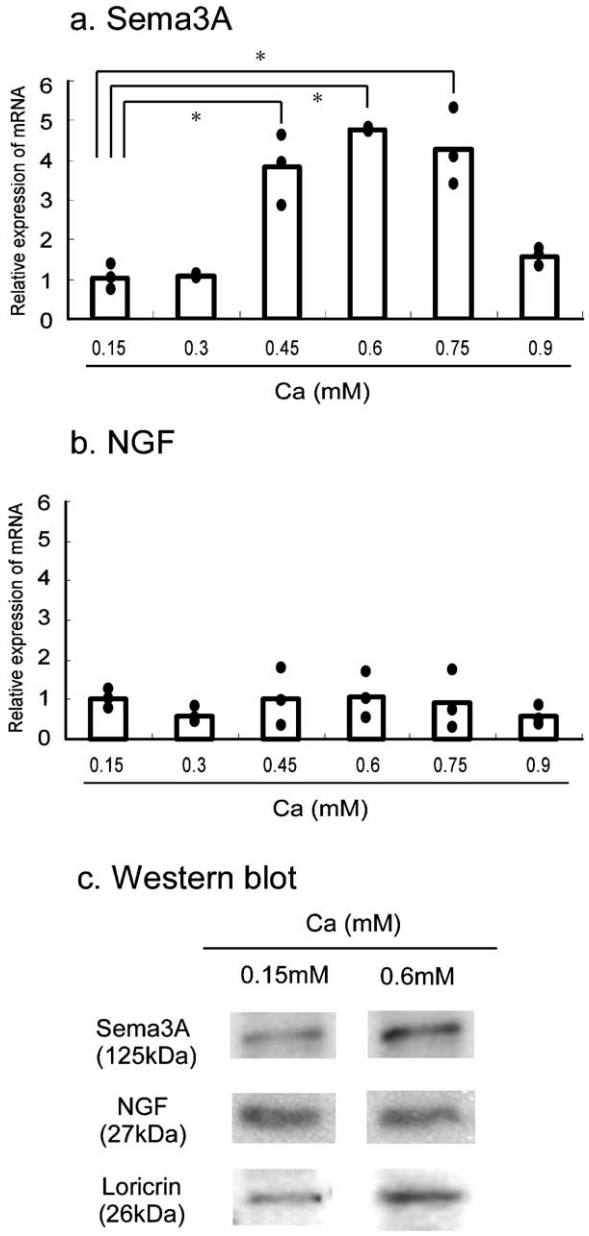

Fig. 1. Effects of calcium concentration on the expression of *SEMA3A* and *NGF* in NHEK. NHEK were cultured under varying concentrations, ranging from 0.15 to 0.9 mM for 2 h. After harvesting, cDNA was synthesized directly and the expression of *SEMA3A* (a) and *NGF* (b) was assessed by real-time PCR. The data are expressed as (expression level of 0.3–0.9 mM calcium group)/(expression level of 0.15 mM calcium group). Bars represent the means of three independent experiments. $^{*}P < 0.05$. The expression levels of Sema3A, NGF, and loricrin in NHEK cultured under 0.15 or 0.6 mM calcium were analyzed by Western blotting (c).