Konjac ceramide (kCer) regulates keratinocyte migration by Sema3A-like repulsion mechanism

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ABSTRACT

Previously, we proposed the following mechanism for konjac ceramide (kCer)-mediated neurite outgrowth inhibition: kCer binds to Nrp as a Sema3A agonist, resulting in Nrp1/PlexA complex formation and activation of the Sema3A signaling pathway to induce phosphorylation of CRMP2 and microtubule depolymerization. The Sema3A/Nrp1 signaling pathway is known to be also expressed in normal human keratinocytes. To determine whether kCer can function in human keratinocytes as it does in neurites, that is, if it can bind to Nrp1 in place of Sema3A, we studied the effect of kCer on HaCaT cell migration activity. Using a trans-well chamber assay, we compared the effects of Sema3A and kCer on serum-derived cell migration activity. kCer showed Sema3A-like suppression of cell migration activity and induction of cellular Cofilin phosphorylation. In addition, kCer and Sema3A inhibited histamine (His)-enhanced migration of immature HaCaT cells. We have demonstrated that kCer does not interact with histamine receptors H1R or H4R directly, but we speculate that kCer may transduce a signal downstream of the His signaling pathway.

1. Introduction

Sphingolipids are comprised of glycosphingolipids, sphingomyelins, and free ceramides. In plants, free ceramides, which we refer to as ceramides herein, occur in low abundance when compared to other sphingolipids such as glucosylceramides (GlcCer) and sphingomyelin, which are enriched in plants. Food sphingolipids such as GlcCer, which is found in cereals, have been reported to be able to benefit health by preventing metabolic syndrome and related diseases. Konjac (Amorphophallus konjac, K. Koch) is a food plant that is rich in GlcCer. The efficacy of konjac GlcCer (kGlcCer) in trans-epidermal water loss in mice and humans has been studied by Uchiyama et al.\cite{1} and used as a preventing metabolic syndrome and related diseases. Konjac is found in cereals, have been reported to be able to benefit health by

Abbreviations: NGF, nerve growth factor; Cer, ceramide; GlcCer, glucosylceramide; kCer, konjac ceramide; C16Cer, N-hexadecanoyl-D-erythro-sphingosine; C18Cer, N-octadecanoyl-D-erythro-sphingosine; C24Cer, N-tetracosanoyl-D-erythro-sphingosine; EGCase I, endoglycoceramidase I; p-Cofilin, phospho-Cofilin; CRMP2, collapsin response mediator protein 2; p-CRMP2, phospho-collapsin response mediator protein 2; Sema3A, semaphorin3A (Sema3A); Nrp1, neuropilin1; hGPCR, histamine G-coupled receptor; His, histamine; H1R, histamine G-coupled receptor 1; H4R, histamine G-coupled receptor 4; AP-Sema3A, alkaline phosphatase-fused Sema3A; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HRs, histamine G-coupled receptors

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maintenance, preventing sensory nerves from extending neurites, which results in skin-hypersensitivity.

We were interested in examining the effect of kCer in keratinocytes, because they also express the Sema3A receptor, neuropilin1 (Nrp1), and kCer can bind Nrp1 in neurons. It has been reported that Sema3A suppresses cell migration of HaCaT cells, a human keratinocyte cell line, against serum-derived chemoattractants (Fig. S1A). This effect is due to activation of the Sema3A signaling pathway.

Histamine (His) stress has been shown to enhance immature keratinocyte migration [3]. In this study, we examined whether kCer can inhibit that enhanced migration similarly to Sema3A.

His plays important roles in inflammation and nervous irritability in allergic disorders, including atopic dermatitis. His has been shown to regulate the expression of pruritic factors such as NGF and Sema3A in skin keratinocytes via His1 receptor (H1R). On the other hand, H4R is highly expressed on keratinocytes from patients with atopic dermatitis, and its stimulation induces keratinocyte proliferation and migration. Immature keratinocyte migration might represent a mechanism that contributes to the epidermal hyperplasia observed in patients with atopic dermatitis. H1R and H4R are also expressed in sensory neurons and are associated with scratching behaviors of mice. In addition to chemotaxis of Sema3A, various chemokines are associated with inducing and regulating keratinocyte migration [4]. Among these chemokines, CCL16 is a His agonist that does not promote cell migratory activity [5], and CCL17 is a non-His agonist that promotes cell migratory activity [6]. To address the regulatory mechanism underlying kCer-induced chemotaxis on His-stressed cells, we investigated the effects of kCer on His receptors (HRs), especially H1R and H4R.

2. Materials and methods

2.1. General

The following materials were commercially obtained: kGlcCer was purchased from Nagara Science Co., Ltd. (Gifu, Japan); 2.5S mouse NGF (N100NF4325) from Alomone Labs; semaphorin 3A (Sema3A, 193-17051) from Wako Co.; C16Cer, C18Cer, and C24Cer from Avanti Polar Lipids; anti-pCofilin (Ser3) polyclonal antibody (pAb) from ABGENT (San Diego, CA); His, anti-CRMP2 pAb, and anti-phospho-CRMP2 pAb (phtr509) from Sigma-Aldrich; anti-GAPDH (3E12) monoclonal antibody (mAb) from Bioss (Woburn, MA); CCL16 (32-193-17051) from Wako Co.; C16Cer, C18Cer, and C24Cer from Avanti Polar Lipids; anti-pCofilin (Ser3) polyclonal antibody (pAb) from ABGENT (San Diego, CA); His; anti-CRMP2 pAb, and anti-phospho-CRMP2 pAb (phtr509) from Sigma-Aldrich; anti-GAPDH (3E12) monoclonal antibody (mAb) from Bioss (Woburn, MA); CCL16 (32-1861) from Abomics (San Diego, CA); and CCL17 (529-TR-025) from R &D Systems (Minneapolis, MN). Alkaline phosphatase (AP) activity was determined using an alkaline phosphatase (ALP) activity assay kit (Nacalai Tesque, Kyoto, Japan). Equal amounts of protein (10 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, WAKO SuperSep™Ace, 5–20%), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp, MA, USA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Berkeley, CA), blocked with Blocking One (Nacalai Tesque, Co., USA) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Berkeley, CA), blocked with Blocking One (Nacalai Tesque) for 1 h, and incubated overnight at 4 °C with primary antibodies, anti-Collin (1:2000), anti-pCofilin (1:1000), or anti-GADPH (1:3000), diluted in 10% blocking One solution with 0.05% Tween 20 and 50 mM Tris-buffered saline (TBST). The next day, each membrane was washed with TBST three times, 10 min each, and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:2000 to 1:4000) for 1 h at room temperature. The membrane was then treated with chemiluminescent reagent (Chemilumi One Super, Nacalai Tesque, Inc., Japan). The antibody-bound protein bands were detected using the Syngene G: BOX Chemi XT4 fluorescence and chemiluminescence gel imaging system (Syngene, Bangalore, India). The relative intensities of developed bands were quantitated using JustTLC software (SWEDAY, Sodra, Sweden) and normalized to the loading control (GAPDH).

2.4. Receptor binding study using AP-Sema3A

Alkaline phosphatase-fused Sema3A (AP-Sema3A) was used as a ligand for receptor binding assays. AP-Sema3A (chick) was a gift from Jonathan Raper (Addgene plasmid # 29448)[7]. Recombinant AP-Sema3A protein was expressed in HEK293 cells that were transiently transfected with an expression plasmid using a Lipofection Kit (ScreenFect™, Plus, Wako Co.). After 2 days of culturing in Opti-MEM, AP-Sema3A protein in the conditioned medium was concentrated, and the enzyme specific activity was determined by AP Activity Kit (LabAssay™-ALP, Wako Co.) and SEMA3A ELISA Kit (ABIN1566604, Aviva Systems Biology, San Diego, CA). According to both assays, a ratio of relative AP activity units (APU, mmol/min/mg protein) of AP per Sema3A protein was calculated as 15.3 APU. For the HaCaT cell receptor binding assay, AP-Sema3A was used at a final concentration of 22.3 nM for 6 × 10⁴ cells per well of a 24-well microplate. After the microplate was incubated at 22 °C for 90 min, the wells were washed with HH-buffer (0.005% BSA/Hank’s balanced buffer + 20 mM HEPES buffer (pH 7.0)), followed by fixation with 3.7% formaldehyde/PBS solution. The fixation medium was exchanged with AP-buffer (100 mM NaCl + 5 mM MgCl₂/50 mM Tris-HCl (pH 9.5)), and then incubated at
65 °C for 30 min. The wells were washed with HH-buffer and treated with a substrate solution of BCIP/NBT Phosphatase Substrate (1-Component, KPL, Gaithersburg, MD, USA). After the coloration reaction with AP-Sema3A, the dye was measured using Imaging-J (1.50 g). The ligand substitution experiment was performed using 22.3 nM AP-Sema3A together with kCer, kGlcCer, C16Cer, C18Cer, and C24Cer dissolved in 0.3% BSA.

2.5. TGFα-shedding assay

The assay procedure was performed according to the method as described by Inoue et. al. [8,9]. Before transfection, HEK293 T cells were seeded in a 12-well plate overnight (5 × 10⁵ cells/mL, 1 mL/well). Each well was transfected in Lipofectamine solution containing plasmids (250 μL per well); a mixture of 125 μL OptiMEM with AP-TGFα (0.25 μg), LGPCR (H1R or H4R, 0.1 μg), and Gαq/11 (for H4R, 0.05 μg) and 125 μL OptiMEM with LFA2000 (1.25 μL). After overnight incubation, the cells were detached from the wells and re-seeded in a 96-well plate and cultured for 30 min. His and other tested reagents (Sema3A, kCer, CCL16, or CCL17) were added and cultured for 1 h. The supernatant and cells were separated by plate centrifugation, and the AP activity in the supernatant and cells was determined using pNPP as the substrate and measuring the absorbance at 405 nm using the iMark™ microplate reader (Bio-Rad). The data represent the ratio of the AP activity in the supernatant to the total AP activity, expressed as a percentage.

2.6. Statistical analysis

The number (n) in each experimental condition is indicated in the figure legends. Data analysis was performed using the commercial program Prism 4.0 (GraphPad, San Diego, CA). When two experimental conditions were compared, statistical analysis was performed using a paired t-test. Otherwise, statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple comparison post-test and Dunnett’s test. A p value less than 0.05 was considered significant. * indicates significantly different results. Ranges of p values are indicated in the figure legends. NS indicates non-significant.

Fig. 1. Binding characteristics of Sema3A to cell surface receptors in HaCaT cells. A: Displacement profile of kCer on AP-Sema3A binding to cell surface receptors. Cells were cultured in a 24-well microplate and treated with 15.3 APU of 22.3 nM AP-Sema3A in the presence of Sema3A (10–100 nM) (upper graph) or kCer (10–100 μM) (lower graph). Cells were washed and incubated at 65 °C for 30 min, and the remaining AP activity was measured using BCIP/NBT Phosphatase Substrate and Imaging-J software. Data are shown as the means ± SD (n = 4). B: AP-Sema3A binding to cells in the presence of Sema3A (0, 10, 50, and 100 nM) or kCer (0, 50, 100 μM). C: Displacement profile of AP-Sema3A binding by Sema3A (50 nM), kCer, kGlcCer, C16Cer, C18Cer, and C24Cer (50 μM, respectively).
All experiments were performed with approval of the regulatory boards of Hokkaido University.

3. Results

3.1. kCer inhibits the binding of AP-Sema3A to a cell surface receptor

To examine whether kCer can associate with Sema3A binding to a cell surface receptor on HaCaT cells, the cells were incubated with AP-Sema3A in combination with non-labeled Sema3A or kCer. As shown in Fig. 1A and B, the binding of AP-Sema3A to cells was clearly attenuated by addition of 10–100 nM Sema3A or 10–100 μM kCer. The inhibitory effect of kCer on Sema3A binding to the cell surface receptor was clearly demonstrated, although the attenuating effect of the displacement was weaker than with Sema3A (Fig. 1A).

We evaluated the displacement reactivity at 50 μM of kCer and other ceramides (kGlcCer, C16Cer, C18Cer, and C24Cer) for AP-Sema3A binding to the cell surface receptor. As compared to kCer (50 μM) and Sema3A (50 nM), the other ceramides did not show any effect on AP-Sema3A binding to the cell surface receptor (Fig. 1C).
3.2. Effect of Sema3A, kCer, His, and chemokines on HaCaT cell migration

To examine the effect of kCer or Sema3A on HaCaT cell migration, cells were exposed to Sema3A (1–200 nM) or kCer (10–100 μM) (Fig. 2). As shown in Fig. 2A and B, increasing the concentration of Sema3A and kCer resulted in an attenuating effect on cell migration by complete serum-containing medium. On the other hand, His induced a stimulating effect on cell migration at low concentration (0.1–1.0 μM), but showed a suppressing effect at high concentration (10–100 μM). The chemokine CCL16 showed no effect but CCL17 showed a stimulating effect on cell migration.

To examine the combined effect of kCer (or Sema3A) and His (or CCL16 or CCL17), cells were exposed to His (1 μM), CCL16 (10 nM), or CCL17 (10 nM) in the presence or absence of kCer (25 μM) or Sema3A (100 nM) (Fig. 3A). As shown in Fig. 3B, Sema3A remarkably decreased the level of His- and CCL-17-induced stimulation of cell migration. kCer showed a similar effect (Fig. 3C). CCL16 showed no effect but CCL17 showed a stimulating effect on cell migration.

To examine the combined effect of kCer (or Sema3A) and His (or CCL16 or CCL17), cells were exposed to His (1 μM), CCL16 (10 nM), or CCL17 (10 nM) in the presence or absence of kCer (25 μM) or Sema3A (100 nM) (Fig. 3A). As shown in Fig. 3B, Sema3A remarkably decreased the level of His- and CCL-17-induced stimulation of cell migration. kCer showed a similar effect (Fig. 3C). CCL16 showed no effect but CCL17 showed a stimulating effect on cell migration.

3.3. Effect on ligand activation of H1R and H4R by kCer

To examine whether kCer (or Sema3A) can activate H1R or H4R by a hGPCR activation manner, we used a TGFα-shedding assay (Fig. S2). As shown in Fig. S2B, C, F, and G, neither kCer nor Sema3A showed any response of TGFα activity with H1R or H4R. On the other hand, CCL16 showed an increasing response for H4R activation, but no response for H1R. His-induced H1R and H4R activation was not influenced by kCer or Sema3A (Fig. S2I, J, M, and N). CCL17 is not a ligand of H1R and H4R and showed no response to the His-induced activation (Fig. S2L and P). On the other hand, specific inhibitors, pyrilamine for H1R and thioperamide for H4R, showed dose-dependent inhibitory responses to His-induced H1R and H4R activation, respectively (Fig. S2K and O).

3.4. Cofilin phosphorylation

NGF treatment induced CRMP2 production in PC12 cells (Fig. S3A) [2], and kCer-induced CRMP2 phosphorylation via binding to Sema3A-receptor was also demonstrated in PC12 cells [10]. To determine if kCer also induced CRMP2 phosphorylation in HaCaT cells, the cells were treated with 100 ng/mL NGF for 24 or 48 h and analyzed by Western blot as previously published [2]. As shown in Fig. 4B, treatment with NGF did not induce CRMP2 expression and phosphorylation in HaCaT cells.

Alternatively, we examined NGF-induced Cofilin expression and phosphorylation in HaCaT cells. Total Cofilin (t-Cofilin) and phosphorylated-Cofilin (p-Cofilin) were expressed in HaCaT cells. There was no effect 0, 24, and 48 h after NGF treatment (Fig. 4A). Treatment with kCer (50 μM) or Sema3A (100 nM) for 0, 30, or 60 min in the absence of NGF showed no effect on t-Cofilin and p-Cofilin (Fig. 4B). However, p-Cofilin was elevated 30–60 min after kCer or Sema3A treatment as compared to untreated cells, as shown in Fig. 4C and D.
4. Discussion

In the peripheral nervous system and epidermis, Sema3A regulates itch sensations from nerve fiber extensions of sensory neurons by reversing NGF activity [11, 12]. At the cell surface, Sema3A forms a hetero-complex with neuropilin1 (Nrp1), and recruits plexin A1 (PlexA), thus enabling signal-transducing activity [13, 14]. Previously, we demonstrated that kCer directly interacts with Nrp1, which activates the Sema3A pathway, causing CRMP2 phosphorylation downstream of Sema3A. Nrp1 is also expressed as a Sema3A receptor by keratinocytes in vitro and in vivo [15]. We investigated the binding of kCer or Sema3A to Nrp1 on the cell surface of keratinocytes by the use of AP-Sema3A and demonstrated that Sema3A could displace AP-labeled Sema3A (AP-Sema3A) (Fig. 1). As shown in Fig. 1C, kCer could also displace the AP-Sema3A, while kGlcCer and other ceramides could not. This result showed that kCer is a weak agonist of Sema3A as also shown in our previous study with PC12 cells [10]. Unexpectedly, NGF did not induce CRMP2 production and phosphorylation by Sema3A in HaCaT cells (Fig. S3B), as it did in PC12 cells as previously reported by us [2]. However, Cofilin expression and phosphorylation occurred in unstimulated HaCaT cells, and downstream activation of the Sema3A pathway by Sema3A or kCer induced phosphorylation of Cofilin, resulting in an increased level of p-Cofilin (Fig. 4C and D). Sema3A is known to inhibit cell migration of HaCaT cells [16]. Nrp1 expression was increased with normal keratinocyte differentiation and migration in the epidermis, resulting in Sema3A-induced inhibition of cell migration. In the present study kCer was demonstrated to work on Nrp1 of HaCaT cell, resulting in signaling pathway of Sema3A as shown in the schematic presentation of Fig. 5.

His treatment induces proliferation [17] but suppresses epidermal keratinocyte differentiation [3]. In human in vitro keratinocyte cultures, it was reported [18] that His reduced keratinocyte differentiation in an H1R-dependent manner, while experiments with His receptor agonists and antagonists showed no effect on keratinocyte differentiation. There is also evidence that H4R knockout mice show reduced epidermal thickness and decreased in vitro proliferation of keratinocytes [18]. Keratinocyte proliferation can be induced in a paracrine or autocrine manner by various mediators. Since His is mainly secreted by mast cells, it regulates keratinocyte proliferation in a paracrine manner, if we assume that His directly affects keratinocyte proliferation. It is assumed that His and Sema3A must be processed by different signaling mechanisms, and keratinocyte migration effects are the outcome of the different upstream cell signaling pathways. We showed that 0.1–1 μM His enhanced the cell migration of HaCaT cells in high Ca²⁺ serum medium (Fig. 2B). This enhancement effect is thought to occur in immature cells in low Ca²⁺ medium (data not shown). To examine the inhibitory mechanism of kCer or Sema3A on HaCaT cell migration, HaCaT cells were exposed to His and the chemokines CCL16 (His...
agonist with absence of cell migratory activity) [5] and CCL17 (non-His agonist with presence of cell migratory activity) [6].

As expected, cell migration was not affected in the presence of CCL16 but was enhanced in the presence of CCL17 (Fig. 2B). As shown in Fig. 3B and C, His-induced stimulation of cell migration was inhibited by Sema3A or kCer, which also inhibited CCL17-induced stimulation, whereas Sema3A or kCer inhibited cell migration independently of CCL16. An alternative possibility is that kCer interacts with H1R or H4R directly and transduces any signal downstream of the His signaling pathway. The interaction of kCer or Sema3A with H1R or H4R was addressed by testing ligand activation in the TGFα-shedding assay. As shown in Fig. S2, kCer did not react with H1R or H4R.

kCer is not associated with the His-H1R (or H4R) binding that occurs at the cell surface in the His signaling pathway, but it remains unknown whether kCer’s inhibitory effect on His-induced stimulation of cell migration occurred downstream of the His signaling pathway.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.11.004.

Appendix A. Supplementary material

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