	Fetus normal	Fetus burn	Lamb normal	Lamb burn
Upper dermis MPM	a	b	c	d
Upper dermis H & E	e	f	g	h
Deep dermis MPM	i	j	k	l
Deep dermis H & E	m	n	o	p

Fig. 3 – Ovine fetus and lamb skin collagen architecture visualized using secondary harmonic generation (upper dermal a–d; lower dermal staining i–l) and hematoxylin and eosin staining (upper dermal e–h; lower dermal staining m–p) in paraffin embedded sections. All images were taken with the same microscope configuration/magnification to compare the differences in signal intensity and collagen distribution arrangement between normal skin and skin after burn. The upper dermal field of view is predominantly comprised of wool follicles with a decreased presence of collagen lower in the mid-dermal tissue. The dashed lines indicate the epidermal-dermal junction with one figure showing epidermal separation after burn wounding (asterisk in h). The H&E stained paraffin embedded sections confirmed the more subtle differences observed using SHG in collagen bundle distribution, length and packing between ovine control and burn tissue samples.

Ovine collagen fibers were also successfully detected in both normal and burn tissue using the same MPM excitation and emission detection settings (Fig. 3). Thin collagen fibers were detected in the papillary dermis (just beneath the dashed lines Fig. 3a and b) of ovine normal control fetal tissue at 140 days gestation (term is 145–150 days) and fetal burn tissue after 60 days post-wounding normal control as well as in lamb tissue (Fig. 3c and d). Thick, SHG-bright collagen bundles were detected in both normal and burn tissues in fetal and lamb deep dermis that correlated with more eosinophilic collagen bundles in H&E sections of burn tissue (Fig. 3m and o verses n and p). Unlike human and porcine

normal skin, the collagen architecture in ovine deep dermis showed relatively longer collagen bundles parallel-aligned in a wavy pattern, but the long, continuous parallel collagen architecture seen in human and porcine scar tissue was not observed in either the adult or fetal ovine tissue. Ovine fetal deep dermis at 60 days post burn demonstrated similar looking collagen bundles to normal control fetal skin (Fig. 3i versus j), however in contrast the collagen bundles were less regularly aligned in parallel. In addition, at 1 day after burn, thick collagen bundles were still visualized in the lamb acute burn deep dermis, but in a much shorter and curled form (Fig. 3i and p).

4. Discussion

Second harmonic generation detection using MPM has been widely used in various dermatological studies in the last few years [15,17,18,24]. It is a non-invasive, high resolution and high contrast laser microscopic technique that allows observation of tissue architecture, identification of skin pathology, and monitoring of drug delivery. Many dermatological studies often require immunohistochemical staining to observe different compartments of skin structure, and these methods include the most commonly used hematoxylin and eosin staining, Masson's Trichrome staining and Sirius red staining viewed using various forms of light microscopy. Antibody-labeled tissue sections or cells viewed by fluorescent or confocal laser microscopy are also frequently used to detect the distribution of specific proteins during molecular trafficking. Transmission electron microscopy can give a high magnification ultrastructural view of the tissue structure, however it is a very slow and time consuming method. Most of these methods require immunohistochemical staining, and are hence time consuming and relatively labor intensive. Second harmonic generation detection using MPM has been used to examine several dermatological conditions including studies observing melanin distribution [1]. The major strength of the SHG detection method is that no immunohistochemical staining is required; therefore long-term monitoring can be performed on the same tissue area without the possibility of image degradation or the need to take skin biopsies. This study demonstrates the feasibility of MPM application to detect collagen SHG in human, porcine and ovine burn tissues, for comparison with normal or control skin.

To verify that the SHG signal detected using MPM was collagen, colocalization of SHG and collagen I by immunohistochemical staining was performed. Although the two signals have similar patterns, the signals did not entirely overlap. One explanation is that a large proportion of the SHG signal detected was collagen III instead of or as well as collagen I. In normal human skin, the collagen I to collagen III ratio is about 3:1, however after burn, the ratio can dramatically alter to 2:1 [25]. After the gradual process of tissue remodeling, collagen III gets replaced by collagen I. Cuttle et al. [26] has described a method for detecting different collagen subtypes in burn tissues using Sirius red staining and polarization microscopy. Thicker and more closely packed collagen fibers such as collagen I exhibited an intense yellow or red birefringence, while relatively thin and looser-packed fibrils such as collagen III often showed a weaker, green tinged birefringence [27]. Although the SHG detection method cannot differentiate between different collagen subtypes, this non-invasive technique still benefits dermatological studies as there are no concerns about sample bleaching. Unfortunately, both the Sirius red method and the SHG collagen detection methods are dependent on collagen fiber density. Newly forming collagen I may be incorrectly identified as collagen III using the Sirius red method, and weak signals from thin fibrils could similarly be missed using the SHG detection method. The observation of mature scars would be expected to exhibit less variability using the SHG detection method, as examination of total collagen architecture is more important than being able to differentiate between different collagen types, and collagen fibers in mature scar would be of sufficient thickness to allow for sufficiently sensitive SHG detection. One advantage of SHG is in examining total global collagen fiber arrangement and architecture rather than detecting collagen subtypes.

Various studies recently published have used excitation wavelengths from 800 nm to 860 nm with an emission wavelength from 393 nm to 436 nm [17,18,24,28]. Our results show that the best SHG signal is detected at an excitation wavelength of 860 nm using the emissions filter that collects laser signals from 390 nm to 465 nm regardless of the tissue processing technique or species tissue origin. Previously, Chen et al. [18] have described the detection of SHG in human hypertrophic scars using 3-dimentiional modeling and Z-stacking methods, and have shown that collagen SHG signal intensity in normal tissue was roughly 1.5 times that in the hypertrophic scar tissue. In Fig. 2, our data did not demonstrate any difference in SHG signal intensity between normal and hypertrophic scar tissues in human samples. This could be due to the difference in sample age such that collagen fibers in children less than one year of age may be thinner and arranged in a different manner compared to teenagers/adolescents. However, it is very difficult to obtain age-, sex-, ethnic background- and body site-matched control human dermal tissue and foreskin tissue was our best option to use as a control in this study.

In this study, although our tissue samples were only 5 μm thick, we demonstrated similar collagen architecture described by Linares et al. [12] and Chen et al. [18] and this was confirmed by subsequent collagen fiber observations using hematoxylin and eosin (H&E) stained tissue in identical samples examined by SHG. Normal dermal architecture shows randomly oriented collagen fibers, while hypertrophic scars show elongated collagen bundles of undulating fibers. No obvious collagen nodules were detected in the hypertrophic scar tissues, possibly due to the relatively young age of the scars and the different body sites, and therefore differences in amounts of collagen cross-linkage and packing. Furthermore, there was a greater frequency of collagen with a more nested arrangement in human Scar sample 2 compared to human Scar sample 1. Again, this may be due to a slightly greater maturation of Scar 2 leading to changes in scar morphology or the different body sites that the scars were formed.

Among the various animal models used in skin wound and particularly burns research, the pig burn model is the most similar to human burn wounds in terms of skin structure, scale, composition, physiology and reaction to injury [29–33]. As shown in Fig. 2, the collagen architecture in porcine skin tissue was similar to that in human tissue, although each of the basket-weave collagen arrangements in normal porcine skin were more widely spaced compared to control human skin, which could be due to the differences in body size or site. Similar but less high resolution changes in collagen bundles were also detected in H&E stained sections in all species examined. Our results therefore provide further evidence that the porcine model is suitable for SHG collagen analysis. Although the ovine model is often used in fetal studies, the lack of species-specific antibodies can limit research using these animals as models. Our results demonstrated that SHG detection using MPM was a quick and powerful method to

study collagen in the ovine model. It was sensitive enough to identify thin collagen fibers in ovine fetal dermal tissue and thin or newly formed collagen fibers after burn as well as the morphological changes occurring in thick, deep dermal collagen bundles. Our success in detecting SHG in porcine and ovine dermal tissues shows the potential of applying this method to study collagen deposition in various diseases and animal models. Other researchers have validated the use of SHG detection in mouse skin and after lung fibrosis [16,34], further demonstrating the potential of SHG detection in dermatological research and research into dermal scarring.

This study demonstrated that high resolution collagen architecture in human, porcine and ovine tissue samples can be detected using SHG and MPM without the need for immunofluorescent staining on frozen tissue or paraffin-embedded tissue sections. Although embedding artifacts present in cryostat and paraffin-embedded sections can still be seen to a lesser extent, tissue architecture using the paraffin-embedded method is better preserved and the samples can be kept for longer periods with only simple storage requirements. Our study highlights the future potential for *in vivo* three-dimensional SHG monitoring of clinical patients to examine collagen architecture from an early stage after injury to identify any early pathogenic signs of hypertrophic scar formation. However, while the benefit and usefulness of *in vivo* application of MPM collagen assessment is theoretically possible, there still remain several issues affecting its usefulness for imaging in living animals. These include the ability of keeping the animals from moving to allow adequate image collection, and secondly the limitations in penetration of MPM microscopy to visualize deep into scar tissue. This method is highly sensitive to movements such as respiratory or involuntary nervous reflex movements, and the imaging depth of MPM in skin is only limited to about 200 μm. Therefore, to allow this method to work in live animal or clinical patients, scanning rates and imaging depth will have to be significantly improved through better the microscope design. Other than its clinical use, this approach also benefits dermatological research that involves animal models or human patients when species-specific antibodies are unavailable and painful biopsies have been refused. It can be an efficient technique clinically, as well as for histologically preserved tissue samples. Future studies should be carried out using this approach to examine a larger sample size of pathogenic scars compared to control tissue, to identify the key differences in collagen fiber organization and potentially the complex pathomechanisms of hypertrophic scarring.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

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Topical Cholecystokinin Depresses Itch-Associated Scratching Behavior in Mice

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Cholecystokinin (CCK) serves as a gastrointestinal hormone and also functions as a neuropeptide in the central nervous system (CNS). CCK may be a downregulator in the CNS, as represented by its anti-opioid properties. The existence of CCK in the peripheral nervous system has also been reported. We investigated the suppressive effects of various CCKs on peripheral pruritus in mice. The clipped backs of ICR mice were painted with CCK synthetic peptides and injected intradermally with substance P (SP). The frequency of SP-induced scratching was reduced significantly by topical application of sulfated CCK8 (CCK8S) and CCK7 (CCK7S), but not by nonsulfated CCK8, CCK7, or CCK6. Dermal injection of CCK8S also suppressed the scratching frequency, suggesting that dermal cells as well as epidermal keratinocytes (KCs) are the targets of CCKs. As determined using real-time PCR, mRNA for CCK2R, one of the two types of CCK receptors, was expressed highly in mouse fetal skin–derived mast cells (FSMCs) and moderately in ICR mouse KCs. CCK8S decreased in vitro compound 48/80-promoted degranulation of FSMCs with a transient elevation of the intracellular calcium concentration. These findings suggest that CCK may exert an antipruritic effect via mast cells and that topical CCK may be clinically useful for pruritic skin disorders.

INTRODUCTION

Itch or pruritus is an unpleasant cutaneous sensation associated with the immediate desire to scratch (Ikoma et al., 2006; Steinhoff et al., 2006). Histamine was long considered the only mediator of pruritus, but this assumption has changed dramatically in the past two decades. Peripheral itch can be evoked in the skin either directly, by mechanical and thermal stimuli, or indirectly, through chemical mediators (Ikoma et al., 2006). Itch may also be generated in the central nervous system (CNS) independent of peripheral stimulation. Different puritic diseases involved different itch mediators, including histamine, neuropeptides, proteases, prostaglandins, serotonin, acetylcholine, cannabinoids, opioids, bradykinins, cytokines, biogenic amines, neurotransmitters, and ion channels (Steinhoff et al., 2006).

Neuromediators such as neuropeptides and neurotrophins, as well as their receptors, have an important role in peripheral itch. Neuropeptides, as represented by pruritogenic neuropeptide substance P (SP), are produced by sensory nerves, but they can also be elaborated by epidermal keratinocytes (KCs), mast cells, fibroblasts, and other cutaneous immunocompetent cells (Ohkubo and Nakanishi, 1991; Scholzen et al., 1998). Many other mediators are also produced by and secreted from skin-constituent cells in close contact to sensory nerves (Steinhoff et al., 2006). They can activate and sensitize pruritic nerve endings and even modulate their growth, as do nerve growth factors and chemorepellents (Yamaguchi et al., 2008; Yosipovitch and Papoiu, 2008). As for the receptor system, it is notable that KCs, mast cells, and fibroblasts express neurokinin-1 receptor (Ohkubo and Nakanishi, 1991; Scholzen et al., 1998), which is the receptor for SP. These complicated neurophysiological interactions yield itch and render its therapeutic control difficult.

Cholecystokinin (CCK) is a peptide hormone in the gastrointestinal tract (Dufresne et al., 2006), but it also serves as a neuropeptide (Ma et al., 2006). Among neuropeptides, CCKs are most abundantly present in the CNS and involved in numerous physiological functions such as anxiety, depression, psychosis, memory, and feeding behavior (Dufresne et al., 2006). CCKs also have anti-opioid properties in the CNS (Pommier et al., 2002; Mollereau et al., 2005) and exert a nociceptive effect in the spinal cord (Wiesenfeld-Hallin et al., 1999). Furthermore, their existence in the peripheral nervous system has been reported (Moriarty et al., 1997). In various CCKs, sulfation of the tyrosine at position 7

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from the C-terminus is a posttranslational modification that makes them biologically active peptides (Ma *et al.*, 2006). Varying lengths of CCKs with or without sulfate have been studied for their activities as gastrointestinal hormones (Bonetto *et al.*, 1999) and as CNS players (Rehfeld *et al.*, 2007). Two types of CCK receptors have been identified: CCK1R (CCK-A receptor) and CCK2R (CCK-B receptor). CCK1R usually requires sulfated CCKs. CCK2R affords a binding site to both sulfated and nonsulfated ligands, but nonsulfated CCKs have affinities that are decreased 10- to 50-fold (Dufresne *et al.*, 2006). CCKs exert their biological functions by interacting with CCK receptors located on multiple target cells in the CNS and on peripheral nerve endings (Dufresne *et al.*, 2006; Rehfeld *et al.*, 2007; Zheng *et al.*, 2009). In the brain, sulfated CCK8 (CCK8S) is most abundant (Rehfeld *et al.*, 2007) and possesses one of the strongest endogenous anti-opioid properties (Ma *et al.*, 2006). Prolonged opioid exposure increases the expression of CCKs and CCK2R in the CNS, where CCK8S negatively modulates opioid responses and maintains homeostasis of the opioid system (Pommier *et al.*, 2002; Agnes *et al.*, 2008). Thus, it seems that CCKs are downregulators of opioid-mediated pruritus in CNS. However, the function of CCKs, the distribution of CCK receptors in the skin, and their effects on peripheral pruritus remain unelucidated (Ma *et al.*, 2006).

In this study, we investigated the antipruritic effects of different lengths of CCKs with or without sulfate, which were topically applied onto the skin of mice. Results suggest that CCKs are capable of reducing SP-induced scratching at least by affecting the function of mast cells. Our findings may lead to the development of previously unreported antipruritic strategies.

RESULTS

Depressive effects of topical application of CCKs on SP-induced itch-associated response

We tested various CCKs for their ability to suppress scratching behavior induced by intradermal SP injection in ICR mice (Andoh *et al.*, 2001). We first examined the effect of percutaneously applied CCK on the response. Because CCK8S has an inhibitory ability in the CNS (Pommier *et al.*, 2002; Dufresne *et al.*, 2006) and gastrointestinal tract (Miyasaka *et al.*, 2004), CCK8S and the other CCK constructs consisting of smaller amino acids with or without sulfate (Table 1) were tested for their antipruritic activities. ICR mice were painted with various CCKs on the clipped, tape-stripped rostral part of the back skin ($\sim 1.8 \, cm^2$) and then injected with SP intradermally at the CCK-painted site.

The topical application of CCK8S significantly reduced the scratching frequency, as compared with the nonapplied control (Figure 1a). Sulfated CCK7 (CCK7S) also reduced the scratching frequency, to a level comparable to that with CCK8S, whereas nonsulfated CCK8, CCK7, and CCK6 had no ability to suppress scratching. These results showed that CCKs have an antipruritic ability, and CCK constructs bearing sulfation of position 7 are required for the ability to suppress the scratching behavior. In this itch-associated-scratching model, we also administered CCK8S intradermally together

with SP to mice. The scratching frequency induced by SP was profoundly suppressed by the simultaneous injection of CCK8S (Figure 1b). When the time course of effects induced by CCK8S was examined, both topical application (Figure 1c) and intradermal injection (Figure 1d) of CCKS reduced the scratching frequency at intervals of 0–5 minutes and 5–10 minutes.
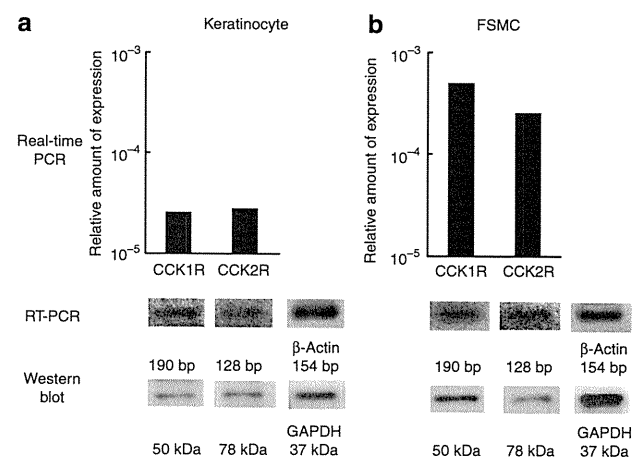
These findings raised the possibility that epidermal and dermal cells participating in the development of itch are affected by CCKs.

Table 1. Amino acid sequences of CCK and its constructs

Sulfated CCK8 (CCK8S)	Asp–Tyr (SO₃−)–Met–Gly–Trp–Met–Asp–Phe–NH₂
Nonsulfated CCK8 (CCK8)	Asp–Tyr–Met–Gly–Trp–Met–Asp–Phe–NH₂
Sulfated CCK7 (CCK7S)	Tyr (SO₃−)–Met–Gly–Trp–Met–Asp–Phe–NH₂
Nonsulfated CCK7 (CCK7)	Tyr–Met–Gly–Trp–Met–Asp–Phe–NH₂
Nonsulfated CCK6 (CCK6)	Met–Gly–Trp–Met–Asp–Phe–NH₂

Abbreviation: CCK, cholecystokinin.
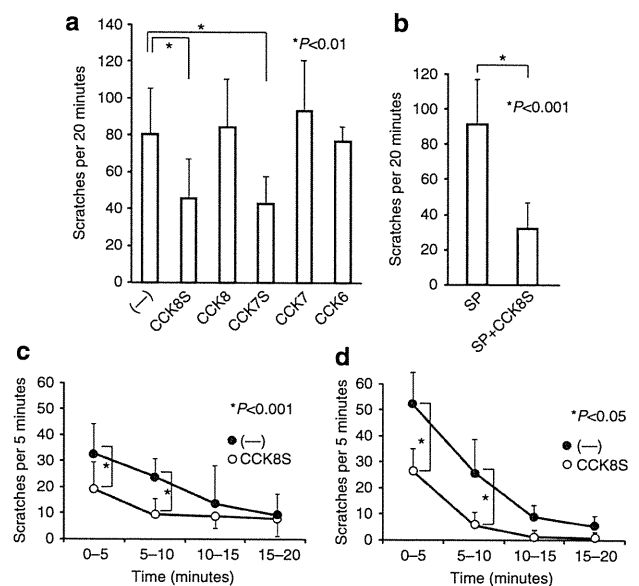
Figure 1. Suppression of substance P (SP)-induced itch by percutaneous application or intradermal injection of cholecystokinins (CCKs). (a) ICR mice were painted on the clipped skin with CCKs (0.5 nmol per site) and injected intradermally with SP (100 nmol per site). The group depicted with "(−)" represents mice injected with vehicle (acetone/olive oil) alone. The numbers of mice were as follows: CCK (—), n = 12; CCK8S, n = 10; CCK8, n = 6; CCK7S, n = 6; CCK7, n = 3; and CCK6, n = 4. (b) ICR mice were injected intradermally with SP or with SP and CCK8S. Each bar represents the mean of scratches per 20 minutes. CCK (−), n = 5 and CCK8S, n = 8. (c) Time course of scratching frequency in mice untreated (n = 12) or topically administered CCK8S (n = 10). (d) Time course of scratching frequency in mice untreated (n = 5) or intradermally injected with CCK8S (n = 8). CCK7, nonsulfated CCK7; CCK8, nonsulfated CCK8; CCK7S, sulfated CCK7; CCK8S, sulfated CCK8.

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CCK2R expression in KCs and mast cells

Both KCs and mast cells are the possible targets of CCKs in the suppression of SP-induced pruritus. It is known that KCs express the receptors for itch-related molecules, neurokinin-1 receptor for SP, H1 receptor for histamine, and protease-activated receptor-2 for tryptase (synthetic agonist, SLIGRL-NH$_2$). Mast cells are well known to possess neurokinin-1 receptor (Scholzen *et al.*, 1998; Liu *et al.*, 2006). We investigated the expression of the CCK receptors CCK1R and CCK2R in KCs and mast cells. Primary cultures of KCs from ICR mice and fetal skin–derived mast cells (FSMCs) were collected, and mRNAs for CCK1R and CCK2R were quantitated by real-time PCR, RT-PCR, and western blotting analyses. The two types of cells expressed both CCK1R and CCK2R at the mRNA and protein levels (Figure 2). When the expression levels of real-time PCR analysis were normalized with the β-actin
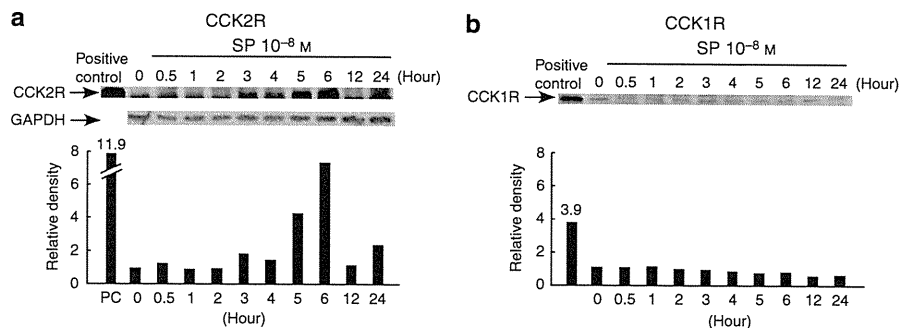

Figure 2. CCK-A receptor (CCK1R) and CCK-B receptor (CCK2R) mRNA expression in keratinocytes and mast cells. Cultured ICR mouse keratinocytes (a) and fetal skin–derived mast cells (FSMCs) (b) were examined for the expression of mRNAs for CCK1R and CCK2R using real-time PCR and RT-PCR and for the expression of their proteins using western blotting analysis. Data are expressed as the amount of expression relative to β-actin. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for western blotting.

level, the two receptors were more highly expressed in FSMCs than in KCs.

In a preliminary DNA microarray analysis of normal human epidermal KCs, we investigated the levels of gene expression from stimulation with SP (10^{-6} M), histamine ($10\,\mu g\,ml^{-1}$), or SLIGRL-NH$_2$ (100 nM) under high calcium (0.6 mM) concentration. The CCK2R expression by SP-stimulated KCs was most elevated; its level was $2^{8.67}$ times that of the control (Supplement Data online). Such an increased CCK2R expression was not observed with histamine or SLIGRL-NH$_2$. This observation suggested that CCK2R has an important role in the suppression of SP-stimulated KCs. To determine the effect of SP on KCs, ICR mouse KCs were incubated with SP for 30 minutes to 24 hours, and the expression of CCK2R and CCK1R was analyzed by western blotting. CCK2R was detected with antibodies as a 78 kDa band (Morisset *et al.*, 2003) and increased in KCs exposed to SP for 5 or 6 hours (Figure 3a). By contrast, CCK1R expression was not augmented by SP (Figure 3b). Thus, the expression of some receptors for CCK is enhanced by SP, depending on the receptor.
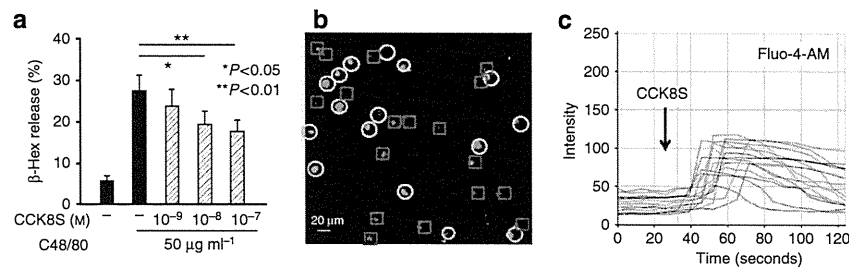
Suppression of FSMC degranulation by CCK8S with transient elevation of intracellular calcium

The above findings suggested that CCKs bind to CCK2R on KCs and mast cells and downmodulate their itch-related biological functions. Given that the level of CCK2R expression was higher in FSMCs than in KCs and the application of CCKs immediately exerted its inhibitory effects on the scratching behavior, it is possible that CCKs directly affect mast cells. We therefore focused on mast cells as the primary target. The addition of compound 48/80 induced degranulation of FSMCs (Figure 4a). This promoted degranulation of FSMCs, which was significantly inhibited by CCK8S at 10^{-8} or 10^{-7} M concentration.

To test whether CCK8S evokes signal transduction in mast cells, we measured the intracellular calcium using confocal recording. The fluorescence intensity of 15 cells positively responding on addition of CCK8S was monitored with computerized color change on a display using a digital image processing system. The mast cells exhibiting green fluorescence of Fluo-4-AM at any time point between 40 and

Figure 3. Western blotting of CCK-A receptor (CCK1R) and CCK-B receptor (CCK2R) in mouse keratinocytes stimulated with substance P (SP). ICR mouse keratinocytes were incubated with SP (10^{-8} M) for 0.5–24 hours and harvested at each time point. The expression levels of CCK2R (a) and CCK1R (b) were analyzed by western blotting. NIH/3T3 whole lysates were used as positive control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for western blotting and exhibited similar intensities in the samples cultured for 0–24 hours. The intensity of each band was subjected to densitometric analysis and expressed as density relative to the nontreated control (0).

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Figure 4. **Suppression of fetal skin-derived mast cell (FSMC) degranulation by sulfated CCK8 (CCK8S) with transient intracellular calcium increase.** (a) FSMCs were incubated with or without CCK8S in the presence of compound 48/80 (C48/80; $50 \mu g \, ml^{-1}$) for 15 minutes. Supernatants and cell lysates were incubated with *p*-nitrophenyl-*N*-acetyl-β-ᴅ-galactosaminide, and β-hexosaminidase (β-Hex) release was determined as the ratio between activity in the supernatant and cell lysate, multiplied by 100. (b) FSMCs were loaded with Fluo-4-AM (green) and stained for CD117 (c-kit, red). The percentage of CCK8S-responding FSMCs was determined from the fluorescence traces of all cells. Circles represent responding FSMCs and squares represent nonresponding FSMCs. Bar $= 20 \mu m$. (c) The addition of CCK8S to FSMC culture induced an increase in intracellular calcium level.

80 seconds after CCK8S addition were defined as positively responding cells (Figure 4b and c); these are marked with a yellow circle in Figure 4b. The addition of CCK8S to the FSMC culture induced a prompt rise of calcium that persisted for more than 2 minutes and then gradually declined, indicating that CCK8S induces signaling via CCK2R in mast cells. The percentage of responding FSMCs was determined from the fluorescence traces of all cells. As shown in Figure 4b, in which circles and squares represent CCK8S-responding and nonresponding FSMCs, respectively, 45% (16 of 36 cells) of FSMCs were typically activated by CCK8S.

DISCUSSION

Despite their original name of cholecyst stimulators, CCKs have been known to serve as CNS modulators (Dufresne *et al.*, 2006) and CCK8S downmodulates opioid responses (Pommier *et al.*, 2002; Agnes *et al.*, 2008). As suggested in our present study, CCKs may also have a function of downmodulator in the peripheral itch. The topical application of CCKs suppressed the itch-related scratching behavior evoked by SP injection. The expression of CCK2R, but not CCK1R, in murine and human KCs was markedly enhanced by exposure to SP. It is likely that CCK2R, whose expression is augmented by SP, has a down-regulatory role in the excess peripheral itch.

Among the CCK constructs consisting of different numbers of amino acids with or without sulfate, CCK8S and CCK7S, but not CCK8, CCK7, or CCK6, exerted an antipruritic action. Therefore, in a comparison between CCK8S and CCK8 and between CCK7S and CCK7, sulfation of position 7 is required for the ability to suppress the scratching behavior. This is in accordance with the observation that CCK8S has an anxiogenic effect (Harro, 2006), a depressant effect, and an anti-opioid effect (Noble *et al.*, 1999), but the effects of CCK are limited by desulfation of the tyrosine (Tokunaga *et al.*, 1993).

Although the exact mode of antipruritic action of CCKs is still speculative, some informative observations were obtained from the study. Both percutaneous application and dermal injection were effective routes for CCK8S in suppressing itch, suggesting that epidermal and/or dermal constituents are the targets of CCKs. Furthermore, the administration of CCK8S rapidly inhibited the SP-induced itch. We thus investigated the mechanism of CCK suppression of itch in

in vitro studies, focusing on mast cells. CCK8S significantly reduced the degranulation of FSMCs with elevation of intracellular calcium concentration, suggesting that mast cell degranulation is inhibited by CCK8S through CCK2R. Alternatively, or additionally, KCs are possibly involved in CCR action. KCs are thought to mediate pruritus by releasing inflammatory mediators and neurotrophins. Therefore, the antipruritic effect of CCK8S on KCs, if any, seems to need a time lag to operate. As the therapeutic action of topical CCK8S is rapid, mast cells may be more likely targeted by CCK8S than KCs. The high expression of CCK2R in mast cells further supports the notion that mast cells are sensitive to CCKs. However, SP was reported to cause scratching behavior in both mast cell–deficient and wild-type mice (Hossen *et al.*, 2003), suggesting a mast cell-independent mechanism of SP-evoked itch. Given this observation, it is possible that another skin constituent, such as the sensory nerves, is affected by CCKs. The target cells and the actions of CCK8S on them warrant elucidation in the future.

The antipruritic effects of CCK8S and CCK7S on this mouse itch model may afford a therapeutic use for the CCKs for the treatment of skin diseases with peripheral itch. Given that the major target of CCKs is mast cells, urticaria, atopic dermatitis, contact dermatitis, and other types of eczematous dermatitis would be successfully treated with CCKs topically applied to the skin. The therapeutic effects of CCKs on peripheral itch may shed light on the mechanisms underlying the physiological regulation of itch, and it is notable that CCKs have potential as a topical drug for pruritic skin diseases.

MATERIALS AND METHODS
Animals
Female ICR mice were obtained from KBT Oriental (Tosu, Japan). These mice were maintained in the Laboratory Animal Research Center of the University of Occupational and Environmental Health under specific-pathogen-free conditions and used at the age of 6–8 weeks. All animal experiments were performed according to the guidelines approved by our university for the care and use of animals.

Reagents
SP, CCK8S, nonsulfated CCK8, CCK7S, and nonsulfated CCK6 were purchased from the Peptide Institute (Osaka, Japan). Nonsulfated

CCK7 was purchased from Funakoshi (Tokyo, Japan). Compound 48/80 was purchased from Sigma (St Louis, MO).

Itch-associated scratching behavior and topical application of CCKs

The hair was clipped over the rostral part of the each mouse's back 1 or 2 days before the experiment. Before behavioral recording, each animal (four per observation) was put into an acrylic cage (18 × 23 × 11 cm) for at least 1 hour for acclimation. An intradermal injection of SP (100 nmol in 50 µl of physiological saline per site) induced scratching of the skin around the injected site with the hindpaws (Andoh *et al.*, 2001). CCKs were topically applied in two ways: percutaneous application and intradermal injection. For percutaneous application, the shaved back skin was stripped with Scotch tape (3 M) six times on the day before the experiment (Nishijima *et al.*, 1997). The clipped backs of the mice were painted (1.8 cm^2, 1.5 cm in diameter) with 20 µl CCK (0.5 nmol per site) in acetone/olive oil (3:1) 3–4 minutes before the SP injection (100 nmol per site). For intradermal application, mice were injected intradermally with CCK (0.5 nmol per site in 20 µl physiological saline), followed immediately by SP injection at the same site (100 nmol per site). Immediately after treatment, the animals were put back into the same cage and their behavior was videotaped for 40 minutes using a digital video camera (Andoh *et al.*, 2001). Using the video, the frequency of scratching toward the injected site by the hindpaws was recorded for the first 20 minutes.

Murine KCs

The skin of ICR newborn mice was peeled within 24 hours after birth and incubated with 0.05% collagenase (collagenase from *Clostridium histolyticum* type 2; Sigma) for 2 days at 37 °C in a 5% CO_2 atmosphere. Epidermal sheets were collected and suspended in culture medium. Cell suspension was plated into six-well plates (Corning Glass Works, Corning, NJ) and grown to subconfluence in CnT-07 medium (Funakoshi).

Murine mast cells

The method for preparation of murine FSMCs has been described previously (Yamada *et al.*, 2003). Day 14 fetal skin was dissociated by 0.25% trypsin for 20 minutes at 37 °C in Hank's balanced salt solution (Gibco, Carlsbad, CA). Erythrocytes were lysed using RBC-lysing buffer (Sigma) and, after washing, cells were resuspended in complete RPMI, which is RPMI-1640 (Gibco) supplemented with 5% fetal bovine serum (Gibco), 1% nonessential amino acids (Gibco), 1% sodium pyruvate (Gibco), antibiotic/antimycotic (Invitrogen, Carlsbad, CA), and 0.1% 2-mercaptoethanol (Gibco). Fifty milliliters of the cell suspension in each 175 cm^2 75-flask (Corning Glass Works) was cultured in the presence of 20 ng ml^{-1} recombinant mouse IL-3 (Invitrogen) and 20 ng ml^{-1} recombinant mouse stem cell factor (Biosource, Camarillo, CA) at 37 °C without changing the medium. Nonadherent and loosely adherent cells were harvested. The cells were centrifuged and resuspended in RPMI, layered on 40% Percoll (GE Healthcare, Buckinghamshire, England), and centrifuged at room temperature. The cell pellet at the bottom was used as FSMCs. For flowcytometric analysis, cells were stained with phycoerythrin-conjugated anti-mouse FcεRI (eBioscience, San Diego, CA) and Alexa Fluor 700-conjugated anti-mouse CD117 (c-kit; eBioscience) for 20 minutes on ice, washed twice, and then resuspended with FACS buffer. Flow cytometric analysis was performed with FACS Canto (BD, Franklin Lakes, NJ) and FlowJo software (TreeStar, San Carlos, CA). The purity of FSMCs was 95%, as determined by both FcεRI and CD117 positivity by flow cytometry.

Real-time quantitative RT-PCR analysis

Total RNA was extracted from the cell pellet using the PureLink RNA Mini Kit (Invitrogen). cDNA was reverse transcribed from total RNA samples using the TaqMan Reverse Transcription reagents (Applied Biosystems, Carlsbad, CA). mRNA expression was quantified by real-time PCR using SYBR Green Dye (PE Biosystems, Foster City, CA) with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Primers were designed using Primer Bank for the human and murine genes, and they were constructed by Invitrogen. The sequences are as follows: murine β-actin: 5'-GGCTGTATTCCCCTCCATCG-3' and 5'-CCAGTTGGTAACAATGCCATGT-3'; murine CCK2R: 5'-GATGG CTGCTACGTGCAACT-3' and 5'-CGCACCACCCGCTTCTTAG-3'; and murine CCK1R: 5'-CACGCTGGTTATCACGGTG-3' and 5'-GC CCATGAAGTAGGTGGTAGTC-3'. The conditions for the real-time PCR were as follows: 2.0 minutes at 50 °C, 10 minutes at 95 °C, and then 50 cycles of amplification consisting of 15 seconds at 95 °C and 1 minute at 60 °C. All heating and cooling steps were performed with a slope of 20 °C per second. Relative expression was calculated using the delta Ct method.

Western blotting

KCs from ICR mice were stimulated with SP (10^{-8} M) in six-well plates (Corning Glass Works) for 0.5–24 hours, harvested with a rubber policeman, and subjected to extraction with RIPA buffer (Wako, Osaka, Japan; 50 mM Tris-HCl (pH8.0), 150 mM sodium chloride, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, and 1.0% (wt/vol) NP-40 substitute). Protein samples (20 ìg) were separated by 8% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes for 2 hours at 180 mA. After blocking with 5% skim milk solution, the membranes were incubated with rabbit anti-mouse CCK1R (SC-33220; 1:1,000, Santa Cruz, Santa Cruz, CA), CCK2R (SC-33221; 1:1,000, Santa Cruz) polyclonal antibodies, or GAPDH (SC-25887; 1:1,000, Santa Cruz), and the reaction was detected with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:3,000, Bio-Rad, Hercules, CA). Immunoblots were visualized using the ECL Plus Western Blotting Detection Reagents (GE Healthcare) according to the manufacturer's protocol. Bands were quantified by densitometry with the help of a CS Analyzer version 2.0 (ATTO, Tokyo, Japan). NIH/3T3 whole cell lysate (Santa Cruz) was used as a positive control.

Mast cell degranulation

Degranulation of FSMCs was assessed by β-hexosaminidase assay (Yamada *et al.*, 2003; Noguchi *et al.*, 2005). FSMCs were washed with Tyrode's buffer (Sigma) and resuspended at a concentration of 2×10^4 cells per well into a 96-well plate. FSMCs were incubated with or without CCK8S in the presence of compound 48/80 (50 µg ml^{-1}) for 15 minutes at 37 °C. The plate was centrifuged for 5 minutes, and the supernatants were placed into another 96-well plate. Cell pellets were resuspended in Tyrode's buffer containing 0.5% triton X-100 (Sigma). Supernatants and cell lysates (50 µl) were incubated with 100 µl of 2.5 mM *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (Sigma),

dissolved in 0.04 mM citrate buffer (pH 4.5), at 37 °C for 90 minutes. The reaction was stopped with 100 μl of 0.4 M glycine (pH 10.7; MP Biomedicals, Solon, OH). The plate was read at 405 nm using a 595 nm reference filter in a microplate reader (Bio-Rad). β-Hexosaminidase release was determined as the ratio between activity in the supernatant and the cell lysate, multiplied by 100.

Calcium influx determined by confocal imaging

Confocal imaging of cells loaded with fluorescent dyes was performed using a Zeiss LSM5 pascal (Carl Zeiss, Jena, Germany). FSMCs were loaded with Fluo-4-AM (Invitrogen; 1 μM) at 37 °C for 20 minutes, washed twice, and set aside for at least 40 minutes. FSMCs were also stained for CD117 (c-kit) with Alexa Fluor 700–conjugated anti-mouse CD117 antibody. The LSM5 multitrack configuration was used for simultaneous measurement of intracellular calcium concentration (excitation, 488 nm; emission, long-pass 505 nm filter) and the expression of CD117 (excitation, 633 nm; emission, long-pass 650 nm filter). Images were recorded (usually recording for 300 seconds) at room temperature, in a 512 × 512-pixel format. As stimulants, SP or CCKs were added directly to the bath as a small drop (10 μl; final concentration, 10^{-6} M SP and 10^{-6} M CCK8S). Image data were analyzed offline using the Zeiss LSM510 analyzing software (LSM Image Examiner, Carl Zeiss). The mast cells exhibiting green fluorescence of Fluo-4-AM at any time point between 40 and 80 seconds after CCK8S addition were defined as positively responding cells.

Gene-array analysis

Normal human epidermal KCs were cultured with SP (10^{-6} M), histamine (10 mg ml^{-1}), and SLIGRL-NH$_2$ (100 nM) for 2 hours and harvested. For DNA microarray analysis, total RNA was extracted from Normal human epidermal KCs with the RNeasy Mini Kit (Qiagen, Valencia, CA). Images were analyzed with the DNASIS Array (DNA Chip Research, Hitachi Software Engineering, Tokyo, Japan) according to the manufacturer's instructions. The results of DNA microarray analysis of the top and bottom 20 genes with respect to expression levels induced by SP, histamine, or SLIGRL-NH$_2$ are shown in the Supplementary Data online.

Statistical analysis

Data were analyzed using an unpaired Student's *t*-test. $P < 0.05$ was considered to be significant.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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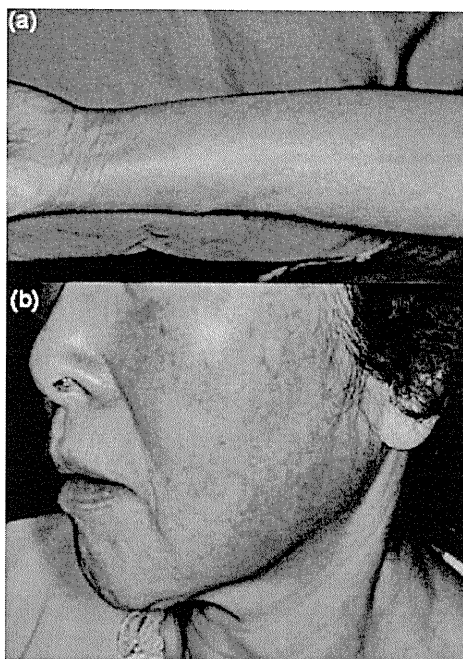
Fig. 1. Recurrent pruritic erythema on the left arm (a) and face (b).

Occupational contact dermatitis caused by asparagus

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Case Report

A 67-year-old Japanese woman presented with a 2-year history of a recurrent pruritic erythema on the face, arms and hands (Fig. 1). She had worked in an asparagus cannery for 20 years. Her medical history indicated no evidence of atopic disease. The dermatitis cleared quickly with courses of anti-histamines and topical steroids but relapsed within days of returning to work. Rubber gloves were initially of benefit, but the pruritic eruption re-appeared when she touched her face with these gloves after working. She was patch-tested to a metals series and a freshly picked shoot of *Asparagus officinalis*. At both D2 and D3, the patch test with asparagus showed a strongly positive reaction (++), whereas all metals and white petrolatum were negative. Skin prick test with raw asparagus showed no skin reaction. Three normal healthy volunteer controls were negative on patch testing to asparagus. In light of the clinical observations and test results, occupational contact dermatitis to asparagus was diagnosed.

Discussion

Asparagus, one of the *Liliaceae* family members, is a vegetable that is cultivated worldwide and consumed when in season. Contact dermatitis to asparagus is rare (1–5). Several cases of allergic reactions by ingestion or inhalation of asparagus have been reported (6). Our patient, working in the cannery, had chronic/seasonally relapsing contact dermatitis to asparagus, which seems to be rare despite the widespread availability and consumption of asparagus. Although an association of contact dermatitis with other *Liliaceae* family members (garlic and onion) has frequently been reported as a result of occupational exposure (7), dermatologists should consider the potential of asparagus as an occupational allergen, particularly if allergic contact dermatitis occurs either chronically or seasonally.

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

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ARTICLE INFO

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.

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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).
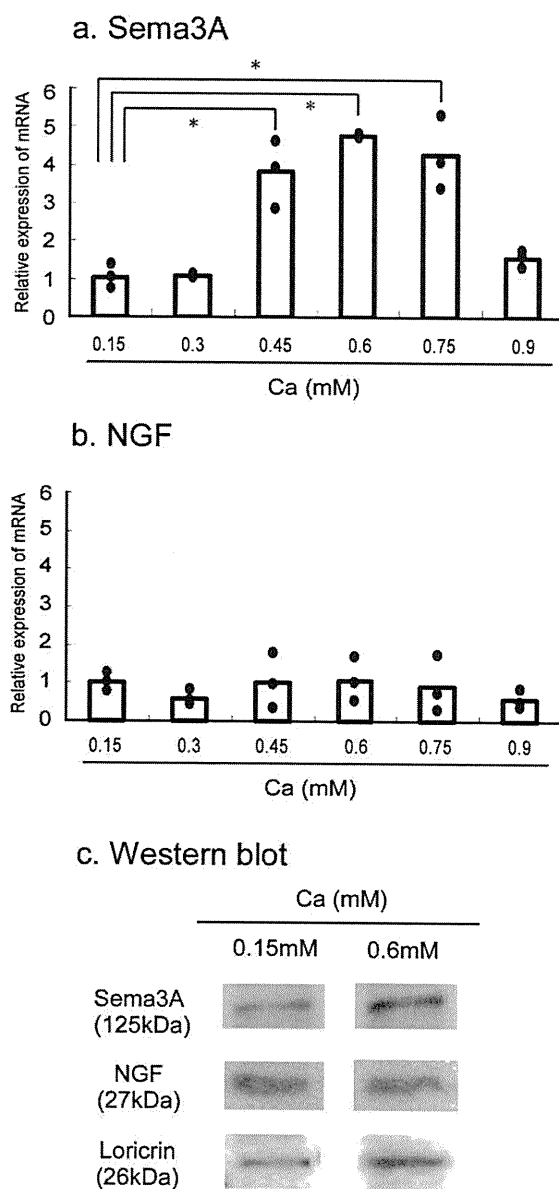
Fig. 1. Effects of calcium concentration on the expression of SEMA3A and NGF in NHEK. NHEK were cultured under varying concentrations, ranging from 0.15 to 0.9 mM for 2 h. After harvesting, cDNA was synthesized directly and the expression of SEMA3A (a) and NGF (b) was assessed by real-time PCR. The data 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).

2.6. Immunohistochemistry

Formalin-fixed and paraffin-embedded 4-μm sections of specimens were deparaffinized and rehydrated through descending alcohol series and in phosphate buffered saline (PBS, pH 7.4). Antigen was retrieved with Antigen Retrieval Citra (BioGenex Laboratories, San Ramon, CA), and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol. After blocking nonspecific antigen binding sites with 3% nonimmune serum, anti-Sema3A rabbit polyclonal IgG antibodies (sc-28867; 1:200; Santa Cruz) was applied overnight at 4 °C. After rinsing with PBS, sections were incubated with biotinylated secondary antibody and then with peroxidase conjugated avidin solution (Vector Laboratories Inc., Burlingame, CA). Peroxidase was visualized with 3-amino-9-ethylcarbazole chromogen, and the sections were lightly counterstained with hematoxylin.

2.7. Statistical analysis

Data were analyzed using an unpaired two-tailed t-test. P-value of less than 0.05 was considered to be significant.

3. Results

### 3.1. High calcium augments the expression of SEMA3A but not NGF in NHEK

Keratinocytes proliferate at a low calcium concentration, such as 0.15 mM, and differentiate at a high calcium concentration in culture [12]. The proliferating and differentiating cells represent the basal cell in the lower most epidermis and the prickle cell in the upper epidermis, respectively. To address the physiological production of Sema3A and NGF by epidermal keratinocytes, NHEK were cultured under varying calcium concentrations, ranging from 0.15 to 0.9 mM, and after 2-h incubation, the expression of SEMA3A and NGF was assessed by real-time PCR. Whereas SEMA3A expression was low at 0.15 or 0.3 mM calcium, its expression was upregulated at higher concentrations of 0.45–0.75 mM (Fig. 1a). Calcium at 0.9 mM or more reduced the expression of SEMA3A. In contrast, calcium concentration did not affect the expression of NGF in NHEK (Fig. 1b). The incubation period of 24 h produced the comparable levels of SEMA3A and NGF expression to the 2-h incubation.

To confirm the above finding at the protein level, we performed a Western blot analysis for Sema3A, NGF, and differentiation marker loricrin in NHEK cultured with 0.15 or 0.6 mM calcium. NHEK incubated with 0.6 mM calcium showed higher levels of Sema3A as well as loricrin than those with 0.15 mM calcium, while the levels of NGF were comparable between them (Fig. 1c). These results indicate that the production of Sema3A but not NGF is dependent on calcium concentration.

3.2. Immunohistochemical staining for Sema3A in normal skin and cutaneous tumors

The above finding suggested that Sema3A is highly expressed in the upper epidermis where keratinocytes are differentiated by high calcium concentration. We therefore investigated Sema3A expression by immunohistochemistry in normal skin, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC). In normal
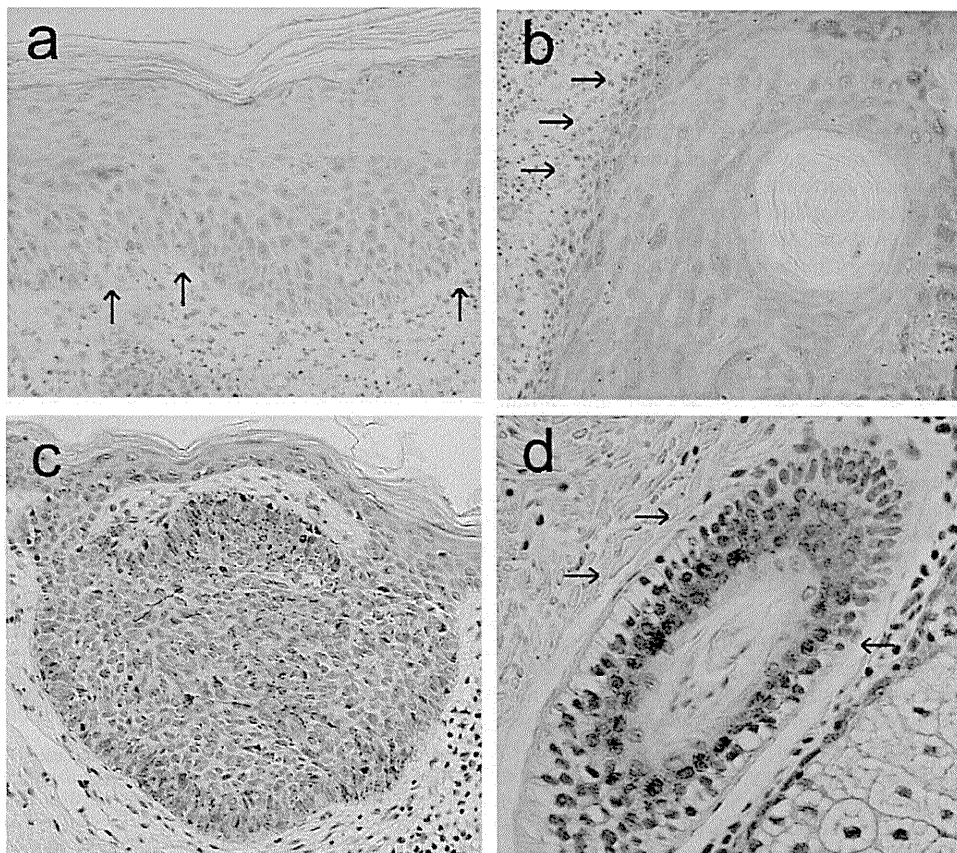
Fig. 2. Sema3A expression in normal skin and SCC. Immunohistochemical staining for Sema3A in normal epidermis (a), tumor nest of SCC (b), tumor nest of BCC (c), and hair follicle (d). Arrows indicate basal and suprabasal cells stained negatively. Original magnification: ×200.

a. GM-CSF

b. IL-8
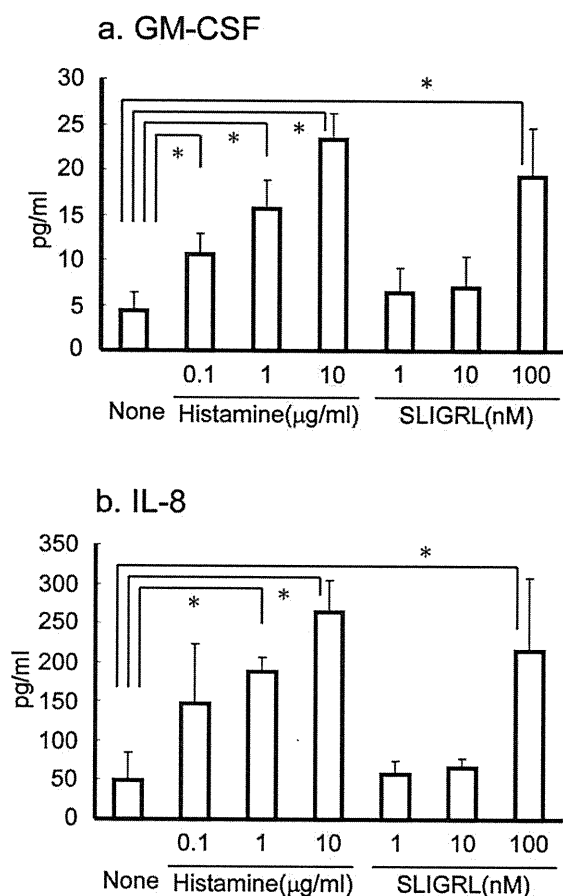
Fig. 3. Effects of histamine and SLIGRL on cytokine/chemokine production by NHEK. NHEK were cultured with various concentrations of histamine or SLIGRL for 48 h. The amounts of IL-8 and GM-CSF in the supernatants was measured using CBA. Bars represent the means ± SD of IL-8/GM-CSF concentration in triplicate. *$P < 0.05$.

skin, keratinocytes of the prickle layer were positive for Sema3A, while cells of the basal and suprabasal layers and the granular layer were negative (Fig. 2a). Likewise, in SCC tissues, squamoid tumor cells were strongly positive for Sema3A except for basal and suprabasal cells of the tumor nests (Fig. 2b). The epidermal cells of BCC were negative for Sema3A (Fig. 2c). In hair follicles of normal skin, inner cells in the outer root sheath were strongly Sema3A-positive, but basal and suprabasal cells in this part were not positively stained (Fig. 2d).

3.3. Histamine and SLIGRL are bioactive for keratinocytes

Since histamine is one of the critical pruritus-related molecules involved in allergic skin disorders, we examined its effect on Sema3A and NGF production by keratinocytes. SLIGRL was also tested in parallel, because signaling via PAR-2 is important as well as signaling via H1 receptor in C-fiber stimulation [15], and keratinocytes express both the receptors [16,17].

In advance of testing the effects of histamine and SLIGRL on the production of Sema3A and NGF, we confirmed their biological activities on keratinocytes and estimated their optimal concentrations in our culture system. NHEK were cultured at 0.15 mM calcium with histamine (0.1–100 μg/ml) and SLIGRL (1–1000 nM) for 48 h. In our preliminary study, we examined the effects of histamine and SLIGRL on the production by NHEK of GM-CSF, IL-8, CXCL9/MIG, CXCL10/IP-10, CCL5/RANTES, CCL22/MDC, and vascular endothelial growth factor. Among these cytokines and

chemokines, GM-CSF and IL-8 were increased by histamine and SLIGRL. We therefore chose these two cytokines in the following experiments. When these two cytokines were quantified in the supernatants, histamine at 0.1 μg/ml or more and SLIGRL at 100 nM increased the production of GM-CSF and IL-8 by NHEK (Fig. 3). Histamine 100 μg/ml and SLIGRL 1000 nM were toxic to NHEK as they abolished cell viability (data not shown). Therefore, histamine at 10 μg/ml and SLIGRL 100 nM were used in the following studies.

3.4. Histamine upmodulates SEMA3A expression and downmodulates NGF expression in keratinocytes
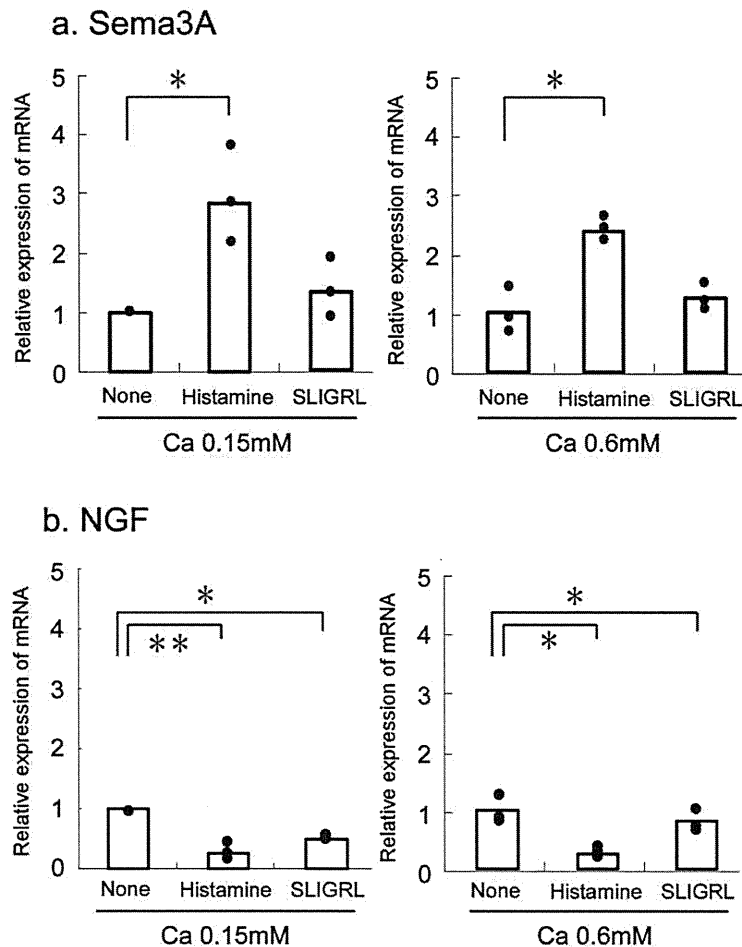
Incubation of NHEK with histamine (10 μg/ml) for 2 h upregulated the expression of SEMA3A under either 0.15 mM or 0.6 mM of calcium concentration, while stimulation with SLIGRL (100 nM) unaffected SEMA3A expression at both calcium concentrations (Fig. 4a). SLIGRL at higher concentrations (up to 10 μM) was also tested, but SEMA3A expression was not increased (data not shown). On the contrary, the expression of NGF was significantly decreased by histamine at 10 μg/ml or SLIGRL at 100 nM, although the former's suppressive activity was higher than the latter's (Fig. 4b). Thus, histamine but not SLIGRL augmented the expression of Sema3A, and both stimulants inhibited NGF expression in keratinocytes. Thus, the opposite effects of histamine on Sema3A and NGF productions are remarkable.

3.5. Histamine and SLIGRL downregulate the expression of both SEMA3A and NGF in fibroblasts

In addition to the epidermis, the dermis is a critical microenvironment where peripheral sensory nerve elongates. Dermal fibroblasts express H1 receptor [18] and PAR-2 [19]. The expression of the axon guidance factors by fibroblasts and the effects of histamine and SLIGRL on their expressions are issues to be explored. By using NHFb, we first examined the fibroblast expression of SEMA3A and NGF and the dependency on calcium concentration. NHFb expressed mRNAs for both factors, and there was no modulatory effect of calcium concentration (data not shown). Next, NHFb were incubated with histamine (1 or 10 μg/ml) or SLIGRL (10 or 100 nM) for 2 h. The expression levels of SEMA3A (Fig. 5a) and NGF (Fig. 5b) were significantly reduced by histamine at 10 μg/ml or SLIGRL at 100 nM, suggesting that the fibroblast production of both axon guidance factors with opposite capacities are depressed by these mast cell-released mediators.

4. Discussion

In this study, we demonstrated the modulation of SEMA3A and NGF expressions by calcium concentration and histamine or SLIGRL. The expression of SEMA3A depended on calcium concentration in NHEK, but not in NHFb. Whereas NHEK cultured at low calcium concentrations of 0.15–0.3 mM, inducible for keratinocyte proliferation, expressed low levels of SEMA3A, cells cultured at high calcium concentrations of 0.45–0.75 mM, suitable for keratinocyte differentiation, expressed high levels of SEMA3A. On the contrary, NGF was constitutively expressed by NHEK irrespective of calcium concentration. Therefore, the expression of SEMA3A but not NGF is markedly regulated by calcium concentration in epidermal keratinocytes, implying that the chemorepellent may control C-fiber elongation in the physiological condition. The mechanisms by which Sema3A production is dependent on the extracellular calcium concentration is not clear. One possible explanation is that Ca^{2+} signaling participates in the transcription of Sema3A gene. The second messenger inositol triphosphate and intracellular

a. Sema3A

Relative expression of mRNA

Ca 0.15mM: None, Histamine, SLIGRL   *

Ca 0.6mM: None, Histamine, SLIGRL   *

b. NGF

Relative expression of mRNA

Ca 0.15mM: None, Histamine, SLIGRL   *, **

Ca 0.6mM: None, Histamine, SLIGRL   *, *

Fig. 4. Effects of histamine and SLIGRL on the expression of SEMA3A and NGF in NHEK. NHEK were cultured with histamine or SLIGRL under different Ca concentrations (0.15 or 0.6 mM) for 2 h. After harvesting, the expression of Sema3A and NGF was assessed by real-time PCR. The data are expressed as (expression level of stimulated group)/ (expression level of no addition control). Bars represent the means of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

calcium are generated in that Ca^{2+}-induced differentiation [20]. Sema3A production might share the signaling pathway with many differntiation-asscoaited molecules. It has been reported that NGF secreted by basal keratinocytes causes hypertrophy of the peripheral nerve [7,21]. Our finding suggests that NGF is homogenously produced throughout the epidermis, and Sema3A determines the location of nerve endings and inhibits excess C-fiber elongation into the epidermis.

In accordance with the results from the NHEK culture study, our immunohistochemical study showed that keratinocytes in the prickle layer of the epidermis and the outer root sheath of the hair follicle were positive for Sema3A, while basal cells and suprabasal cells were negative in these tissues. The similar staining pattern was obtained in SCC, where Sema3A was expressed by squamoid epithelial cells in the tumor nests, sparing two to three layers of basal or adjacent suprabasal cells. Notably, cells of the sebaceous glands and arector pilli bore Sema3A, providing a possibility that sensory nerves cannot invade these apparatus tissues.

Histamine and SLIGRL not only stimulated NHEK to produce IL-8 and GM-CSF, but also modulated the expression of Sema3A and NGF in NHEK and NHFb. In NHEK, histamine increased Sema3A expression but decreased NGF expression, and SLIGRL did not increase Sema3A expression and decreased NGF expression to some degree. Thus, the modulatory effects of histamine on NHEK are greater than those of SLIGRL. These in vitro results suggest that histamine unexpectedly inhibits C-fiber elongation in the epider-

mis. The release of histamine from mast cells occurs upon antigenic stimulation via IgE and Fcε receptors and upon substance P stimulation via NK1 receptor [3,4,22]. Since these stimulatory events take place in itch-related allergic diseases, the histamine-enhanced SEMA3A and -reduced NGF expressions in keratinocytes might be a feedback phenomenon to suppress exaggerated pruritus. However, a recent paper has reported that H1 antagonist olopatadine hydrochloride increased the expression of Sema3A in the skin of NC/Nga mice with atopic dermatitis [23], providing a contradictory finding. Considering that the histamine-augmented Sema3A expression in NHEK was not inhibited by H1 blocker pyrilamine maleate salt (data not shown), the discrepancy between their and our findings might be due to different usage of the types of histamine receptors. In NHFb, histamine and SLIGRL depressed the expression of both SEMA3A and NGF. Considering the opposite actions of these two axon guidance factors, the final outcome remains unclear in this study. Concerning the chemor-epellent, however, histamine seems to allow sensory fibers to elongate in the dermis. It should be noted that the opposite effects of histamine on NHEK and NHFb in the expression of SEMA3A may induce positive and negative sensory fiber elongation in the dermis and epidermis, respectively.

Sema3A may play a crucial inhibitory role for C-fiber elonga-tion/sprouting in the upper layers of the epidermis. Disruption of the physiological calcium gradient may induce the disordered Sema3A production by keratinocytes, resulting in C-fiber elonga-

a. Sema3A

b. NGF
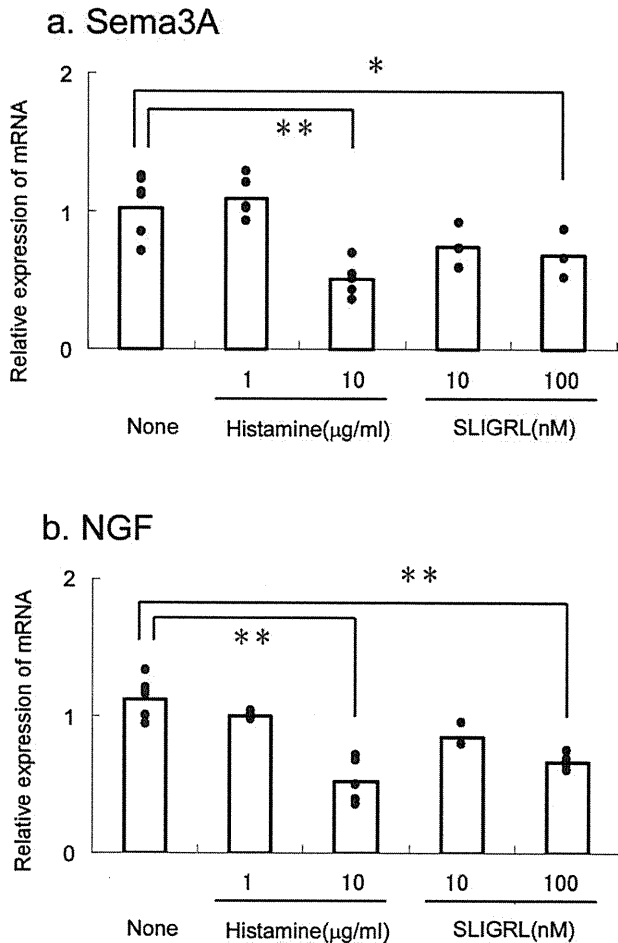
Fig. 5. Effects of histamine and SLIGRL on the expression of *SEMA3A* and *NGF* in NHFb. NHFb were cultured with histamine and SLIGRL at the indicated concentration for 2 h. After harvesting, the expression of *SEMA3A* (a) and *NGF* (b) was assessed by real-time PCR. The data are expressed as (expression level of stimulated group)/(expression level of no addition control). The bars represent the means of three to five independent experiments. *$P < 0.05$, **$P < 0.01$.

tion. In the epidermis of patients receiving hemodialysis and suffering from uremic pruritus, calcium concentration is not elevated from the inside to the outside of the skin, but distributed equally in all layers of the epidermis [24]. Considering that Sema3A production is regulated by calcium concentration, the disordered calcium gradient in such a pruritic disease might result in the elongation of C-fiber in the epidermis. In atopic dermatitis, C-fiber elongates into the upper epidermis as a result of a reduced production of Sema3A [9–11]. Our finding suggests that this epidermal elongation and sprouting of nerve endings is not promoted by histamine. It has been reported that mast cell-derived tumor necrosis factor (TNF) promotes nerve fiber elongation in the epidermis and dermis during contact hypersensitivity in mice [25]. This provides a possibility that mast cells contribute to nerve elongation by secreting TNF but not histamine. Future investigation may clarify this important issue.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2010.11.012.

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Original Article

Protective effects of platinum nanoparticles against UV-light-induced epidermal inflammation

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Abstract: Intracellular reactive oxygen species (ROS) and apoptosis play important roles in the ultraviolet (UV)-induced inflammatory responses in the skin. Metal nanoparticles have been developed to increase the catalytic activity of metals, which is because of the large surface area of smaller particles. Platinum nanoparticles (nano-Pt) protected by poly acrylic acid were manufactured by reduction with ethanol. A marked increase in ROS production was observed in UV-treated HaCaT keratinocytes cell lines, while a decrease in ROS production was observed in nano-Pt-treated cells. Pretreatment of the cells with nano-Pt also caused a significant inhibition of UVB- and UVC-induced

apoptosis. Furthermore, we found that mice treated with nano-Pt gel prior to UV irradiation showed significant inhibition of UVB-induced inflammation and UVA-induced photoallergy compared to UV-irradiated control mice. These results suggest that nano-Pt effectively protects against UV-induced inflammation by decreasing ROS production and inhibiting apoptosis in keratinocytes.

Key words: apoptosis – platinum nanoparticles – reactive oxygen species – skin – ultraviolet

Introduction

Skin is a major target for toxic insult by a broad spectrum of ultraviolet (UV) radiation that is capable of altering its structure and function. Photodamaged skin exhibits wrinkles, pigmented spots, dryness, and tumors. UV radiation has been reported to generate reactive oxygen species (ROS), such as singlet oxygen, superoxide radicals, hydroxyl radicals, and hydrogen peroxide, in a variety of cells (1), and intracellular ROS are thought to play an important role in UV-induced inflammatory responses in the skin. Both short-wavelength UVC (200–280 nm) and UVB (280–320 nm) as well as long-wavelength UVA (320–400 nm) produce ROS, which can lead to cell damage when the bal-

ance between the prooxidant stimuli and cellular antioxidant defenses is impaired (2,3).

The skin has a complex defense system of enzymatic and non-enzymatic components to counter the adverse biological effects of ROS. As the first defense, ROS are reduced by antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, as well as endogenous and exogenous small molecules, including glutathione and vitamins C and E. When biomolecules are oxidized, they are repaired or replaced by biological protective systems. Nevertheless, biomolecules are gradually irreversibly oxidized, and accumulation of these biomolecules over time impairs biological functions, eventually leading to ageing and age-related diseases (4,5). Sunscreen use is a widely accepted method of primary prevention against skin cancer, sunburn, and photoageing. In fact, antioxidant compounds used in these products seem to function as protective substances against UV-induced ROS in the skin. Topical antioxidants, such as the flavonoid quercetin, have been reported to diminish UV radiation–mediated oxidative damage to the skin (6,7). Ascorbic acid (AA), which is the

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Abbreviations: AA, ascorbic acid; IL, interleukin; MIF, macrophage migration inhibitory factor; MMP, mitochondrial membrane potential; nano-Pt, platinum nanoparticles; ROS, reactive oxygen species; TNF, tumor necrosis factor; UV, ultraviolet; LFLX, lomefloxacin hydrochloride.

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most abundant antioxidant in the dermis, has exhibited a protective effect against oxidative stress.

Metal nanoparticles have been developed to increase the catalytic activity of metals because of the large surface area of smaller particles. Some noble metal nanoparticles are reducing catalysts that may be useful as antioxidants acting to reduce ROS in a living body. Nanotechnology is of interest because of the wide application of nanoparticles not only in material science and engineering but also in medical science and clinical use. Platinum nanoparticles (nano-Pt) protected by polyacrylic acid were recently manufactured by reduction with ethanol for 2 h (8). Nano-Pt are known to function as reductive catalysts; thus, they may be used as antioxidants to reduce ROS in living organisms. In fact, nano-Pt have the ability to scavenge O_2^- and H_2O_2, indicating that nano-Pt can act as a SOD/catalase mimetic (8). However, only a few studies have reported the antioxidant effects of nano-Pt in living organisms.

To elucidate the possible role of nano-Pt in UV-induced skin damage, we examined their protective effect on UV-induced ROS production and apoptosis using cultured keratinocytes. We also investigated the role of topically applied nano-Pt against UV-induced skin damage in mice.

Materials and methods

Reagents

Anti-Bcl-X_L monoclonal antibody (mAb), anti-Bax polyclonal antibody (pAb), anti-caspase-3 pAb, and anti-β-actin Ab were obtained from Santa Cruz Biotechnology, Inc.(Santa Cruz, CA, USA); nano-Pt were prepared as described previously (8). Nano-Pt gel was formulated by mixing 0.02% nano-Pt in water (corresponding to approximately 1 mM) with water and carboxyvinyl polymer (WAKO, Tokyo, Japan) (nano-Pt:water:carboxyvinyl polymer = 1:8.8:0.2) to yield a 0.002% w/w gel. Other reagents were of analytical grade.

Cell culture

HaCaT cells, an immortalized, non-tumorigenic human keratinocyte cell line (9), were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum and 1% antibiotics at standard cell culture conditions (37°C, 5% CO_2 in a humidified incubator).

Mice

Eight-week-old male Balb/c mice were purchased from Japan Clea (Shizuoka, Japan). These mice were maintained under specific pathogen-free conditions. All experiments involving mice were performed in accordance with the guidelines for the care and use of laboratory animals at the Hokkaido University Graduate School of Medicine and University of Toyama.

UV irradiation

The UVA irradiation source was a FL20S/BLB fluorescent lamp (Clinical Supply, Tokyo, Japan) that emitted an energy spectrum with high fluency in the UVA region (300–430 nm), with a peak at 352 nm. A 6-mm-thick glass plate was used to block UVB emissions. The UVB light source was a FL20SE30 (Clinical Supply Co., Tokyo, Japan) fluorescent lamp that emitted 1.0 mW/cm² of UV radiation between 280 and 370 nm (peak 305 nm) at a distance of 25 cm, as measured by a UV radiometer (Torex Co., Tokyo, Japan). The UVC light source was a 615T8 (Sanyo Denki., Tokyo, Japan) fluorescent lamp that emitted 15 W of UV radiation between 190 and 280 nm (peak 254 nm) at a distance of 25 cm, as measured by a UV radiometer (Funakoshi Co., Tokyo, Japan).

Assessment of intracellular ROS

Intracellular ROS were measured using hydroethidine (HE) (Molecular Probes, Eugene, OR, USA) to detect intracellular superoxide (O_2^-) (10). HE is oxidized by O_2^- within the cells to produce ethidium bromide, which fluoresces when it intercalates into DNA. After 30 min of incubation following different treatments, the cells were stained with either 2 μM HE and incubated for 15 min at 37°C in darkness. Fluorescence emission of the resulting dyes was then analysed by flow cytometry.

Assay of DNA fragmentation

For the detection of apoptosis, the percentage of DNA fragmentation was assessed 24 h post treatment using the method of Sellins and Cohen (11) with minor modifications. In brief, approximately 3×10^6 cells were lysed using 200 μl of lysis buffer and centrifuged at 13 000 g for 10 min. Subsequently, DNA from each sample in the supernatant, and pellet was precipitated in 12.5% trichloroacetic acid (TCA) at 4°C overnight and quantified using the diphenylamine reagent after hydrolysis in 5% TCA at 90°C for 20 min. The percentage of fragmented DNA for each sample was calculated as the amount of DNA in the supernatant divided by the total DNA for that sample (supernatant plus pellet).

Assessment of early apoptosis and secondary necrosis

To determine the amount of early apoptosis and secondary necrosis, cells were collected 24 h post treatment and simultaneously stained with propidium iodide- and fluorescein isothiocyanate (FITC)-labelled annexin V, according to the instructions of the annexin V-FITC kit (Immunotech, Marseille, France) and finally, analysed with a flow cytometer (Beckman-Coulter EPICS XLTM) (12).

Measurement of mitochondrial membrane potential (MMP)

To measure changes in MMP, control cells and cells treated with nano-Pt (100 μM), HT, and a combination of both were collected at 6 h post treatment. The cells were then stained with 10 nM tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR) for 15 min at 37°C in phosphate-buffered saline (PBS) containing 1% FBS. The fluorescence of TMRM was analysed using flow cytometry (excitation at 488 nm; emission at 575 nm) (13).

Assay of UVB-induced inflammation and apoptosis in vivo

The mice were placed in individual compartments of specially designed cages, and their ears were exposed once to 350 mJ/cm^2 of UVB radiation. One group was treated with nano-Pt ointment on the earlobes (0.3 g on each earlobe) 24 h and immediately (two times) prior to UVB irradiation, while the other group was treated with carboxyvinyl polymer gel alone. The thickness of each ear was measured using a spring-loaded micrometre (Mitutoyo, Tokyo, Japan) to determine the baseline thickness. Ear swelling was calculated as the difference in ear thickness between the 24 h and the baseline values (14).

TUNEL assay

After 24 h, 350 mJ/cm^2 of UVB-irradiated mouse skin was obtained and analysed by TUNEL assay according to the manufacturer's instructions (R&D Systems). For statistical analysis, apoptotic cells were counted by light microscopy (100×) and expressed as the mean number (±SD) of apoptotic cells per section. Five random fields per section (one section per mouse, five mice per group) were analysed.

Assay of fluoroquinolone photoallergy

Fluoroquinolone photoallergy is inducible in Balb/c mice when they are sensitized by systemic administration of lomefloxacin hydrochloride (LFLX) (2 mg/0.2 ml, i.p.) followed by 12 J/cm^2 UVA irradiation of the shaved abdomen and challenged with systemic quinolone and UVA exposure of the earlobes (20 J/cm^2) (15). One group was treated with nano-Pt ointment on the earlobes (0.3 g on each earlobe) 24 h before and immediately (two times) prior to UVA challenge, while the other group was treated with carboxyvinyl polymer gel alone. Ear thickness was measured using an engineer's micrometre 6–10 days after the challenge and compared with ear thickness before the challenge.

Histological observation

Ears were fixed in 10% PBS-buffered formalin, and paraffin sections were stained with haematoxylin and eosin and examined by light microscopy to assess histological changes.

Northern blot analysis

Total cellular RNA was isolated from epidermis using the Isogen extraction kit according to the manufacturer's instructions. The epidermis was separated from the dermis by incubation in 0.5% dispase in RPMI-1640 at 37°C for 1 h. RNA was quantified spectrophotometrically, and equal amounts of RNA (10 μg) from samples were loaded on a formaldehyde-agarose gel. The gel was stained with ethidium bromide to visualize the RNA strands, and the RNA was transferred onto a nylon membrane. Fragments obtained by restriction enzyme treatments for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, macrophage migration inhibitory factor (MIF), and GAPDH were labelled with [α-^{32}P]dCTP using a DNA random primer labelling kit. Hybridization was performed using the mouse MIF cDNA probe as described previously. The cDNA probes for TNF-α were 5'-TCTCATCAGTTCTATGGCCC-3' (forward) and 5'-GGGAGTAGACAAGGTACAAC-3' (reverse) and for IL-1β were 5'-CCCATACTTTAGGAAGACACGGATT-3' (forward) and 5'-TCATGGGATGATGATGATAACCTGCT-3' (reverse). The membrane was washed twice with 2× SSC (16.7 mM NaCl, 16.7 mM sodium citrate) at 22°C for 5 min, twice with 0.2× SSC containing 0.1% SDS at 65°C for 15 min, and twice with 2× SSC at 22°C for 20 min prior to autoradiography. The density of cytokine bands was normalized by the GAPDH intensities.

Western blot analysis

Cells were collected and washed with cold PBS. They were lysed at a density of 1 × 10^6 cells/50 μl of RIPA buffer (1 M Tris-HCl, 5 M NaCl, 1% Nonidet P-40 (v/v), 1% sodium deoxycholate, 0.05% SDS, 1 mM phenylmethyl sulfonyl fluoride) for 20 min. Following brief sonication, the lysates were centrifuged at 12 000 g for 10 min at 4°C, and the protein content in the supernatant was measured using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Protein lysates were denatured at 96°C for 5 min after mixing with 5 μl SDS-loading buffer, applied on an SDS-polyacrylamide gel for electrophoresis, and transferred to nitrocellulose membranes. Western blot analysis was performed to detect Bcl-X$_L$, Bax, and caspase-3 expression using specific antibodies. Band signals were visualized on X-ray film using chemiluminescence ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK) (16). Band density was quantified by a BIO-ID image analyzer, and the relative amounts of proteins associated with specific antibodies were normalized according to the intensities of β-actin.

Measurement of intracellular Pt content

The cells (1 × 10^7 cells) treated for 24 h with or without nano-Pt (100 μM) were collected by centrifugation, and the pellets were frozen. After thawing, each sample was placed in a glass vessel and reconstituted in nitric acid and hydrogen