Peripheral endomorphins drive mechanical alloknesis under the enzymatic control of CD26/DPPIV

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Background: Mechanical alloknesis (or innocuous mechanical stimuli–evoked itch) often occurs in dry skin–based disorders such as atopic dermatitis and psoriasis. However, the molecular and cellular mechanisms underlying mechanical alloknesis remain unclear. We recently reported the involvement of CD26 in the regulation of psoriatic itch. This molecule exhibits dipeptidyl peptidase IV (DPPIV) enzyme activity and exerts its biologic effects by processing various substances, including neuropeptides.

Objective: The aim of the present study was to investigate the peripheral mechanisms of mechanical alloknesis by using CD26/DPPIV knockout (CD26KO) mice.

Methods: We applied innocuous mechanical stimuli to CD26KO or wild-type mice. The total number of scratching responses was counted as the alloknesis score. Immunohistochemical and behavioral pharmacologic analyses were then performed to examine the physiologic activities of CD26/DPPIV or endomorphins (EMs), endogenous agonists of µ-opioid receptors.

Results: Mechanical alloknesis was more frequent in CD26KO mice than in wild-type mice. The alloknesis score in CD26KO mice was significantly reduced by the intradermal administration of recombinant DPPIV or naloxone methiodide, a peripheral µ-opioid receptor antagonist, but not by that of mutant DPPIV without enzyme activity. EMs (EM-1 and EM-2), selective ligands for µ-opioid receptors, are substrates for DPPIV. Immunohistochemically, EMs were located in keratinocytes, fibroblasts, and peripheral sensory nerves. Behavioral analyses revealed that EMs preferentially provoked mechanical alloknesis over chemical itch. DPPIV-digested forms of EMs did not induce mechanical alloknesis.

Conclusion: The present results suggest that EMs induce mechanical alloknesis at the periphery under the enzymatic control of CD26/DPPIV.

Key words: CD26, dipeptidyl peptidase IV enzyme, endomorphin, mechanical alloknesis, mechanical itch, peripheral µ-opioid receptor, skin

In many skin disorders with chronic itch, including xerosis, atopic dermatitis (AD), and psoriasis, there often occurs a vicious itch-scratch cycle in which scratching behaviors themselves aggravate the itch sensation by exacerbating skin lesions.1-3 These skin conditions often concomitantly display itch hypersensitivity, in which the threshold for itch is lower than in healthy controls and sensitivity to pruritogens is increased.4-5

The phenomenon of itch hypersensitivity, which is caused by normally innocuous mechanical stimuli, is referred to as mechanical alloknesis and has been reported in various mouse models and patients with dry skin–based skin diseases such as AD.6-10 Accumulating evidence indicates that innocuous mechanical stimuli–evoked itch (mechanical alloknesis) is mediated by neural pathways distinct from those of chemical itch, which is caused by chemical mediators, including histamine and proteases, released from cutaneous cells or exogenous sources.9,12

Chemical itch is transmitted to the spinal cord by various chemical mediators through C- prurceptors expressing Mas-related GPR A3 (MrgrpA3), natriuretic peptide B (Nppb), and gastrin-releasing peptide (GRP) at the peripheral level.11,12 At the spinal cord level, a subpopulation of excitatory interneurons (INs) expressing the GRP receptor (GRPR) or natriuretic peptide receptor A (Npra) convey chemical itch signals,13,14 whereas inhibitory INs expressing the transcription factor BHLHB5 negatively regulate these signals.15,16 Regarding the innocuous mechanical stimuli–evoked itch pathway at the spinal cord, a subpopulation of inhibitory neuropeptide Y-expressing (NPY+) INs was found to negatively modulate this sensation without affecting chemical itch.12 Moreover, NPY 1 receptor (Y1)-expressing neurons and...
Abbreviations used

AD: Atopic dermatitis
CD26KO: CD26/DPPIV knockout
CGRP: Calcitonin gene–related peptide
DPPIV: Dipeptidyl peptidase IV
EM: Endomorphin
GRP: Gastrin-releasing peptide
GRPR: Gastrin-releasing peptide receptor
IN: Interneuron
MOR: μ-Opioid receptor
Mrgpr: Mas-related gastrin-releasing peptide
NF200: Neurofilament 200
NK1r: Neurokinin 1 receptor
Nppb: Natriuretic peptide B
NprA: Natriuretic peptide receptor A
NPY: Neuropeptide Y
sDPPIV: Soluble intact CD26/DPPIV
sDPPIV: Soluble mutant CD26/DPPIV
SP: Substance P
TrkB: Tropomyosin-related tyrosine kinase B
Ucn3: Urocortin 3
WT: Wild-type
Y1: NPY Y1 receptor

urocortin 3-expressing (Ucn3+) neurons were identified as excitatory neurons gated by NPY Y1 INs. 21,22 Although evidence obtained from patients with AD suggests involvement of both the central and peripheral nervous systems, 3,4,7,8,23,25 the cellular and molecular mechanisms underlying innocuous mechanical stimuliv–evoked itch at the periphery remain unknown, except for Merkel cells in the touch dome being important for negative regulation. 25

CD26 is a 110-kDa multifunctional glycoprotein that is expressed on various cell types, including T cells, epithelial cells, endothelial cells, fibroblasts, and various tumor cells. CD26 exhibits dipeptidyl peptidase IV activity (DPPIV [EC 3.4.14.5]) in its extracellular domain and is capable of cleaving the N-terminus of peptides with L-proline or L-alanine at the penultimate position. 26,28 This enzyme is involved in the activation and inactivation of a number of cytokines, chemokines, and neuropeptides. 29 We recently reported that DPPIV is associated with psoriatic itch by regulating the cleavage of substance P (SP). 30 However, the involvement of the CD26 molecule or DPPIV in mechanical allknesis remains unclear.

In the present study, we investigated the role of CD26/DPPIV in the regulation of mechanical allknesis at the periphery by using CD26 knockout (CD26KO) mice. We focused on substrates for DPPIV, namely, the endomorphins (EMs) EM-1 and EM-2, which are selective ligands for μ-opioid receptors (MORs), and we identified them as pruritogens that preferentially provoke mechanical alknesis over chemical itch. Herein, we have demonstrated that EM–MOR signaling and its degradation pathway by DPPIV play a pivotal role in the peripheral mechanisms of mechanical allknesis.

METHODS

Animals

C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan) or Oriental BioService (Kyoto, Japan). CD26KO (CD26−/−) mice developed from C57BL/6 mice were kindly gifted from the laboratory of Dr Takeshi Watanabe at Kyusyu University (Fukuoka, Japan). 31 These mice were bred in-house and used at 8 to 16 weeks of age. They were kept under controlled temperature (23°C – 25°C) and light (exposure to light from 8:00 AM to 8:00 PM) conditions. Food and water were freely available to the mice. All experiments on animals were approved by the animal ethics committee at Juntendo University (authorization nos. 280038, 290132, 300024, 310029, 2020063, and 2021079).

Recombinant proteins, antibodies, and reagents

Recombinant EMs (EM-1 and EM-2) were purchased from the Peptide Institute (Osaka, Japan). Truncated peptides of EMs [YP [common N-terminal dipeptides], WF-NH2 [C-terminal side amidated dipeptides of EM-1], and FF-NH2 [C-terminal side amidated dipeptides of EM-2]) were purchased from BEX Co, Ltd (Tokyo, Japan). All peptides were dissolved in 2.5% dimethyl sulfoxide physiologic saline to make a stock solution. Recombinable soluble CD26/DPPIV (sDPPIV) and sDPPIV lacking DPPIV enzyme activity mutated at catalytic site (Ser630 was replaced by Ala) (soluble mutant DPPIV [sDPPIV]) were produced according to a previously described method. 32

The primary antibodies used in the present study were as follows: goat anti–mouse CD26/DPPIV (1:1000; R&D Systems, Minneapolis, Minn), rabbit anti–EM-1 (1:200; Phoenix Pharmaceuticals, Inc, Burlingame, Calif), rabbit anti–EM-2 (1:200; Phoenix Pharmaceuticals, Inc), guinea pig anti–cytoketarin 10 (anti–CK10,1:200; Progen Biotechnik GmbH, Heidelberg, Germany), guinea pig anti–CK14 (1:200; Progen Biotechnik GmbH), and chicken anti–vimentin (1:200, Abcam, Cambridge, United Kingdom). A cyanine 3–conjugated rabbit polyclonal anti–β-III tubulin antibody was purchased from Merck Millipore (Temecula, Calif) and used at a dilution of 1:500 to 1:800. A secondary rabbit antibody conjugated with Alexa 488 was obtained from Thermofisher Scientific (Rockford, Ill). Other secondary antibodies conjugated with Alexa 488 or Alexa 594 were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pa). All secondary antibodies were used at a 1:300 dilution. The peripheral MOR antagonist naloxone methiodide was purchased from Merck (Darmstadt, Germany) and dissolved, stocked, and used in saline.

Mechanical allknesis assay

Mechanical allknesis assays were performed by using a previously described method with some modifications. 9,22,25 The rostral back of each mouse was shaved at least 2 days before the test. On the day of the test, each mouse was placed in a new cage and habituated for at least 1 hour. Mechanical stimuli were delivered with von Frey filaments (Bioseb, Chaville, France) with bending forces ranging between 0.008 and 1.4 g. Unless otherwise noted, von Frey filaments with bending forces of 0.07 and 0.16 g were used for the test, and data obtained with a force of 0.16 g were shown. Each mouse received 3 innocuous mechanical stimuli on the rostral back by using this filament with longer than 5–second intervals (average 20 seconds). Within a 3-minute interval, this sequence was repeated 10 times (30 stimulations in total). Mechanical allknesis scores were calculated as the total number of scratching responses. To test the effects of reagents or peptides on mice, CD26KO or control wild-type (WT) mice received a 50-μL intradermal injection of recombinant sDPPIV, smDPPIV, or naloxone methiodide into the center of the shaved area through use of a 29G Myjector syringe (Thermo, Tokyo, Japan). Mechanical allknesis assays were then immediately conducted. To test EM–evoked mechanical allknesis, WT mice received an intradermal injection of EM (EM-1, EM-2, or their fragments) with or without naloxone methiodide under the same conditions as used for the CD26KO mouse test, and to prevent scratching behavior caused by spontaneous itch affecting mechanical allknesis scores, mechanical allknesis assays were performed 30 minutes after the intradermal injection. Each experiment was performed with 6 or more mice in all groups.

Scratching bout counting assay

The rostral part of the back was shaved at least 2 days before the test. Before behavioral recording, the mice (4 animals per observation) were placed in an acrylic cage (19.5 × 24 × 35 cm) for at least 1 hour for acclimation. The frequency of scratching bouts of the rostral back was analyzed by using the
SCLABA-Real system (NOVETEC, Kobe, Japan) for the indicated time intervals, with observers being kept out of the experimental room. To test EM-evoked scratching bouts, each mouse received an intradermal injection under the same conditions as used for the EM-evoked mechanical alloknesis assay. After the injection, the mice were immediately placed back in the acrylic cage, and behavioral recording using the SCLABA-Real system was started. "One-time" scratching behavior by mice was defined as scratching occurring from the initiation of scratching with the hind limb to cessation thereof. Each experiment was performed with 6 or more mice in all groups.

**Evaluation of skin conditions**

Transdermal water loss and stratum corneum hydration were evaluated in each mouse by using the Tewameter TM300 and Corneometer CM825 (Courage and Khazawa, Cologne, Germany), respectively (at a room temperature of 25.1°C ± 0.6°C and relative humidity of 43.4% ± 1.3%). At the time of both measurements, each measuring device was placed on the surface of murine back skin for approximately 20 to 30 seconds after the achievement of isoflurane anesthesia. Each experiment was performed with 7 WT mice and 8 CD26KO mice.

**Immunohistochemistry**

Frozen blocks were prepared by embedding the unfixed upper back skin of the mice in optimal cutting temperature (OCT) compound (Sakura Finetechical Co, Ltd, Tokyo, Japan). Next, 10-μm-thick cryosections were made by cutting the blocks using a CM1850 cryostat (Leica, Wetzlar, Germany). Skin sections were air-dried and fixed with ice-cold acetone for 10 minutes. After rehydration with PBS solution, the sections were blocked with blocking buffer (PBS solution with 5% normal donkey serum, 2% BSA, and 0.2% Triton X-100) at room temperature for 2 hours and then incubated with each primary antibody at 4°C overnight. After washing with wash buffer (PBS solution with 2% BSA and 0.05% Tween 20), secondary antibodies were added to the sections and incubated at room temperature for 2 hours with shading. As negative control experiments, the primary antibodies were either omitted or replaced with normal IgG. After washing with wash buffer with shading, VECTOSHIELD Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Labs, Burlingame, Calif) was added, the sections were covered with coverslips, and images were taken by using a Keyence BZ-X800 microscope (Osaka, Japan). Even in cases without statistical processing, 2 or more mice were examined and 3 to 9 visual fields per sample were photographed.

**Semiquantification of β-III tubulin–immunoreactive fibers**

There were 8 mice in each group; 3 skin specimens from each mouse were incubated with an anti–β-III tubulin antibody. A BZ-X800 all-in-one fluorescence microscope was used to scan 10-μm-thick sections at a thickness of 1.0 μm in the z-axis of the stained samples, and images were reconstructed in 3 dimensions by using the BZ-X800 viewer (Keyence). The entire fluorescence intensity on the field and its nerve fiber–positive areas (superficial measure) were assessed in 9 fields of view for each mouse by using the BZ-X800 analyzer (Keyence). By dividing the intensity of fluorescence in the whole field by the nerve fiber–positive areas in the field, the fluorescence intensity of each neuronal marker per unit area was calculated. All values are reported as means plus or minus SEMs.

**Statistical analysis**

Data were expressed as mean values plus or minus SEMs and analyzed by the 2-tailed Student t-test for 2 group comparisons or by ANOVA for multiple comparisons followed by the Tukey-Kramer post hoc test. P values of <.05 or less were considered significant. Calculations were performed and graphed by using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, Calif).

**RESULTS**

CD26KO mice display mechanical alloknesis

We initially conducted mechanical alloknesis assays to clarify susceptibility to innocuous mechanical stimuli in CD26KO mice (Fig 1, A). The frequency of hind limb scratching evoked by von Frey filaments (mechanical alloknesis score) with a low bending force (0.04-0.6 g) was significantly higher in CD26KO mice than in WT mice (Fig 1, B). Only slight differences were observed between these mice at each of the other forces tested (Fig 1, B). In contrast, spontaneous scratching with no mechanical stimuli was similar between CD26KO and WT mice (Fig 1, C).

**Characterization of mechanical alloknesis in CD26KO mice**

We examined the skin condition of CD26KO mice, including barrier function and innervation. No significant differences in the degree of transdermal water loss (Fig 2, A) or stratum corneum hydration (Fig 2, B) were observed between the WT and CD26KO mice. To assess innervation, the fluorescence intensity of the neuronal marker β-III tubulin per area in the skin was evaluated immunohistochemically. No significant differences were noted in the fluorescence intensity of β-III tubulin between the WT and CD26KO mice (Fig 2, C and D).

Immunohistochemically, CD26 was expressed in the skin of the WT mice but not in the skin of the CD26KO mice, and it was more strongly expressed in the dermis (Fig 2, E) than in nerve fibers (see Fig E1 in this article’s Online Repository at www.jacionline.org). The expression of CD26 was markedly weaker in the spinal cord, except for the meninges, than in the skin (see Fig E2 in this article’s Online Repository at www.jacionline.org).

We then investigated whether a CD26 deficiency in skin was responsible for the induction of mechanical alloknesis. CD26KO mice were injected intradermally with 0.2 nmol soluble intact CD26/DDPPIV<sup>+</sup> (sDDPPIV), and mechanical alloknesis assays were performed. Mechanical alloknesis scores were significantly lower in sDDPPIV-injected CD26KO mice than in vehicle-injected CD26KO mice. In contrast, the smCD26/DDPPIV soluble form (0.2 nmol) did not affect the mechanical alloknesis scores of CD26KO mice (Fig 2, F). Moreover, mechanical alloknesis in CD26KO mice was almost completely abrogated by the intradermal injection of 30 μg naloxone methiodide (Fig 2, G). These results strongly suggest that DDPPIV enzyme activity in the dermal layer of mouse skin negatively regulates MOR-mediated mechanical alloknesis.

**EMs are distributed in mouse keratinocytes, nerve fibers, and fibroblasts**

EM-1 and EM-2 are selective ligands for MOR and substrates for DDPPIV.<sup>13,34</sup> Because these ligands evoke scratching behavior in mice following their intracisternal injection,<sup>35</sup> we focused on whether these EMs are significant pruritogens that are regulated by DDPPIV enzyme activity in the skin. Although previous studies reported that these MOR ligands are both expressed in the central nervous system, such as in the brain and spinal cord,<sup>36,37</sup> there is currently no information on whether these ligands are expressed in mouse skin. Therefore, we immunohistochemically examined the expression and distribution patterns of the EM-1 and EM-2 proteins in mouse skin (Fig 3). In murine skin, both EMs were strongly detected in the epidermis, both in the squamous cell layer (Fig 3, A and B) and basal layer (Fig 3, C and D) of keratinocytes. In addition to distinct expression in some cutaneous nerve fibers (Fig 3, E and F), these EMs were also detectable in dermal fibroblasts (Fig 3, G and H).
To further identify subpopulations of EM-containing sensory nerve fibers, costaining and triple staining of mouse skin were conducted by using each EM antibody, C- and A-fiber markers (for costaining and triple staining), and peptidergic neuron and Aβ-fiber markers (for triple staining), respectively.38,39 The results of costaining revealed EMs in both C- and A-fibers (see Fig E3, A-D in this article’s Online Repository at www.jacionline.org). Triple staining showed that EM-1 and EM-2 were mainly expressed in peptidergic C- and Aβ-fibers and weakly expressed in Aδ-fibers (see Fig E4, A-F in this article’s Online Repository at www.jacionline.org).

EM preferentially induce mechanical alloknesis over chemical itch

To clarify whether EM-1 or EM-2 induces innocuous mechanical stimulus–evoked itch, we used WT mice and conducted mechanical alloknesis assays following the intradermal injection of EM-1 or EM-2 at various concentrations (Fig 4, A). To exclude the effects of EM-derived spontaneous itch (nonmechanical itch) from mechanical alloknesis scores, we also performed scratching bout counting assays under the same administration conditions (Fig 4, A). The results obtained showed that only high concentrations of each EM (eg, 100 or 200 nmol per mouse) evoked scratching bouts compared with when the control vehicle was used under nonmechanical conditions (Fig 4, A). However, the frequency of scratching bouts under these conditions peaked approximately 0 to 20 minutes after administration and then converged to the level of the vehicle within 30 to 40 minutes (see Fig E5, A and B in this article’s Online Repository at www.jacionline.org). Therefore, we performed mechanical alloknesis assays 30 minutes after the subsidence of EM-evoked itch. We found that intradermal injection of EM at a concentration of 25 to 200 nmol caused mechanical alloknesis in a dose-dependent manner (Fig 4, D and E). Mechanical alloknesis was induced in the presence of mechanical stimuli (Fig 4, D and E) even at low concentrations at which scratching behaviors hardly occurred in the absence of mechanical stimuli (eg, 25 and 50 nmol) (Fig 4, B and C).

EM-induced mechanical alloknesis is mediated by MOR and also regulated by DPPIV

To confirm whether EMs evoke mechanical alloknesis via peripheral MORs, mice were coadministered 100 nmol of each EM (EM-1 or EM-2) with 30 μg of naloxone methiodide, after which mechanical alloknesis scores were assessed (Fig 5, A). Pharmacologically, naloxone methiodide inhibited EM-evoked mechanical alloknesis to a level similar to that observed with the vehicle (Fig 5, B and C). We investigated the effects of DPPIV enzyme activity on this alloknesis. Because both EMs comprise 4 amino acids and have a DPPIV cleavage site at the penultimate position, we synthesized 3 types of dipeptides as estimated forms of EM-1 and EM-2 cleaved by the enzyme DPPIV (ie, YP, WF-NH₂, and FF-NH₂ [Fig 6, A]). Using a mouse MOR-expressing cell line, we confirmed that the cleaved forms of these peptides did not bind to MOR, whereas full-length EMs did (on the basis of the functional changes that occurred when EM bound to MOR [see Figs E6 and E7 in this article’s Online Repository at www.jacionline.org]). We coadministered 100 nmol of these peptides (mixtures of YP and WF-NH₂ or YP and FF-NH₂, as components that make up full-length EM-1 or EM-2, respectively) to WT mice (Fig 6, B). None of the combinations of truncated EM peptides induced mechanical alloknesis compared with its intact full-length EM (100 nmol), respectively (Fig 6, C and D).
FIG 2. Characterization of mechanical allkinesis in CD26KO mice. A and B, Transepidermal water loss (TEWL) (A) and stratum corneum (SC) hydration (B) were evaluated in the skin of WT and CD26KO mice. C, Representative immunofluorescence image of the back skin from a WT or CD26KO mouse using a cyanine 3-labeled β-III tubulin antibody (red indicates a neuronal marker). Arrowheads indicate β-III tubulin-immunoreactive fibers. Each image between the dotted lines indicates the epidermis. Scale bar = 100 μm. D, The fluorescence intensity per unit area of the β-III tubulin antibody was measured for WT or CD26KO mice by using Keyence software (BZ-X800). E, Representative immunofluorescence images of the back skin of WT and CD26KO mice obtained by using the anti-CD26 antibody (green). Each image between the dotted lines indicates the epidermis. Scale bar = 100 μm. F, Effects of sDPPIV and smDPPIV on mechanical allkinesis in CD26KO mice. Mechanical allkinesis in CD26KO mice was inhibited by an intradermal injection of sDPPIV but not smDPPIV. G, Naloxone methiodide (a peripheral MOR antagonist) inhibited mechanical allkinesis in CD26KO mice. **P < .01. n.s., Not significant.
To clarify whether the mechanisms underlying EM-induced itch differ between mechanical and nonmechanical conditions, we also conducted scratching bout counting assays under nonmechanical conditions by using the same administration protocol as that used for the mechanical alloknesis assays. Similar to what we observed in the case of mechanical conditions, neither naloxone methiodide (see Fig E5, C and D) nor the mixture of each truncated EM peptide (see Fig E5, E and F) induced more frequent scratching bouts than did intact full-length EM alone. Furthermore, when WT or CD26KO mice were intradermally administered 100 nmol of EM-1 or EM-2, the frequency of scratching bouts within 1 hour was significantly higher in CD26KO mice than in WT mice (see Fig E5, G and H).

**DISCUSSION**

The present results suggest that a peripheral EM (EM-1 or EM-2)-MOR system mediates mechanical-induced itch (mechanical alloknesis) and that this pathway is modulated by CD26/DPPIV in mouse skin (Fig 7). Our immunohistochemical analyses showed that EM-1 and EM-2 were both expressed in nerve fibers, keratinocytes, and fibroblasts (Fig 3). Therefore, sensory nerve fibers and cutaneous cells, such as keratinocytes and fibroblasts, are considered to be sources of EMs. We also found that an intradermal injection of EM induced mechanical alloknesis in a dose-dependent manner (Fig 4) and that CD26KO mice exhibited mechanical alloknesis, which was rescued by an intradermal injection of the active form of soluble intact CD26/DPPIV (sDPPIV) (Fig 2, F). Mechanical alloknesis was significantly inhibited by the peripheral MOR antagonist naloxone methiodide (Figs 2, G and 5, B and C). Thus, although further analyses of molecular and cellular mechanisms are needed to obtain a more detailed understanding of mechanical alloknesis, cutaneous EM-MOR may play a pivotal role in its induction under the enzymatic control of DPPIV. This concept may also be supported by the present results showing that cleaved EMs (YP + WF-NH₂
and YP + FF-NH₂ did not induce mechanical allokinesis (Fig 6, C and D).

CD26 was expressed in the dermal layer of WT mouse skin but not in the dermal layer of CD26KO mouse skin (Fig 2, E). Mechanical allokinesis in CD26KO mice was almost completely abrogated by the intradermal injection of sDPPIV but not by the enzymatic mutant (Fig 2, F). Thus, although we cannot exclude the possibility of other degradation systems for EM, such as aminopeptidase M⁴⁰ or dipeptidyl peptidase III,⁴¹ in CD26KO mice, these results suggest that DPPIV enzyme activity in the dermal layer is at least partially responsible for the negative regulation of mechanical allokinesis in normal mouse skin.

We also found that intradermal injection of EM caused mechanical allokinesis in WT mice in a dose-dependent manner (Fig 4, D and E). This phenomenon occurred even at low EM concentrations at which scratching behaviors hardly occurred in the absence of mechanical stimuli (Fig 4, B and C). This may explain why CD26KO mice exhibited only mechanical allokinesis (Fig 1, B and C).

A previous study also reported that CD26KO mice displayed high susceptibility to nociceptive stimuli, which was restored by a substance P (SP) receptor (NK-1R) antagonist.⁴² We recently reported that DPPIV exaggerated itch in psoriasis by cleaving SP.⁴³ Mechanical allokinesis in CD26KO mice was markedly inhibited (to a level that was not significantly different from that in WT) by the peripheral MOR inhibitor naloxone methiodide (Fig 2, G). In contrast, when we examined the effects of SP on mechanical allokinesis induced in CD26KO mice by an intradermal injection of the SP receptor antagonist QWF (Boc-Gln-D-Trp(Formyl)-Phe benzyl ester trifluoroacetate salt, which is an inhibitor of the SP receptors NK1R, the enzyme DPPIV MrgrprA1, and MrgrprB2),⁴³ the mechanical allokinesis score was partially
attenuated by the highest dose of QWF (see Fig E8 in this article’s Online Repository at www.jacionline.org). Although previous findings showed that SP is an important itch mediator and also a substrate for DPPIV in a psoriasis model,30 SP and its receptors did not appear to make a significant contribution to the present results. The reason for this may be differences in skin SP levels between previous psoriasis model mice and the CD26KO mice in the present study. In the psoriasis model, SP levels were systemically elevated. SP levels of nontreated CD26KO were significantly lower than those in the WT psoriasis model (unpublished observation, 2017). We speculate that the effects of cutaneous SP were weaker in CD26KO mice than in the WT psoriasis model. Furthermore, SP was shown to play a role in mechanical itch at the spinal level.2 However, because DPPIV levels in the spinal cord were markedly lower than those in the skin (see Fig E2), the effects of DPPIV on SP in the spinal cord were considered to be limited. Therefore, these results suggest that MOR ligands, rather than SP, play a central role in the regulatory mechanisms of DPPIV-associated mechanical alloknesis at the periphery, and also that the SP-NK1R system may be one of the important signaling pathways for mechanical alloknesis at the spinal level.10,64

EM-1 and EM-2 are endogenous opioid peptides that have high affinity and selectivity for MOR.45 Although EM-1 and EM-2 expression patterns differ in different brain regions, both are strongly expressed in the central nervous system, in which MOR is concentrated.36,37 Furthermore, an intracisternal injection of both EMs elicited scratching behavior that was inhibited by an MOR antagonist.35 In the present study, we showed that an intradermal injection of EM elicited mechanical alloknesis, and that this was inhibited by a peripheral MOR antagonist (Fig 5, B and C). Our histologic analyses revealed that EM-1 and EM-2 were expressed in some sensory nerve fibers (Fig 3, E and F), which is consistent with previous findings on EM-2 expression in rat skin.46 We also provided the first evidence for EMs in keratinocytes (Fig 3, A-D) and fibroblasts (Fig 3, G and H). Because general peripheral itch sensations are transmitted and induced through sensory nerve fibers, it appears to be important for EM to act on nerves. EM-1 and/or EM-2 located in nerve fibers may be digested by DPPIV around nerves and involved in the induction of itch and mechanical alloknesis. However, in addition to the nerve fibers themselves potentially secreting these EMs, we were unable to exclude the possibility that EM-1 and/or EM-2 located at nerves are secreted by keratinocytes, which are their most potent expressors in the skin, or fibroblasts localized around nerves in the present study. Similarly, previous findings showing that MORs are located in nerve endings38,47 and keratinocytes48,49 imply that EM mediated the induction of mechanical alloknesis via MORs expressed in sensory nerves; however, we cannot completely exclude the possibility of an indirect pathway via MOR-expressing keratinocytes. This concept appears to be supported by the present results showing that
mechanical alloknesis was strongly affected by DPPIV in the dermal layer, which closely surrounds nerve fibers (Fig 2, E and F and see Fig E1).

In addition to mechanical alloknesis, chemical itch began to appear in normal mice at high concentrations of EMs (>100 nmol) (Fig 4, B and C and see also Fig E5, A and B). Our pharmacologic analyses showed that chemical itch was also mediated via MORs and controlled by DPPIV (see Fig E5, C-H). Currently, the mechanisms by which MOR agonists induce chemical itch remain unclear. MOR generally suppresses nerve activation by stimulating the heterotrimeric Gi/o protein. Whole-cell patch recordings revealed that EM-1 and EM-2 acted on spinal neurons and attenuated excitatory and inhibitory synaptic currents via MORs. Wang et al recently reported that itch induced by the intrathecal administration of MOR agonists was caused by the suppression of vesicular γ-aminobutyric acid transporter (Vgat) inhibitory neurons in the spinal cord. Furthermore, Liu et al demonstrated that one of the isoforms of MOR, MOR-1D, activated nerves by forming a heterodimer with the GRPR. Accumulating evidence recently showed that similar to the involvment of C-fibers in chemical itch, Aβ-fibers play a pivotal role in mechanical itch. Multiple staining of mouse skin with neuronal fiber markers revealed the presence of both EMs in Aβ-fibers (neurofilament 200 [NF200]/TrkB) and peptidergic C-fibers (Peripherin/CGRP), and to a lesser extent, in Aδ-fibers (NF200/CGRP) (see Fig E4); however, their expression profiles in other subpopulations remain unknown. Therefore, we performed a functional silencing experiment on Aβ- and C-fibers and found that the functional silencing of Aβ-fibers suppressed EM-evoked alloknesis, whereas silencing of C-fibers did not (unpublished observation, 2020). Moreover, the functional silencing of Aβ-fibers did not attenuate spontaneous scratching behavior (unpublished observation, 2020). Although further studies are needed, these results indicate that EM-1 and EM-2 evoke chemical itch by suppressing the activation of C-fiber neurons innervating inhibitory spinal neurons or activating those innervating excitatory spinal neurons (eg, subpopulation of peptidergic neurons) through a heterodimer of MOR with other G protein–coupled receptors, such as a MOR1D-GRPR heterodimer in the spinal cord, whereas EM-evoked alloknesis is due to the suppression of Aβ-fiber neurons innervating inhibitory spinal neurons. Because Merkel cells are mechanoreceptors with Aβ-fibers that suppress mechanical itch, they are promising targets for EM-1 and EM-2. Although we did not obtain such data in this study, EM-1 and EM-2 may have also been expressed in these cells, because it has been reported that various neuropeptides and opioid receptors are expressed in Merkel cells.

On the basis of the finding showing that many opioids cause mast cell degranulation, in addition to the direct pathway via nerve fibers, a mast cell–mediated pathway is also conceivable as a chemical itch– and/or mechanical itch–inducing pathway. The results of the toluidine blue staining experiment revealed that the mast cell degranulation ratio was significantly higher in CD26KO mice than in WT mice, whereas the number of mast cells in the skin was very low and similar to that in WT.
mice (see Fig E9, A-D in this article’s Online Repository at www.jacionline.org). Furthermore, our β-hexosaminidase assay on mouse bone marrow–derived mast cells revealed that the highest dose (such as 1 mM) of full-length EM-1 or EM-2, but not the mixture of dipeptides, significantly induced mast cell degranulation (see Fig E10, B and C in this article’s Online Repository at www.jacionline.org). However, in contrast to the results regarding EM-evoked chemical and/or mechanical itch, this degranulation was not inhibited by naloxone (see Fig E10, D). Therefore, the contribution of mast cell degranulation to EM-induced chemical and/or mechanical itch is estimated to be small.

We investigated the expression of CD26, EM-1, and EM-2 in the skin of patients with several diseases and in mouse models for which allodynia has been reported. Although allodynia in AD has been reported both in human and mice models, the expression of CD26 was not reduced in human AD skin. 

In addition, no significant differences were observed in skin EM-1 and/or EM-2 levels between WT and CD26KO mice (see Fig E13 in this article’s Online Repository at www.jacionline.org). These results may highlight the difficulties associated with using immunohistochemistry to detect alterations in EM-1 and EM-2 levels in skin with allodynia induced by EM-1 or EM-2. This may also be due to transient or local increases in EM level in skin around the site that received the mechanical stimulus or the low concentration of EM that induced mechanical itch (Fig 4). In contrast to these data, although the number of samples was small, in cutaneous T-cell lymphoma skin, a disease associated with severe itch, the expression of CD26 decreased whereas that of EM-1 and/or EM-2 increased in cancer cells and fibroblasts (see Fig E11, D). These data indicate that there are definitely cases of itchy diseases in which there is a significant increase in the expression of EM-1 and EM-2 with the decreased activity of DPPIV. In addition, increased expression of EM-2 was also observed in patients with bullous pemphigoid (a well-known skin disease characterized by itching, in which DPPIV inhibitors, first-line drugs for diabetes, are suspected to be among the causes of its development) who are taking a DPPIV inhibitor (bullous pemphigoid + DPPIV inhibitor [see Fig E11, C]).

Although as far as we know there are no reports that DPPIV inhibitors directly induce itch or allodynia, our preliminary results

**FIG 7.** A model for the regulation of EM-induced mechanical allodynia in mice. Schematic diagram of EM-induced mechanical allodynia in WT or CD26KO mice. EMs are produced from keratinocytes, parts of nerve endings, and fibroblasts in the skin, and EM may preferentially evoke mechanical allodynia in a dose-dependent manner over nonmechanical scratching bouts. In WT mice, mechanical allodynia induced by EM was normally controlled because EM in the skin may have been degraded by DPPIV (one of the functions of CD26). Because EM may not be degraded by DPPIV in CD26KO mice, the sensitivity of innocuous mechanical stimuli–evoked itch (i.e., mechanical allodynia) may be increased via the MOR expressed in skin components, such as sensory nerve fibers.
showing scratching bouts under nonmechanical conditions in WT mice after an intradermal injection of sitagliptin provide support for this concept (Komiya et al, unpublished observations, 2016). These results indicate that in at least some clinical cases, DPPIV enzyme regulates spontaneous itch, and possibly alloknesis, through the degradation of EMs.

In conclusion, the present study showed that EM-1 and EM-2 in the skin preferentially induced mechanical alloknesis over chemical itch and that CD26/DPPIV is the regulatory enzyme for mechanical alloknesis at the periphery. These results at least partly support the role of the peripheral nervous system in the alloknesis-inducing mechanism that has been demonstrated in clinical studies on skin diseases, 7,8 and they also suggest that in addition to being effective in studies of the central nervous system, peripheral approaches are effective for diseases associated with itch hypersensitivity.

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Key messages
- EM-MOR signaling provoked mechanical alloknesis at the periphery under the enzymatic control of CD26/DPPIV.
- Topically applied MOR antagonists and CD26/DPPIV are promising treatments for mechanical alloknesis.

REFERENCES


