Possible Roles of Epidermal Opioid Systems in Pruritus of Atopic Dermatitis

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The μ-opioid (β-endorphin/μ-opioid receptor) and κ-opioid (dynorphin A (DynA)/κ-opioid receptor) systems play pivotal roles in the modulation of pruritus in the central nervous system. The μ-opioid system has also been identified in human epidermis, raising the possibility that the system controls the peripheral itch. However, the precise distribution of the κ-opioid system has not yet been clarified in human epidermis. To address this issue, reverse transcription-PCR and immunohistochemical analyses were performed on cultured keratinocytes and normal skins from humans. The analyses revealed that epidermal keratinocytes express κ-opioid receptor and its ligands, DynA (1–17) and DynA (1–8). Moreover, expression for μ- and κ-opioid systems was examined immunohistochemically in skin biopsies from healthy volunteers and patients with atopic dermatitis (AD) before and after psoralen-ultraviolet A (PUVA) therapy. Our expression analyses showed that only the κ-opioid system, not the μ-opioid system, was downregulated in the epidermis of AD patients. The downregulation of the μ-opioid system and the restoration of the κ-opioid system by PUVA therapy were observed in the AD patients, concomitant with a decrease of VAS (visual analogue scale) scores. These results suggest epidermal opioid systems are associated with the modulation of pruritus in AD. This new finding may help us to understand the control mechanism of peripheral itch.

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INTRODUCTION

Endogenous opioid peptides and opiates, like morphine, transmit their pharmacological effects through membrane-bound opioid receptors, which belong to the family of G-protein-coupled receptors (Tseng, 1995). Pharmacological studies and molecular cloning have led to the identification of three major types of opioid receptors, μ-type (MOR), κ-type (KOR), and δ-type (Satoh and Minami, 1995; Zadina *et al.*, 1999; Stefano *et al.*, 2000). They primarily differ in the affinity for various opioid ligands such as MOR is a receptor for β-endorphin (β-end), KOR is a receptor for dynorphins, and δ-type is a receptor for enkephalins (Goldstein and Naidu, 1989; Tseng, 1995).

Opioid-induced pruritus is a well-known side effect in pain treatment with morphine and other MOR agonists in humans (Cousins and Mather, 1984; Ballantyne *et al.*, 1988). In contrast, MOR antagonists (e.g., naloxone and naltrexone) are known to suppress pruritus in patients with chronic cholestasis, chronic renal failure, and atopic dermatitis (AD) (Bergasa *et al.*, 1992; Peer *et al.*, 1996; Metze *et al.*, 1999; Greaves, 2005). These findings suggest that the μ-opioid system has a role in the itching. In addition, it has been recently reported that the activation of KOR antagonizes various MOR-mediated actions but not the analgesic action (Bodnar and Klein, 2006). Nagase *et al.* (1998) have discovered a novel KOR agonist, nalfurafine/TRK-820. When the compound is administered subcutaneously or orally, the antipruritic activity was demonstrated in morphine-, histamine-, and substance P-induced animal scratching models (Togashi *et al.*, 2002; Umeuchi *et al.*, 2003; Wakasa *et al.*, 2004). Therefore, these studies suggest that the μ-opioid system is itch-inducible, whereas the κ-opioid system is itch-suppressive.

Expression analyses of opioid receptors show that normal human epidermal keratinocytes (NHEKs) express a functionally active MOR (Bigliardi *et al.*, 1998; Bigliardi-Qi *et al.*, 2000) as well as primary afferent neurons (Stander *et al.*, 2002). Additionally, the MOR expression alters in epidermis and nerve endings of chronic AD (Bigliardi-Qi *et al.*, 2005). It has been speculated that internalization of the MOR in the epidermis of patients with AD may lead to the availability of free opioid ligands, which then induce chronic pruritogenic signals via altered unmyelinated nerve C-fibers. However, the role of non-neuronal opioid receptors for the modulation of pruritus is as yet unclear. On the other hand, KOR is detected in fibroblast and mononuclear cells of normal human skin (Salemi *et al.*, 2005) but has not yet been identified in the epidermis.

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Abbreviations: AD, atopic dermatitis; β-end, β-endorphin; DynA, dynorphin A; KOR, κ-opioid receptor; MOR, μ-opioid receptor; NHEK, normal human epidermal keratinocyte; PDYN, prodynorphin; POMC, proopiomelanocortin; PUVA, psoralen-ultraviolet A; RT-PCR, reverse transcription-PCR ; VAS, visual analogue scale

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This study examines the distribution of KOR and dynorphin A (DynA) in normal human epidermis. Moreover, this report describes the immunohistological features of μ- and κ-opioid systems in skin biopsies obtained from AD patients before and after psoralen-ultraviolet A (PUVA) therapy.

RESULTS

Expression of MOR and KOR in cultured keratinocytes

To examine whether keratinocytes express transcripts for *MOR* and *KOR* genes, reverse transcription-PCR (RT-PCR) analysis was conducted on cultured HaCaT cells and NHEK (Figure 1a). Transcripts for *MOR* and *KOR* genes were expressed in both HaCaT cells and NHEK. In addition, these genes were expressed in normal human skin.

Immunocytochemical analysis on cultured HaCaT cells and NHEK was carried out to evaluate that MOR and KOR were expressed on the protein level. The specificities of antibodies to these opioid receptors were validated in Figure S1. In MOR staining, the strong immunoreactivity was observed in plasma membrane of HaCaT cells (Figure 1b) and NHEK (Figure 1c). Immunoreactivity for KOR was detected in HaCaT cells (Figure 1d) and NHEK (Figure 1e), and the receptor was primarily localized in plasma membrane of the cells. The immunoreactivities for MOR and KOR were also faintly detected in the cytoplasm of these cultured cells.

Distribution of MOR and KOR in normal human skin

RT-PCR analysis showed that both *MOR* and *KOR* genes were expressed in normal human skin. Therefore, the distribution of MOR and KOR was examined immunohistochemically in normal human skin. MOR expression was observed throughout the epidermis (Figure 2a). Similarly, KOR was expressed in epidermal keratinocytes of the skin (Figure 2b). Some cells expressing MOR or KOR were presented in the dermis. The omission of the primary antibodies but inclusion of secondary antibodies did not produce any specific staining (data not shown). However, the immunoreactivities in the stratum corneum were detected with normal rabbit IgG and normal goat IgG (Figure 2c and d). Therefore, the staining pattern may be nonspecific immunoreaction by the primary antibodies.

Distribution of β-end and DynAs in human epidermis

Proopiomelanocortin (POMC) is the precursor of β-end (Smith and Funder, 1988; Autelitano *et al.*, 1989), while prodynorphin (PDYN) is the precursor of DynA (1–17) and DynA (1–8) (Civelli *et al.*, 1985). In RT-PCR analysis, the transcripts for *POMC* and *PDYN* genes were detected in normal human skin and cultured NHEK (Figure S2).

β-end and DynAs bind with high affinity to MOR and KOR, respectively (Gilmore and Weiner, 1989; Nock *et al.*, 1990; Kinouchi and Pasternak, 1991; Konkoy and Childers, 1993; Bigliardi-Qi *et al.*, 2000). Therefore, the distribution of β-end, DynA (1–17), and DynA (1–8) in normal epidermis was examined by double-immunolabeling studies with antibodies to keratin 14 (K14) and K10. No immunoreactivity for β-end was observed in the basal layer (Figure 3a), whereas it was strongly detected in the suprabasal layer (Figure 3b).

Immunoreactivity for DynA (1–17) was confined to the basal layer (Figure 3c) but not in the suprabasal layer

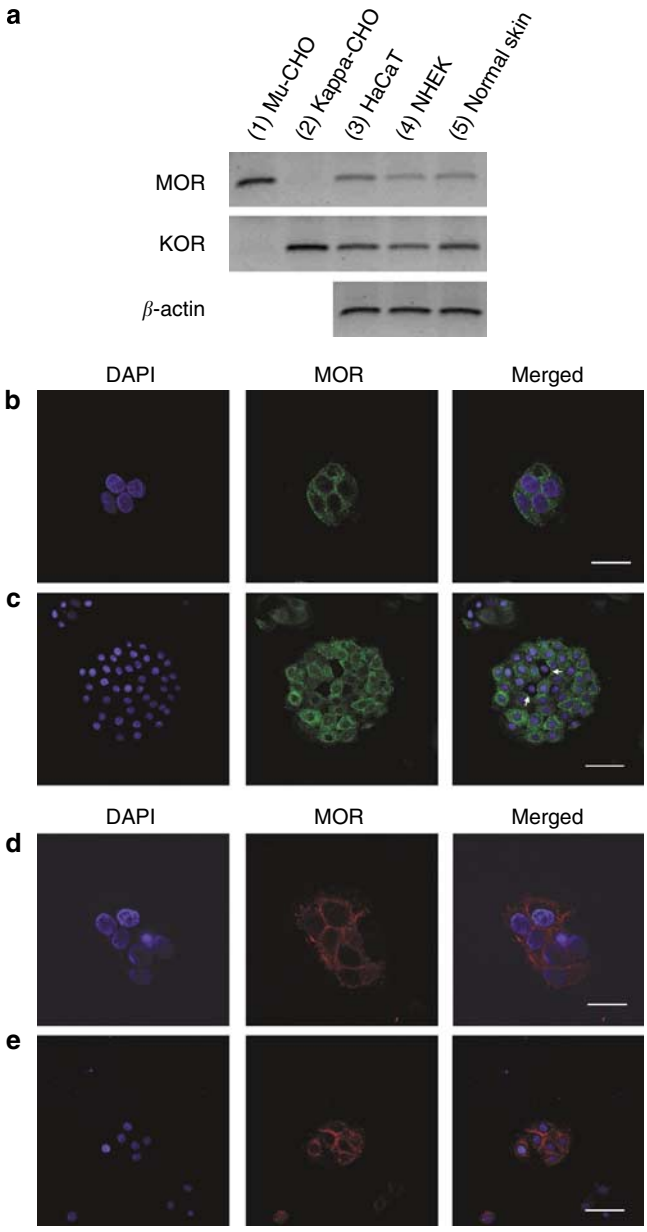

Figure 1. Expression analyses of MOR and KOR in cultured keratinocytes. (**a**) Total RNA was isolated from cultured keratinocytes or normal human skin, and RT-PCR analysis was conducted with primers specific for *MOR* and *KOR* genes. Lanes 1 and 2 were positive controls for expression of these genes (Supplementary Data). Lane 1, Mu-CHO; lane 2, Kappa-CHO; lane 3, HaCaT cells; lane 4, NHEK; lane 5, normal human skin. *β-actin* was used as an internal control for mRNA intensity and quality. Immunostaining for MOR (green) showed that the immunoreactivity was primarily detected in the plasma membrane of (**b**) HaCaT cells and (**c**) NHEK. Some cells were negative for anti-MOR antibody (arrows in panel **c**). Immunostaining for KOR (red) showed that the receptor was localized in the plasma membrane of (**d**) HaCaT cells and (**e**) NHEK. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) (blue). Bars (**b**, **d**) = 30 μm; (**c**, **e**) = 75 μm.

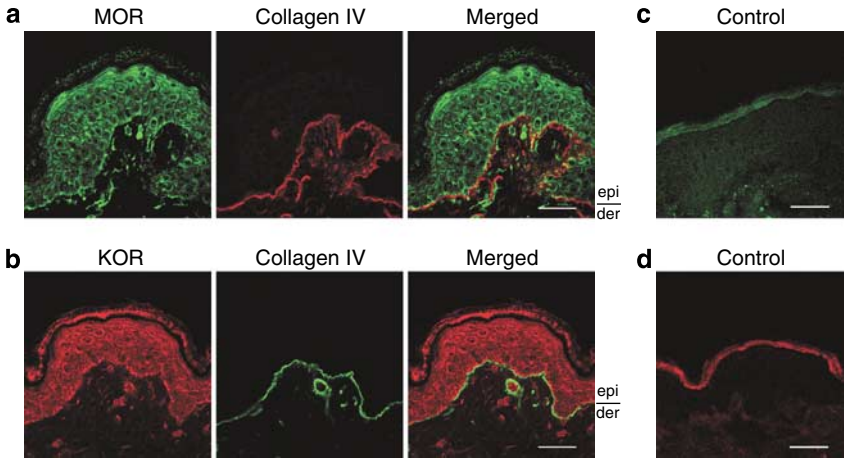

Figure 2. Immunostaining for MOR and KOR in normal human skin. (**a**) Double-labeling for MOR (green) and collagen type IV (red) was performed in normal human skin. Immunoreactivity for MOR was mainly detected in epidermal keratinocytes and some dermal cells. (**b**) Double-labeling for KOR (red) and collagen type IV (green) in normal human skin showed that the receptor was expressed in epidermal keratinocytes and some dermal cells. In negative control experiments using (**c**) normal rabbit IgG and (**d**) normal goat IgG, nonspecific immunoreactivities were detected in the stratum corneum. Bar = 37.5 μm. epi: epidermis; der: dermis.

(Figure 3d). In contrast, immunoreactivity for DynA (1–8) was present throughout the epidermis but was more prominent in the suprabasal layer than the basal layer (Figure 3e and f).

Expression changes of epidermal opioid systems in AD patients before and after PUVA therapy

Expression of MOR and KOR in skins of AD patients before and after PUVA therapy, an established treatment for many skin disorders (Henseler *et al.*, 1981), was examined by immunohistochemistry. Skin biopsies used in this experiment were taken from AD patients before and after PUVA therapy, and visual analogue scale (VAS) scores were significantly decreased in the treated group (Figure 4a). In comparison with healthy volunteers, expression level of MOR was unchanged in the epidermis of AD patients. On the other hand, the MOR expression was decreased in the epidermis after PUVA therapy (Figure 4b and d). Contrary to the MOR expression, the epidermal KOR level was decreased in the AD patients compared with that in healthy volunteers. The epidermal KOR level was unchanged in the AD patients before and after PUVA therapy (Figure 4c and e).

We investigated additionally if expression patterns of β-end and DynAs alter in the epidermis of AD patients before and after PUVA therapy. In comparison with healthy volunteers, the expression level of epidermal β-end was unchanged in the AD patients before and after PUVA therapy (Figure 5a and d). On the other hand, expression levels of DynA (1–17) and DynA (1–8) were decreased in the epidermis of AD patients compared with those in healthy volunteers, and they tended to return to the normal control levels by PUVA therapy (Figure 5b, c, e, and f).

DISCUSSION

In this study, we found that cultured human keratinocytes expressed the transcripts for *KOR* genes as well as *MOR* genes. Additionally, immunofluorescence studies using a specific anti-KOR antibody showed that the receptor was expressed in epidermal keratinocytes *in situ* and *in vitro*. The expression was also observed in some dermal cells except for keratinocytes, and they may be dermal fibroblast and mononuclear cells, as described previously (Salemi *et al.*, 2005).

PDYN, the precursor of DynAs, has a signal peptide at the N terminus (Civelli *et al.*, 1985). Our RT-PCR analysis showed that the transcripts for *PDYN* gene were detected in cultured keratinocytes. These data suggest that PDYN are produced and secreted from epidermal keratinocytes. The secreted PDYN is cleaved by prohormone convertases, and thereby DynA (1–17) is produced (Tseng, 1995). The formation of DynA (1–8) is also specifically due to the cleavage of its precursor DynA (1–17) by prohormone convertase 2 (Day *et al.*, 1998). We revealed that these prohormone convertases are expressed in epidermal keratinocytes (data not shown). In addition, DynA (1–17) and DynA (1–8) were distributed in the epidermis. These results imply that keratinocytes can produce the DnyAs in the skin.

The DynAs bind with high affinity to KOR, a G-protein-coupled receptor (Kinouchi and Pasternak, 1991; Konkoy and Childers, 1993). Several reports have characterized the signal-transduction events initiated by KOR activation. By coupling to the G-protein $G\alpha_{i/o}$, KOR inhibits adenylate cyclase, increases potassium conductance, decreases calcium conductance, and mobilizes intracellular calcium (Tseng, 1995). Recently, KOR has been recognized to activate the extracellular signal-regulated kinase in neural cells (Bohn *et al.*, 2000; Belcheva *et al.*, 2005). This activation has been demonstrated to persist for several hours following agonist treatment, suggesting a role for KOR in long-term growth and gene regulation. Moreover, KOR can activate extracellular signal-regulated kinase via a protein kinase C isoform, secondary messengers, and a heterologous desensitization event different from MOR in neural cells (Bohn *et al.*, 2000; Belcheva *et al.*, 2005). We found that both KOR and MOR are coexpressed in keratinocytes of normal skins (data not shown). Therefore, these data may suggest the

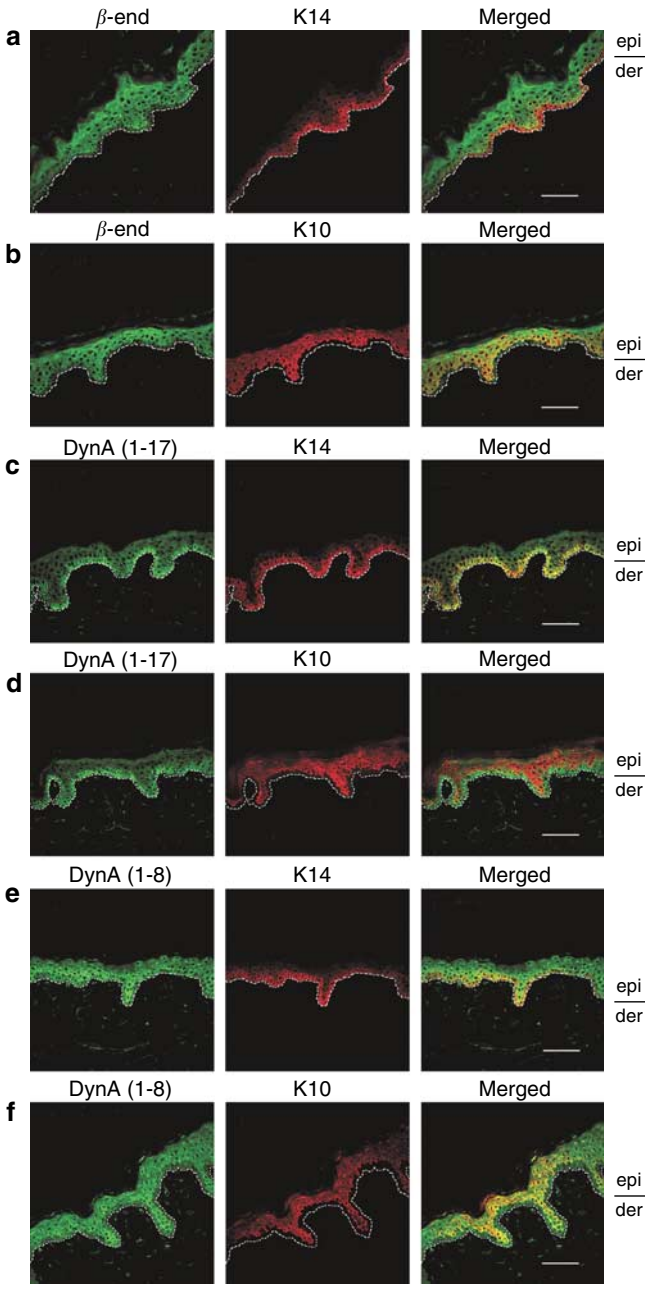

Figure 3. Distribution patterns of β-end and DynAs in normal human skin. Double-labeling with antibodies to opioid peptides (β-end, DynA (1–17), and DynA (1–8)) and keratins (K14 and K10) was carried out in normal human skin. (**a**) The K14-positive cell layer was negative for β-end in the epidermis. (**b**) The pattern of β-end staining was overlapped with the pattern of K10 staining in the epidermis. Immunoreactivity for DynA (1–17) was mainly detected (**c**) in the K14-positive cell layer but not (**d**) in the K10-positive cell layer. (**e**) Immunoreactivity for DynA (1–8) was weakly detected in the K14-positive cell layer, (**f**) whereas the staining pattern corresponded predominantly to that of K10. The white dotted line in each panel indicates the border between the epidermis and the dermis (basement membrane). Bar = 75 μm. epi: epidermis; der: dermis.

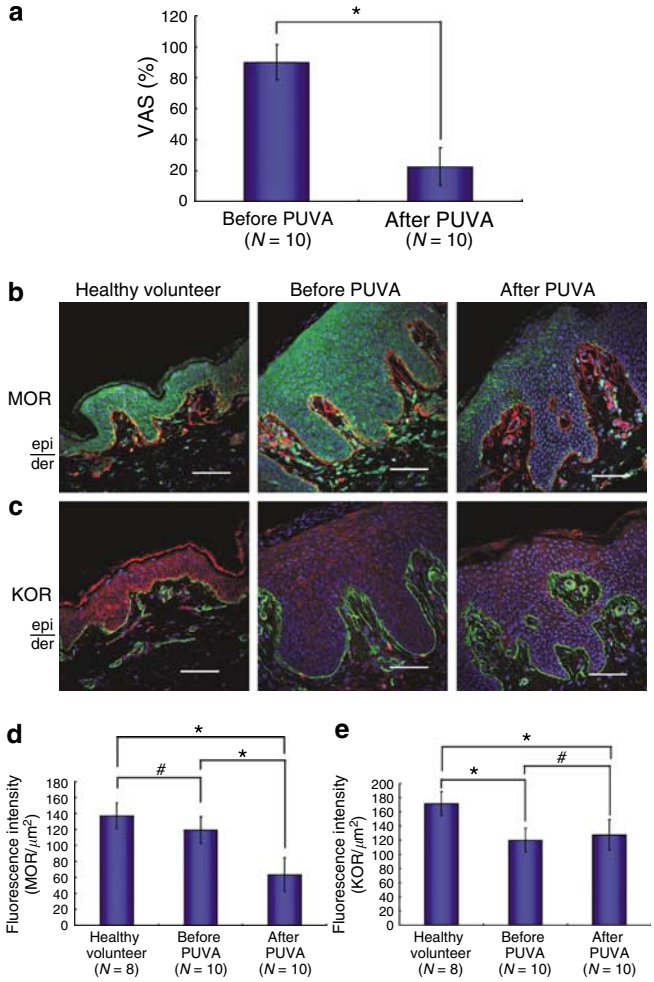

Figure 4. Expression patterns of MOR and KOR in skins of AD patients before and after PUVA therapy. (**a**) VAS scores were significantly decreased in the AD patients after PUVA therapy, compared with before the therapy (*$P<0.01$). (**b**) Double-labeling for MOR (green) and collagen type IV (red) was performed in skins of healthy volunteers and AD patients before and after PUVA therapy. Expression of MOR in the epidermis of AD patients was at the same level as healthy volunteers. After PUVA therapy, decreased expression of the receptor was observed in the epidermis. (**c**) Double-labeling for KOR (red) and collagen type IV (green) was performed in skins of healthy volunteers and AD patients before and after PUVA therapy. In comparison with healthy volunteers, KOR expression was decreased in the epidermis of AD patients. The expression level of KOR was unchanged in the AD patients before and after PUVA therapy. Nuclei in each panel were counterstained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) (blue). Bar = 75 μm. epi: epidermis; der: dermis. Fluorescence intensity per unit area of epidermal (**d**) MOR and (**e**) KOR was calculated in each group, and statistical analysis was performed (*$P<0.01$; $^{\#}P>0.05$).

distinct signaling mechanisms between these opioid receptors in epidermal keratinocytes.

In our experiments, MOR and β-end were mainly distributed in normal epidermis, as described previously (Bigliardi *et al.*, 1998; Kauser *et al.*, 2003; Bigliardi-Qi *et al.*, 2004). The β-end stimulates the keratinocyte migration *in vitro* (Bigliardi *et al.*, 2002). More recently, Bigliardi-Qi *et al.* (2007) reported that epidermal hypertrophy and densities of peripheral nerve fibers were altered in MOR and KOR knockout mice. These observations suggest that μ- and κ-opioid systems function in not only the nervous system but also skin tissue.

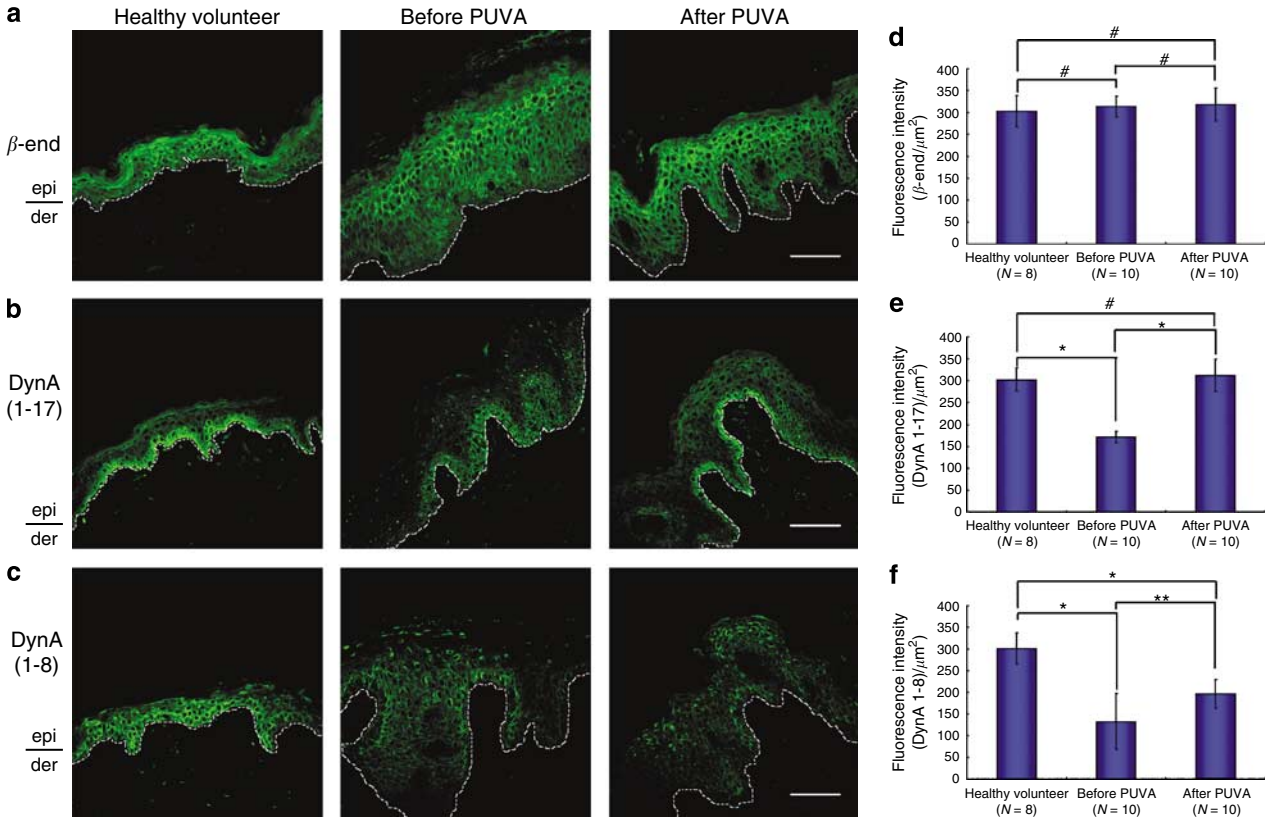

Figure 5. Expression patterns of β-end and DynAs in skins of AD patients before and after PUVA therapy. Skins of healthy volunteers and AD patients before and after PUVA therapy were stained with antibodies to β-end, DynA (1–17), and DynA (1–8). (**a**) The expression level of β-end in the epidermis of AD patients before and after PUVA therapy remained unchanged compared with that of healthy volunteers. (**b**, **c**) In contrast, DynA (1–17) and DynA (1–8) expressions were decreased in the epidermis of AD patients, and the expression levels tended to return to the normal control levels after PUVA therapy. The white dotted line in each panel indicates the border between the epidermis and the dermis (basement membrane). Bar = 75 μm. epi: epidermis; der: dermis. Fluorescence intensity per unit area of epidermal (**d**) β-end, (**e**) DynA (1–17), and (**f**) DynA (1–8) was calculated in each group, and statistical analysis was performed (*$P<0.01$; **$P<0.05$; #$P>0.05$).

The pharmacology of nalfurafine/TRK-820 has been examined extensively. It is characterized as a chemically novel, centrally penetrating, selective agonist at KOR. Non-sedating doses of nalfurafine/TRK-820 inhibited the scratching behavior in mice elicited by histamine, substance P (Togashi *et al.*, 2002), or morphine (Umeuchi *et al.*, 2003). The antipruritic activities were antagonized by pretreatment with nor-BNI, a selective KOR antagonist (Togashi *et al.*, 2002; Umeuchi *et al.*, 2003). It is therefore reasonable to conclude that KOR is involved in mediating the antipruritic effects of nalfurafine/TRK-820.

Chloroquine, a popular antimalarial agent, provokes an often-intolerable side effect-generalized pruritus. Inan and Cowan (2004) have validated the antipruritic effects of nalfurafine/TRK-820 against chloroquine-induced scratching in mice. Interestingly, the scratching behavior was also antagonized by the peripherally restricted KOR agonist, ICI 204448. In addition, our preliminary experiments using NC/Nga mice with AD-like dermatitis induced by 2,4-dinitrofluorobenzene showed that the topical application of nalfurafine/TRK-820 in the skin inhibited the scratching behavior in treated mice, but no sedating effects were observed (unpublished observations). These data raise the possibility of the antipruritic effects of KOR agonists at the peripheral level. On the other hand, it is now generally accepted that the μ-opioid system is centrally itch-inducible (Metze *et al.*, 1999; Greaves, 2005). A recent study has demonstrated that the topical application of MOR antagonists in the skin inhibited the pruritus in AD patients (Bigliardi *et al.*, 2007), indicating that MOR antagonists have antipruritic effects on peripheral itch.

Our expression analyses showed that only the κ-opioid system, not the μ-opioid system, was downregulated in the epidermis of AD patients. Bigliardi-Qi *et al.* (2005) have described the downregulation of epidermal MOR expression in AD patients. This discrepancy may be due to the different immunohistochemical methods or the different antibody reactivities. The authors have also demonstrated the MOR internalization in the epidermis of AD patients by immunostaining with a permeabilization method. In our immunostaining method, the treatment was not conducted when the skin was stained with antibodies, which recognizes the extracellular domains of opioid receptors. Therefore, although we could not exclude the penetration of antibodies into the cells completely, many opioid receptors detected by our immunostaining are localized on the plasma

membrane of epidermal keratinocytes, suggesting that they are functionally active.

Moreover, the downregulation of MOR expression was observed in the epidermis after PUVA therapy. Epidermal KOR expression was unchanged in the AD patients before and after PUVA therapy. However, elevated levels of epidermal DynAs were observed in the treated group. This result raises the possibility that the function of the epidermal κ-opioid system is restored by PUVA therapy. The downregulation of the μ-opioid system and the restoration of the κ-opioid system were also concomitant with the decrease of VAS scores in the treated group. Therefore, although the roles of opioid systems for pruritus are controversial (Bigliardi-Qi *et al.*, 2007, it could be speculated that the μ-opioid system has a role in the induction of itch and the κ-opioid system has a role in the suppression of itch in the peripheral level. This may help to explain why the pruritus is controlled by treatments with opioid agonists or antagonists at the peripheral level. Another possibility is that the opioid receptors on peripheral nerve fibers are directly linked to the modulation of peripheral itch, as well as the central itch. This idea is supported by previous studies that suggest sensory neurons express MOR and/or KOR (Stander *et al.*, 2002; Bigliardi-Qi *et al.*, 2005; Rau *et al.*, 2005).

The expression of the epidermal opioid system controlled by PUVA was observed in this study. In mammalian neurons, gene expression of opioid receptors is regulated by chromatin remodeling and various transcription factors such as activator protein-1 and poly(C) binding protein (Borner *et al.*, 2002; Kim *et al.*, 2004, 2005; Park *et al.*, 2005). The psoralen functions by interfering with activator protein-1 in murine keratinocytes, and thereby inhibits the DNA binding of activator protein-1 (Martey *et al.*, 2005). Others have reported that the chromatin structure in human epithelial cells is affected by PUVA (Ree *et al.*, 1981; Gasparro *et al.*, 1997), and the change of chromatin structure influences the DNA binding activity of transcription factors (Park *et al.*, 2005). These studies may explain the mechanism of PUVA-regulated gene expression in epidermal keratinocytes.

The non-neuronal opioid receptors possibly influence the cytokine pattern of keratinocytes (Slominski *et al.*, 2000) or the production of pruritogenic mediators from keratinocytes (Andoh *et al.*, 2004). In preliminary experiments using cultured keratinocytes, we examined with quantitative RT-PCR analysis whether expression levels of pruritogenic cytokines such as IL-1, IL-2, and tumor necrosis factor-α are changed by opioid peptides, but no significant differences in these expressions between the cells cultured with and without opioid peptides were observed (unpublished observations). Therefore, it will be necessary to carry out comprehensive screenings for other cytokines and pruritogens, which are controlled by these opioid systems.

In conclusion, the precise distribution of the κ-opioid system in human epidermis was demonstrated for the first time in this study. Additionally, our data suggest that epidermal opioid systems are involved in the modulation of pruritus in AD. These findings may expand the knowledge of the effective treatment for pruritus of AD.

MATERIALS AND METHODS

Skin biopsies

Three-millimeter punch biopsies were taken with informed consent from normal abdominal skins of 8 healthy male volunteers (age range, 20–42 years; mean age, 30.6 years) and lesional abdominal skins of 10 male patients (age range, 20–44 years; mean age, 29.1 years) with clinical appearance of chronic AD.

The AD patients were treated with PUVA, and skin biopsies were also obtained from the same subjects. The therapy was performed with oral administration of 0.6 mg/kg of 8-methoxypsoralen, and UVA irradiation was given by a DERMARAY (Toko Electric Corporation, Tokyo, Japan) at 2–6 J/cm^2. The clinical skin conditions were evaluated by clinical and VAS scores. The medical ethical committee of the Juntendo University Urayasu Hospital approved all described studies. This study was conducted according to the Declaration of Helsinki Principles.

Cell culture

HaCaT cells (Boukamp *et al.*, 1988) were maintained as monolayers in DMEM (Sigma, St Louis, MO) containing heat-inactivated fetal bovine serum at 10% supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C, 5% CO_2. NHEK was purchased from Cambrex (Walkersville, MD). They were maintained according to the company's recommendations.

Antibodies

Primary antibodies used in this study were as follows: rabbit anti-MOR (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-KOR (1:100; Santa Cruz Biotechnology), rabbit anti-β-end (1:200; Chemicon, Temecula, CA), rabbit anti-DynA (1–17) (1:200; Bachem, Bubendorf, Switzerland), rabbit anti-DynA (1–8) (1:200; Bachem), mouse anti-collagen type IV (1:20; Progen Biotechnik GmbH, Heidelberg, Germany), mouse anti-K10 (1:10; Progen Biotechnik GmbH), guinea-pig anti-K14 (1:200; Progen Biotechnik GmbH). Secondary antibodies conjugated with Alexa 488 or Alexa 594 used in this study were obtained from Molecular Probes (Eugene, OR).

Immunofluorescence staining

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 10 minutes. They were washed three times with phosphate-buffered saline (PBS, pH 7.4) for 5 minutes and were blocked in PBS with 5% normal donkey serum (Chemicon) and 2% BSA (Sigma) for 1 hour at room temperature. Cells were overlaid with primary antibodies at working dilutions and incubated for 16 hours at 4°C. After washing with PBS, the cells were incubated with secondary antibodies for 1 hour at room temperature. After washing with PBS, they were mounted in vectashield mounting medium with 4′,6′-diamidino-2-phenylindole hydrochloride (Vector Laboratories Ltd, Peterborough, UK). Immunolabeling controls were performed by omitting primary antibodies in the procedure. Immunoreactivity was viewed with a confocal laser-scanning microscope DMIRE2 (Leica, Wetzlar, Germany).

Immunohistochemistry. Human skins were fixed with 4% paraformaldehyde in 0.1 M PB for 4 hours. After washing with PBS, they were immersed successively in PBS solution containing 10, 15, and 20% sucrose. After the skins were embedded in optimal cutting temperature (O.C.T.) compound (Sakura Finetechnical Co., Ltd.,

Tokyo, Japan) and frozen in liquid nitrogen, cryosections (thickness 7 μm) were cut using a CM1850 cryostat (Leica). They were mounted on silane-coated glass slides. After blocking in PBS with 5% normal donkey serum and 2% BSA, the sections were incubated with antibodies against opioid receptors for 16 hours at 4°C. In immunofluorescence staining for opioid peptides, cryosections were fixed with ice-cold acetone for 10 minutes at −20°C. They were rehydrated in PBS, and blocked in PBS with 5% normal donkey serum and 2% BSA. The sections were incubated with antibodies against opioid peptides for 16 hours at 4°C. After washing with PBS, secondary antibodies were added to the sections for 1 hour at room temperature, as described above. As negative control experiments, the primary antibodies were either omitted or replaced with normal IgG.

Total RNA preparation

Cultured cells were suspended in TRIZOL solution (Invitrogen, Carlsbad, CA) and processed to purify total RNA following the manufacturer's protocol. Total RNA from human skins was isolated with Rneasy® Fibrous Tissue Mini Kit (Qiagen KK, Tokyo, Japan), according to the manufacturer's protocol. Universal Human Reference RNA (UHR RNA, Stratagene, La Jolla, CA) was used as a positive control for RT-PCR.

RT-PCR analysis

RT reaction was performed with random primers and ExScript Rtase (all from TaKaRa, Kyoto, Japan). RT-PCR analysis was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster, CA). The primers used in this study are listed in Table S1. They were designed to meet specific criteria and were synthesized by Perfect Real Time support system (TaKaRa). The RT-PCR mixture consisted of 0.2 μM of each primer, 1 × SYBR® Premix EX Taq™ (Perfect Real Time) premix reagent (TaKaRa), and 50 ng cDNA to a final volume of 25 μl. The PCR was performed with 40 cycles (denaturation at 95°C for 5 seconds, primer annealing and elongation at 60°C for 30 seconds). The PCR specificity was confirmed by dissociation curve analysis and gel electrophoresis. *β-actin* was used as an internal control for mRNA intensity and quality. The RT-PCR analyses were repeated at least three times.

Semiquantitative measurements and statistical analyses

To semiquantify fluorescent intensity levels, at least five confocal images were analyzed per skin biopsy in each experiment. Exposure and acquisition setting were fixed and were such that no signal saturation occurred. The total fluorescence intensity in the epidermis of each skin biopsy was measured using Leica Confocal Software (Leica), and fluorescence intensity per unit area was calculated. All values were presented as mean ± SD from three experiments. Statistical analyses were performed by two-tailed Student's *t*-test and one-way analysis of variance with the Bonferroni's multiple comparison test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Figure S1. An assay for specific antibodies to opioid receptors.

Figure S2. RT-PCR analysis for *POMC* and *PDYN* gene expressions.

Table S1. Sequences of primer pairs used for RT-PCR and predicted size of PCR products.

Supplementary Data. Additional results and methods.

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