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ORIGINAL ARTICLE

Protease-Activated Receptor-2 Is Associated with Terminal Differentiation of Epidermis and Eccrine Sweat Glands

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Background: Protease-activated receptor 2 (PAR-2) participates in various biological activities, including the regulation of epidermal barrier homeostasis, inflammation, pain perception, and melanosome transfer in the skin. **Objective:** To evaluate the basic physiological role of PAR-2 in skin. **Methods:** We investigated PAR-2 expression in human epidermis, skin tumors, and cultured epidermal cells using western blot and immunohistochemical analysis. Additionally, we examined the effect of the PAR-2 agonist, SLIGRL-NH2, on cultured keratinocytes. **Results:** Strong PAR-2 immunoreactivity was observed in the granular layer of normal human skin and the acrosyringium of the eccrine sweat glands. In contrast, weak PAR-2 immunoreactivity was seen in the granular layer of callused skin and in the duct and gland cells of the eccrine sweat glands. Interestingly, PAR-2 immunoreactivity was very weak or absent in the tumor cells of squamous cell carcinoma (SCC) and syringoma. PAR-2 was detected in primary keratinocytes and SV-40T-transformed human epidermal keratinocytes (SV-HEKs), an immortalized keratinocyte cell line, but not in SCC12 cells. SV-HEKs that were fully differentiated following calcium treatment displayed higher PAR-2 expression than undifferentiated SV-HEKs. Treatment of cultured SV-HEKs with PAR-2 agonist increased loricrin and filaggrin expression, a terminal differentiation marker. **Conclusion:** Our data suggest that PAR-2 is associated with terminal differentiation of epidermis and eccrine sweat glands. **(Ann Dermatol 27(4) 364～370, 2015)**

-Keywords-
Eccrine sweat glands, Epidermis, Keratinocytes, Terminal differentiation

INTRODUCTION

Protease-activated receptor 2 (PAR-2) is a G-protein-coupled receptor with seven transmembrane domains and is expressed on the membrane of many cell types, including keratinocytes. PAR-2 is a sensor for endogenous and exogenous proteases, playing numerous physiological and pathological roles in the skin. PAR-2 plays an important role in the maintenance of epidermal permeability barrier homeostasis via serine protease activation[1-4], regulation of inflammation[5-7] and pain perception[8,9]. In addition, PAR-2 expression is up-regulated by ultraviolet irradiation and is involved in melanosome transfer from melanocytes to keratinocytes in the epidermis[10-12].

In the skin, PAR-2 is expressed in epidermal keratinocytes, endothelial cells, fibroblasts, sensory neurons, and inflammatory cells[13,14]. PAR-2 is also expressed in the suprabasal layer of the epidermis, most prominently in the granular layer, implying that PAR-2 expression may be associated with the state of epidermal differentiation[3,15].

While PAR-2 signaling negatively affects permeability barrier homeostasis by inhibiting the restoration of the lipid

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barrier (lamellar body secretion), it also acts as a positive regulator of permeability barrier recovery by accelerating cornification[1,2,15]. *In vitro* agonist activation of PAR-2 provokes transient intracellular calcium mobilization in primary keratinocytes, suggesting that PAR-2 could regulate the proliferation and differentiation of keratinocytes[16-19].

The eccrine sweat glands are the major sweat glands of the human body, producing a clear, odorless substance, consisting primarily of water and NaCl. These glands are composed of an intraepidermal spiral duct, the acrosyringium, a dermal duct, and a secretory tubule. PAR-2 has been reported to be involved in the regulation of secretion of sweat in the eccrine sweat glands. PAR-2 agonists increased $[Ca^{2+}]_i$ in the eccrine sweat glands and induced anion secretion in a sweat gland cell line, NCL-SG3[20,21]. PAR-2 is expressed in human sweat gland secretory cells, where it is functionally active and can induce changes associated with secretory activities in the eccrine glands.

PAR-2-deficient mice displayed a significantly increased number of skin tumors in comparison to wild type mice. Stimulation of PAR-2 in HaCaT keratinocytes demonstrated the involvement of extracellular signal-regulated kinase 1/2 and profound epidermal growth receptor transactivation, leading to secretion of the tumor-suppressing factor, and transforming growth factor-β1 (TGF-β1). These data indicate that PAR-2 has a tumor-protective role[22] in the skin.

We studied the precise expression pattern of PAR-2 in human epidermis, eccrine sweat glands, skin tumors, and cultured epidermal cells. Additionally, we investigated the effect of a PAR-2 agonist on cultured keratinocytes to determine the basic role of PAR-2 in the skin.

MATERIALS AND METHODS

Skin specimens

Normal skin was dissected from the dorsum of the hand and palm of three fresh cadavers, donated for medical research and education to the Department of Anatomy, Chungnam National University School of Medicine. Tumor tissues were obtained from the biopsy specimens of three patients with squamous cell carcinoma (SCC) and three patients with syringoma. The tumor specimens and prepuce of the penis were obtained during dermatologic surgery in accordance with the guidelines of the ethics committee of Chungnam National University Hospital (IRB No. 2012-08-023).

Preparation of cells used in the culture experiment

To generate SV-40T-transformed human epidermal keratinocytes (SV-HEKs), primary epidermal keratinocytes were cultured according to a previous method[23]. In SV40 transformation, the retroviral vector pLXIN-SV40T was stably transfected into PT67 cells (Clontech Laboratories, Mountain View, CA, USA), a recombinant retrovirus-packaging cell line. Retrovirus-containing medium was collected, filtered through a 0.22-μm low-protein-binding filter (Millipore, Billerica, MA, USA), and transferred to primary cultured keratinocytes. Following overnight infection, the retrovirus-containing medium was replaced with fresh medium and the cells were incubated for 2 days. Transfectants were selected in medium containing G418 (Sigma-Aldrich, St. Louis, MO, USA) (1 mg/ml) for 4 weeks, as reported previously[23]. SV-HEKs were maintained in keratinocyte serum-free medium supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Life Technologies Corporation, Grand Island, NY, USA). SCC12 cells were purchased from Invitrogen (Carlsbad, CA, USA). The SCC12 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Life Technologies Corporation).

Western blot analysis

Cellular proteins were separated using SDS-PAGE, transferred to nitrocellulose membranes, and incubated with appropriate antibodies overnight at 4°C with gentle agitation. The blots were incubated with peroxidase-conjugated secondary antibodies for 2 h at room temperature, and the signals were visualized using enhanced chemiluminescence (Intron, Daejeon, Korea). We used primary antibodies against PAR-2, phosphorylated-ERK (p-ERK), filaggrin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and loricrin (Covance Research Products, Denver, PA, USA).

Immunohistochemistry

Paraffin-embedded tissue was cut into 4-μm sections and mounted on slides. The tissue sections were deparaffinized and antigen retrieval was performed by heating the slides for 4 min in 10 mmol L^{-1} citrate buffer (pH 6.0) in a pressure cooker. Subsequent procedures were conducted at room temperature. The sections were pretreated with 1% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. The tissue sections were treated with PAR-2 antibody (Santa Cruz Biotechnologies) for 1 h followed by treatment with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA). Immunoreactivity was visualized by incubation for 1 h with an avidin-biotin-peroxidase complex (Vectastain ABC system; Vector Laboratories) in phosphate-buffered saline (PBS) and for 5~10 min in 0.05% 3,3′-diaminobenzidine, 0.01% H_2O_2 in 0.1 mol L^{-1} PBS. The immunostained tissue

slides were counterstained with 0.1% methyl green. The immunolabeled sections were dehydrated though graded ethanol solutions, cleared in xylene, and mounted. Negative control sections were treated as described above, but the primary antibody was omitted.

RESULTS

Strong PAR-2 immunoreactivity was observed in the granular layer of the epidermis in the dorsum of the hand (Fig. 1A). In contrast, very weak PAR-2 immunoreactivity was detected in the granular layer in the palm with thicker, horny layered, callused skin, compared to the dorsum of the hand (Fig. 1B).

Moderate PAR-2 immunoreactivity was detected in the duct of the eccrine sweat glands (Fig. 1C), while weak immunoreactivity was observed in the apical surface of the sweat gland cells projecting into the lumen in the palm (Fig. 1C). Strong PAR-2 immunoreactivity was observed in the acrosyringium, the portion of the sweat gland duct in the epidermis of the palm (Fig. 1D).

Although strong PAR-2 immunoreactivity was found in the granular layer of the epidermis, no PAR-2 immunoreactivity was observed in the epithelia of the syringoma (Fig. 2A). Immunoreactivity for PAR-2 was absent or very weak in SCC tumor cells (Fig. 2B).

PAR-2 was expressed in the primary keratinocytes and SV-HEKs. The bands of the upper region in Fig. 3A indicated glycosylated PAR-2. PAR-2 expression, particularly that of the glycosylated form, was increased in the fully differentiated SV-HEKs obtained via calcium treatment for 2 weeks. In contrast, PAR-2 expression was very low in the SCC12 cells (Fig. 3A, C).

To investigate the effect of PAR-2 activation, we treated

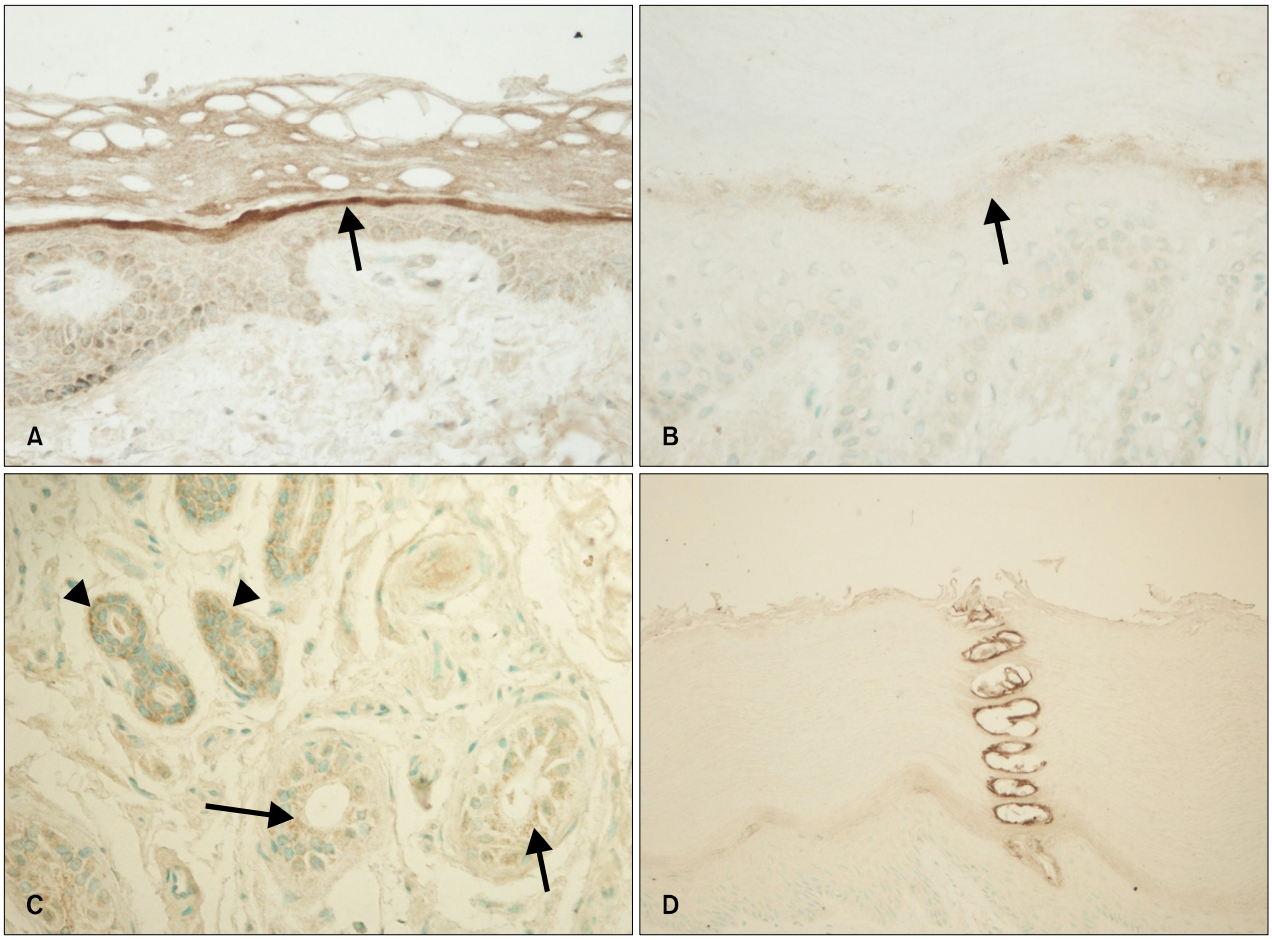

Fig. 1. Immunohistochemistry for protease-activated receptor 2 (PAR-2) in the human epidermis and eccrine sweat glands (A~C: ×400, D: ×200). (A) Strong PAR-2 immunoreactivity in the granular layer (arrow) of the epidermis in the dorsum of the hand. (B) Weak PAR-2 immunoreactivity in the granular layer (arrow) of the epidermis in the palm. (C) Weak PAR-2 immunoreactivity in the apical portion of the gland cells (arrows) and moderate immunoreactivity in the duct (arrowheads) of the eccrine sweat glands. (D) Strong PAR-2 immunoreactivity in the acrosyringium of the eccrine sweat glands.

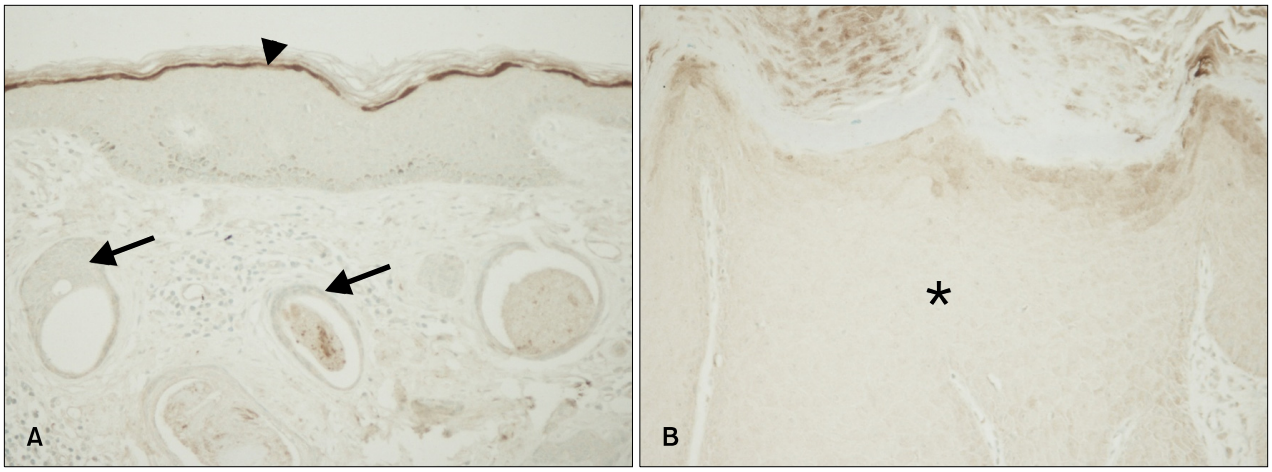

Fig. 2. Immunohistochemistry for protease-activated receptor 2 (PAR-2) in the syringoma and squamous cell carcinoma (SCC) (×200). (A) No PAR-2 immunoreactivity in the tumor cells (arrows) of syringoma. Strong immunoreactivity in the granular layer (arrowhead). (B) Weak or no PAR-2 immunoreactivity in the tumor cells (asterisk) of SCC.

SV-HEKs with SLIGRL-NH2, a PAR-2 agonist. Consequently, the expression of PAR-2, loricrin (a terminal differentiation marker)[24], filaggrin, and p-ERK (an important signaling molecule in keratinocyte differentiation) was increased after SLIGRL-NH2 treatment (Fig. 3B, C).

DISCUSSION

We observed strong PAR-2 expression in the granular layer of the epidermis and the acrosyringium of the eccrine sweat glands. PAR-2 expression was weak or absent in SCC and syringoma tumor cells. Calcium-induced differentiation of keratinocytes to a fully differentiated state resulted in increased PAR-2 expression. PAR-2 agonist treatment of cultured keratinocytes increased loricrin and filaggrin expression, which are known terminal differentiation markers.

Strong immunoreactivity for PAR-2 in the granular layer of the epidermis and acrosyringium of the eccrine sweat glands suggested that PAR-2 is expressed in the fully or terminally differentiated skin regions. We have shown previously that incomplete cornification or differentiation was associated with callused skin, such as on the sole of the foot and palm[25]. In the present study, we observed a decreased expression of PAR-2 in the granular layer of the palm skin, supporting the hypothesis that PAR-2 expression reflects the keratinocyte differentiation status.

We observed that PAR-2 expression was location dependent in the eccrine sweat glands, with higher expression observed in the acrosyringium while moderate to low expression was observed in the gland duct cells. These data further support the idea that PAR-2 expression is strongly associated with the differentiation status of epithelial cells, even in the epithelial appendage organs, including the eccrine sweat glands. Bovell et al.[21] showed that PAR-2 localized to the secretory coil and reabsortive duct. PAR-2 agonists increased $[Ca^{2+}]_i$ in eccrine sweat glands and induced anion secretion in a sweat gland cell line, NCL-SG3. Our immunohistochemical data showed PAR-2 expression in the apical portion of the gland cells projecting into the lumen. Therefore, PAR-2 expression in the gland cells may be associated with secretion in the eccrine sweat glands.

PAR-2 expression was very weak or absent in the tumor cells in the syringoma derived from the acrosyringium and in SCC derived from squamous cells. These data suggest that epithelial skin carcinogenesis is associated with the loss of keratinocyte differentiation, as reported earlier[26]. Rattenholl et al.[22] have demonstrated the role of PAR-2 as an inhibitor of keratinocyte-derived skin tumor development in vivo, possibly by regulating K10 expression, suppressing angiogenesis, and stimulating TGF-β1 secretion. These data suggest that PAR-2 activation may suppress skin tumors by inducing terminal differentiation. However, epigenetic modulation of gene expression also occurs during keratinocyte differentiaion[27]. Further study on the anti-tumor effect of PAR-2, via epigenetic modulation or other signal transduction pathway is required.

The *in vitro* study of PAR-2 expression using cells was consistent with that of the in vivo study using human skin sample. PAR-2 expression in SCC12 cells decreased compared with that in primary keratinocytes. In contrast, PAR-2 expression in fully differentiated keratinocytes was increased compared to undifferentiated keratinocytes. The

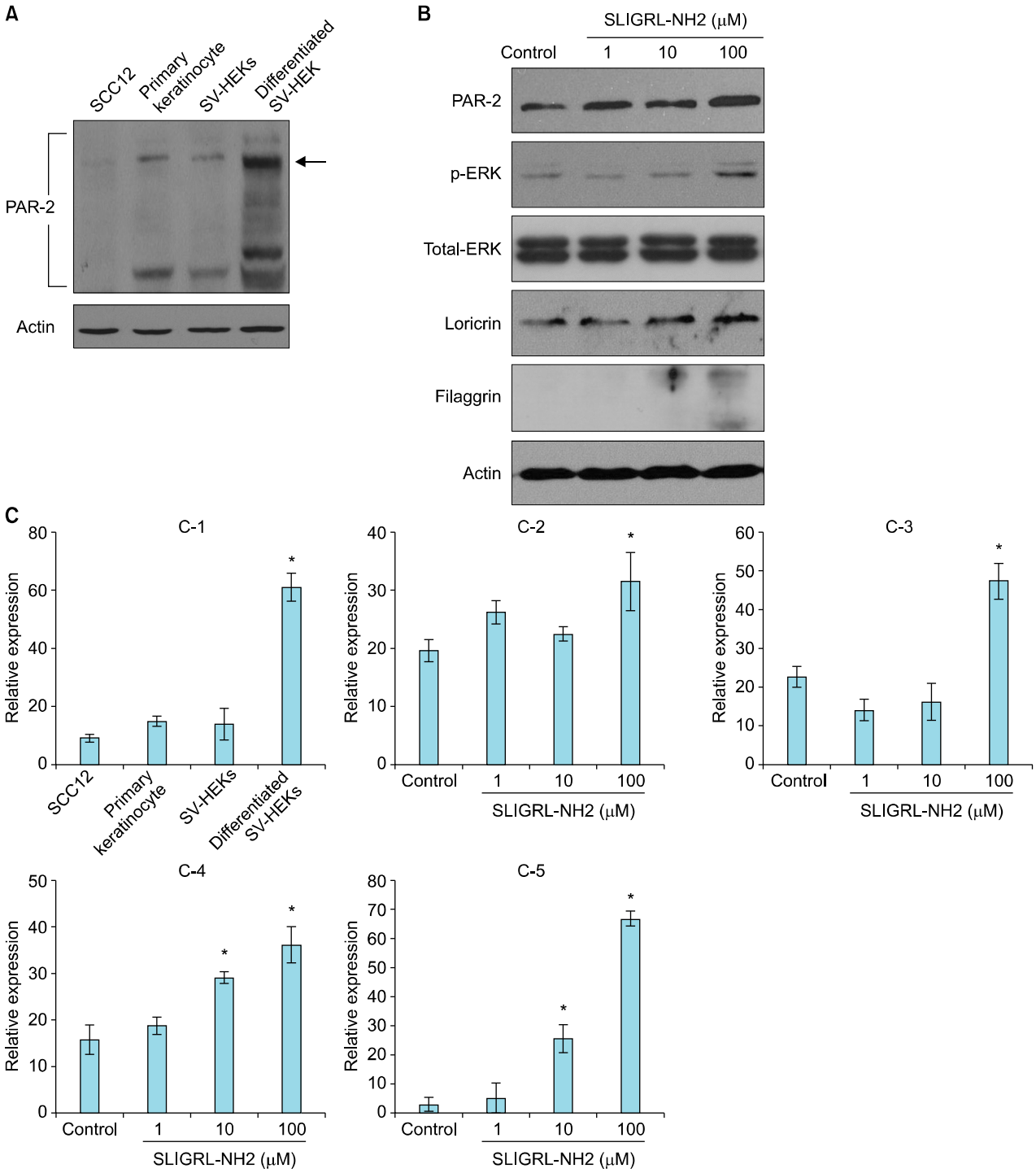

Fig. 3. (A) Protease-activated receptor 2 (PAR-2) expression in skin cells. Western blotting for PAR-2 in primary keratinocytes, squamous cell carcinoma 12 (SCC12) cells, SV-40T-transformed human epidermal keratinocytes (SV-HEKs), and SV-HEKs differentiated by calcium treatment for 2 weeks. (B) Effect of PAR-2 agonist, SLIGRL-NH2, treatment for 48 h on loricrin, filaggrin, and ERK expression in SV-HEKs. The PAR-2 band in the western blot is glycosylated PAR-2 (arrow in Fig. 3A). (C) The graph of relative protein expression in Fig. 3A and 3B. (C-1) PAR-2 in Fig. 3A, (C-2) PAR-2 in Fig. 3B, (C-3) phosphorylated-ERK (p-ERK) in Fig. 3B, (C-4) loricrin, and (C-5) filaggrin in Fig. 3B. Results are expressed as the mean±standard deviation of three independent experiments (n=3). *Significantly different ($p<0.05$) from SCC12, primary keratinocyte, SV-HEK in C-1, and from control (C) in C-2, C-3, C-4, and C-5.

PAR-2 agonist, SLIGRL-NH2, increased loricrin expression, a terminal differentiation marker, in cultured keratinocytes.
Epidermal keratinocytes respond to extracellular influences by activating cytoplasmic signaling transduction pathways that alter gene expression. ERK is one of the important regulators in epidermal differentiation, proliferative and inflammatory skin diseases[28]. p-ERK upregulation may be associated with activation of signal transduction in epidermal keratinocytes.
PAR-2 is involved in formation of the corneocyte[1]. This implied that PAR-2 might be involved in keratinocyte differentiation. However, Derian et al.[19] reported that PAR-2 activation inhibit cell differentiation. The discrepancy between previously reported and our studies may be related to the difference in cell line, culture media, and target molecules. Our data showed that PAR-2 is associated with the regulation of keratinocyte differentiation, but precise functional mechanism remains to be elucidated.
In conclusion, our data suggest that PAR-2 is associated with terminal differentiation of epidermis and eccrine sweat glands.

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