suggested that opsin is expressed in the epidermis (data not shown).

Although the mechanisms involved have not been clarified, our findings indicate that visible light exerts wavelength-dependent effects upon epidermal barrier homeostasis.

MATERIALS AND METHODS

Materials

All experiments were performed on 7- to 10-week-old male hairless mice (HR-1, Hoshino, Japan). All procedures in the measurement of skin barrier function, disruption of the barrier, and application of test sample were carried out under anesthesia. All experiments were approved by the Animal Research Committee of the Shiseido Research Center in accordance with the National Research Council Guide (National Research Council, 1996).

Visible light radiation

To obtain radiation of each wavelength range, we used arrays of 50 light-emitting diodes (Nakamura 1998) (lamp type 5 mm series, Nichia, Tokushima, Japan). The power level was set at 20 W (W = joules of energy per second) with a variable resistor. Radiation was applied for 1 hour from the light-emitting diodes placed at 5 cm from the surface of the skin or skin section, immediately after barrier disruption by tape stripping (Denda *et al.*, 1998). During the radiation, the temperature at the surface of the skin or skin section was kept at 37°C by the use of a heat pad.

Cutaneous barrier function

Permeability barrier function was evaluated by measurement of transepidermal water loss with an electric water analyzer (Meeco, Warrington, PA) as described previously (Denda *et al.*, 2007).

Organ culture study

Immediately after euthanasia of hairless mice by pentobarbital application, flank skin (2×2 cm) was taken and the barrier was disrupted by acetone treatment as previously described (Denda *et al.* 1998). Then the skin sections were incubated with DMEM (Cellgro Mediatech, Herndon, VA) at 37°C under different conditions of light exposure for 1 hour. At the end of the incubation, the transepidermal water loss was evaluated and tissue was taken for electron-microscopic study.

Electron-microscopic study

Full-thickness skin samples for electron microscopy were cut into pieces ($<0.5\,mm^3$) and fixed overnight in modified Karnovsky's fixative. They were then post-fixed in 2% aqueous osmium tetroxide or 0.2% ruthenium tetroxide as described previously (Denda *et al.*, 1998). Parameters were evaluated from photographs of randomly selected sections at a constant magnification, using computer software (NIH Image).

Reverse transcription-PCR assays

We used four mice for the assay. Epidermis of the skin tissue was removed by incubation in a 10 mM EDTA phosphate-buffered saline solution at 37°C for 30 min and total RNA was isolated by ISOGEN (Wako, Osaka, Japan), containing phenol and guanidine thiocyanate, according to the manufacturer's instructions. The resulting pellet was suspended in 10 µl of water, and 2 µl was analyzed by PCR. For opsin5 analysis, primer 1 (AGTCTGTGATCTGGGG ATATCAGG region 228–402, 175 bp) and primer 2 (ACAGAT CTTCAG ATA GCGG TCCAG region 228–377, 150 bp).

Statistics

Results are expressed as the mean ± SD. The statistical significance of differences were determined by analysis of variance with Fisher's protected least significant difference.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Identification of Skn-1n, a Splice Variant Induced by High Calcium Concentration and Specifically Expressed in Normal Human Keratinocytes

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TO THE EDITOR

POU domain transcription factors are a superfamily of homeodomain proteins that regulate cell differentiation and proliferation (Scholer, 1991; Wegner *et al.*, 1993) in a promoter-dependent and cell type-specific manner. Three POU proteins, Oct-1, Oct-6, and *Skn-1a*, are known to be expressed in the epidermis. Among them, the *Skn-1a* gene is primarily, if not exclusively, expressed in the epidermis (Andersen

Abbreviations: RT-PCR, reverse transcription-PCR; NHEK, normal human epidermal keratinocyte

et al., 1993). Previous *in vitro* studies demonstrated that Skn-1a binds to and transactivates the promoter regions of keratinocyte genes involved in epidermal differentiation, such as human keratin 10 (Andersen *et al.*, 1993) and *SPRR-2A* (Fischer *et al.*, 1996). Thus, Skn-1a appears to regulate, both temporally and spatially, the expression of keratinocyte-specific genes during differentiation, playing a role in the development and differentiation of the epidermis. In this study, we provide early evidence for an alternative splice variant Skn-1n that is induced by high calcium concentration and specifically expressed in normal human epidermal keratinocytes.

To determine the 5′ end of Skn-1a mRNA, 5′ rapid amplification of cDNA ends PCR was performed and two bands of PCR product in the 300–400 bp range were noted (Figure 1). The shorter rapid amplification of cDNA ends product was specifically shown to contain the 5′ end of Skn-1a cDNA by direct sequence. Sequencing of the longer rapid amplification of cDNA ends product revealed that there was a 112-bp insertion in the cDNA sequence when compared with the published human Skn-1a cDNA sequence (GenBank accession no. AF162278, Figure 1b). This cDNA clone, designated as Skn-1n, contained two in-frame translational start sites, one at nucleotide position −124 (distal ATG codon) and the other at the position 1 (proximal ATG codon). However, the use of the distal ATG codon in Skn-1n created a termination codon at −42, while if the proximal ATG codon was used, it is predicted to create an open-reading frame of 432 amino acids. Reverse transcription-PCR (RT-PCR) was used to clone the full-length human Skn-1n cDNA. Comparison of open-reading frame of both Skn-1n and Skn-1a revealed that the deduced N-terminus of Skn-1n was shorter than the N-terminus of Skn-1a by four amino-acid residues, while downstream of the N-terminus of Skn-1n the sequences were identical with

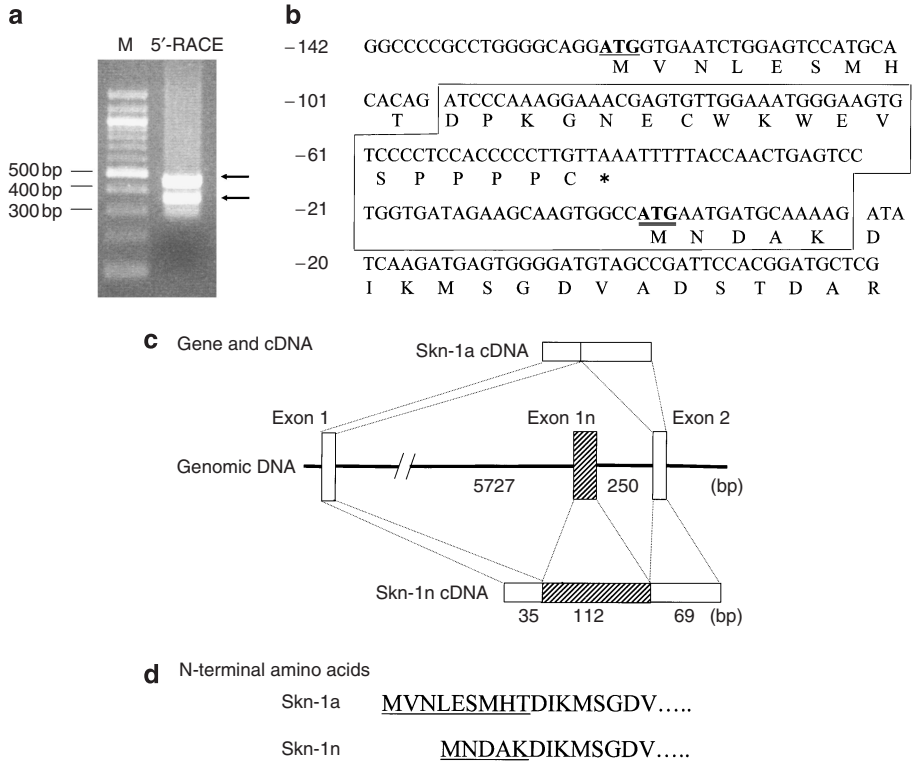

Figure 1. Identification of a splice variant Skn-1n. (**a**) 5′Rapid amplification of cDNA ends of human Skn-1a was performed using 5′-rapid amplification of cDNA ends System for Rapid Amplification of cDNA Ends, Version 2.0 (Life Technologies, Gaithersburg, MD) according to the instruction manual. The Skn-1a cDNA was reverse transcribed from 1 μg total NHEK RNA using a gene-specific primer (5′-GAGACCGCTTTGTTGCTGTG-3′) corresponding to position 503–527 of the Skn-1a mRNA, and subjected to PCR. Using the PCR products, the secondary PCR with the supplied anchor primer and nested gene-specific primer (5′-GGTTTCCGGACATCATGGCAGGTCCTT-3′) was performed. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The major two bands were indicated by arrows. M, molecular weight marker. (**b**) To isolate a full-length Skn-1n clone, we performed RT-PCR using total RNA prepared from NHEK. The resultant cDNA was amplified with primer pairs specific for Skn-1n: sense primer (5′-CGAGTGTTGGAAATG GGAAGT-3′) and antisense primer (5′-AAAATGGGGTGAGATGAAGAT-3′), followed by the secondary PCR with nested sense primer (5′-CTGAGTCCTGG TGATAGAAGC-3′) and nested antisense primer (5′-AGGAAGGTGAAAATGGTAAGC-3′). The PCR products were subcloned and sequenced with a DNA analyzer. Numbers to the left of each row of the nucleotide sequence refer to the nucleotide position. The distal ATG codon (at nucleotide position −124) is underlined and the proximal ATG codon (at nucleotide position +1) is double underlined. Asterisk, a termination codon (TAA) at nucleotide position −42. The alternatively spliced exon 1n is boxed. (**c**) The human *Skn-1a* genomic organization is depicted schematically with the exon shown as boxes and the intron shown as horizontal lines. The exon 1n is shown as a hatched box. (**d**) Comparison of deduced amino-acid sequences of Skn-1a and Skn-1n. The differences in the amino-terminal sequences of the two proteins are highlighted by underlining.

that of Skn-1a (Figure 1b and d). Comparison of the Skn-1a and Skn-1n cDNA sequences with those in the genomic DNA revealed that the 112-bp insert detected in Skn-1n is encoded by sequences embedded within the intron 1 (Figure 1c). The exon within the Skn-1a gene is designated as exon 1n (Figure 1c). Thus, Skn-1n is an alternative splice variant resulting in generation of a protein different of Skn-1a due to utilization of a different translation initiation codon (Figure 1b and c). This variant is distinct from the previously identified Skn-1 isoforms, the human Skn1d1 and Skn1d2 (Cabral et al., 2003) and the mouse Skn-1i (Andersen *et al.*, 1993).

Both Skn-1a and Skn-1n transcripts were specifically expressed in normal human epidermal keratinocytes (NHEK), but not detected in the immortalized human keratinocytes HaCaT, or in the selected cell lines (Figure 2a). The absence of Skn-1a expression in HaCaT is in agreement with the previous data (Hildesheim *et al.*, 1999). This keratinocyte cell line is known to maintain some characteristics of the terminal differentiation of epidermis, such as keratinization and involucrin induction. Interestingly, Skn-1a knockout mice do not exhibit obvious phenotypic changes in the skin (Andersen *et al.*, 1997). These findings suggest that Skn-1 is not prerequisite for the terminal differentiation of epidermal keratinocytes and that there are compensatory POU molecules for coordinated differentiation of the epidermis. Indeed, double knockout of Skn-1a and Oct-6, another POU member expressed in the skin, resulted in abnormal differentiation and wound healing of the epidermis (Andersen *et al.*, 1997). Semiquantitative RT-PCR analysis showed that the relative amount of Skn-1a mRNA was higher than that of Skn-1n, implying that the former is predominately expressed in NHEK (Figure 2b). In NHEK cultured with the high calcium concentrations for 48 hours, the level of Skn-1a expression was approximately 25-fold higher than that in cells with low calcium (Figure 2c, upper panel). This is consistent with the previous *in vivo* and *in vitro* studies (Andersen *et al.*, 1997; Cabral *et al.*, 2003). The Skn-1n mRNA level was also significantly increased in NHEK stimulated with high calcium (Figure 2c, middle panel). Although the precise function of these epidermal-specific POU transcription factors remains to be clarified, genetic alterations of these factors may cause congenital epidermal diseases reflecting disorganized epidermogenesis. In this context, cloning and characterization of the structures of epidermal POU genes should enhance our ability to identify such skin diseases.

This study protocol was approved by the Institutional Review Board.

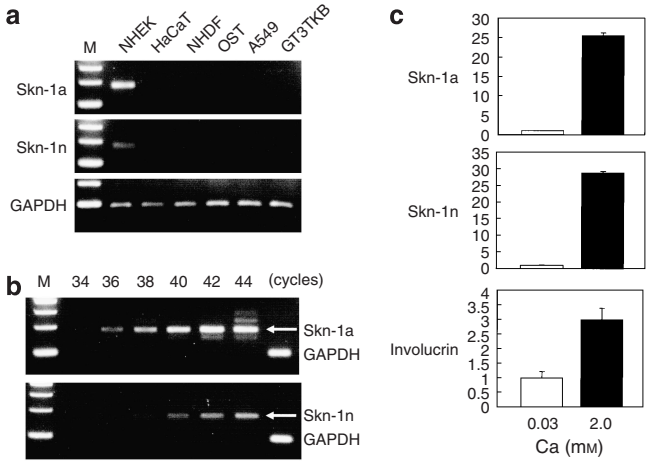

Figure 2. Effect of high calcium concentration on the expression of Skn-1a and -1n transcripts in NHEK. (**a**) RT-PCR analysis of Skn-1a, Skn-1n, and glyceraldehyde-3-phosphate dehydrogenase expression in various human cultured cells. The primers for detection of Skn-1a and Skn-1n transcripts, designed with OLIGO Analysis Software (Molecular Biology Insights, Cascade, CO), were as follows: Skn-1a: forward: 5′-GAGTCCATGCACACAGATAT-3′, reverse: 5′-ATGGCCGATGGGAGAGGGTC-3′ (product size; 175 bp). Skn-1n: forward: 5′-GTCCCCTCCACCCCCTTGTT-3′, reverse: TCTAGGCCATTTCGATCATT (product size; 169 bp). cDNA was prepared by reverse transcription of 0.5 μg total RNA. PCR was performed with 40 cycles. NHEK, normal human epidermal keratinocytes; HaCaT, spontaneously immortalized human keratinocyte cell line; OST, human osteosarcoma cell line; NHDF, normal human dermal fibroblasts; A549, human lung carcinoma cell line; GT3TKB, human gastric adenocarcinoma cell line; M, molecular weight marker. (**b**) Semiquantitative RT-PCR analysis of Skn-1a and Skn-1n expression in NHEK. Reverse transcription and PCR were performed as described above with 34–44 PCR cycles. For glyceraldehyde-3-phosphate dehydrogenase, only the 40-cycle product is shown. (**c**) Skn-1a and Skn-1n mRNA expression in NHEK cultured in low (0.03 mM) and high (2.0 mM) calcium-containing medium was determined by quantitative real-time RT-PCR analysis. Total RNA was extracted from keratinocytes using the RNeasy kit (Qiagen, Hilden, Germany). cDNA was prepared by reverse transcription of 0.5 μg total RNA. The primers used to detect the expression levels of Skn-1a, Skn-1n, and involucrin transcripts, designed with OLIGO Analysis Software, were as follows: Skn-1a: forward: 5′-GAGTCCATGCACAC AGATAT-3′, reverse: 5′-TCTAGGCCATTTCGATCATT-3′. Skn-1n: the same primer pair as (**a**). Involucrin: forward: 5′-CCAGTCAATACCCATCAGGA-3′, reverse: 5′-CCTTTACAGCAGTCATGTGC-3′. Continuous quantitative measurement of the PCR products was achieved by incorporation of SYBR Green fluorescent dye (Opiticon 2, Bio-Rad, Hercules, CA). All real-time PCR were performed in triplicate. The transcript level of each gene was normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase. The results are expressed as mean ± SD. Data are representative of three independent experiments.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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