Activation of Vascular Endothelial Growth Factor Receptor 2 in a Cellular Model of Loricrin Keratoderma

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Loricrin is a major constituent of the epidermal cornified cell envelope. Recently, heterozygous loricrin gene mutations have been identified in two dominantly inherited skin diseases, Vohwinkel syndrome with ichthyosis and progressive symmetric erythrokeratoderma, collectively termed loricrin keratoderma. We generated stable HaCaT cell lines that express wild-type (WT) loricrin and a mutant form found in Vohwinkel syndrome with ichthyosis, using an edcsyne-inducible promoter system. The cells expressing the mutant loricrin grew more rapidly than those expressing WT loricrin after induction for 5 days. Confocal immunofluorescence microscopy revealed that phospho-Akt occurred in the nucleolus where the mutant loricrin was also located. The level of activity of Akt kinase was about nine times higher in cells with the mutant than in those with WT loricrin. ERK1/2, the epidermal growth factor receptor, vascular endothelial growth factor (VEGF) receptor 2 and Stat3 were all phosphorylated in cells with the mutant loricrin. The docking proteins, Gab1 and c-Cbl, were also tyrosine-phosphorylated in these cells. Furthermore, chromatin immunoprecipitation assays showed that Stat3 protein bound to the VEGF promoter in cells with the mutant. Thus, this study suggests that VEGF release and the subsequent activation of VEGF receptor 2 link loricrin gene mutations to rapid cell proliferation in a cellular model of loricrin keratoderma.

The stratum corneum functions as a barrier both to protect against environmental insults and prevent water loss. These functions are mainly attributed to cornified cell envelope (1, 2) formed beneath the plasma membrane in terminally differentiating stratified squamous epithelia. It provides a vital physical barrier in mammals and consists of a 10-nm-thick layer of highly cross-linked insoluble proteins. Its components are several epidermis-specific structural proteins, involucrin, cystatin A, and loricrin (OMIM 152445); several small proline-rich proteins, trichohyalin, profilaggrin, repetin, hornerin, elafin, and profilaggrin-related proteins; S100 family proteins, and some desmosomal proteins and keratins, assembled by the catalytic action of transglutaminases.

Loricrin (Latin for “lorica,” a protective shell or cover) is incorporated into the scaffold formed with involucrin, envoplakin, and periplakin. Loricin consists of many tandem quasi-repeats in the form of aliphatic (glycine/serine/cysteine)n loops, which are interspaced by glutamine-serine-rich domains (3–5). Recently, unique heterozygous, insertional mutations in the loricrin gene have been found to cause some congenital skin abnormalities (6–14). Clinically, the diagnosis for such a condition can be Vohwinkel syndrome with ichthyosis (OMIM 604117), progressive symmetric erythrokeratoderma (OMIM 602036), or congenital ichthyosiform keratoderma born as a collodion baby. The clinical features originally described by Vohwinkel in 1929 include the following: (i) honeycomb-like palmoplantar keratoderma accompanying small “honeycomb” depressions; (ii) starfish-like hyperkeratosis and hyperkeratotic knuckle pads on dorsal parts of hands; and (iii) pseudoainhums of the fingers and/or toes leading to autoamputation. If these signs are associated with hearing impairment, the diagnosis is classic (hearing loss-associated) Vohwinkel syndrome (OMIM 124500: deafness, congenital, with keratopachydermia and constrictions of fingers and toes) caused by a mutation in the connexin 26 gene (GJB2). Vohwinkel syndrome caused by an insertional loricrin mutation is currently termed loricrin keratoderma (LK)2 (OMIM 604117) (15–17). Patients from nine families with four different mutations have been reported so far. The most frequent mutation, 730insG, has been found in families from the United Kingdom, Japan, and Italy.

We have previously shown that the expression of wild-type (WT), but not a mutant, loricin causes programmed cell death in HaCaT keratinocytes (18). We have demonstrated that WT loricin-transfected HaCaT keratinocytes are susceptible to programmed cell death caused by the activation of caspase-14. Although such a function of WT loricin is plausible, it was not possible to quantitate biochemical changes occurring in these...
cells due to the low frequency of transient transfections. Hence, we created stable human keratinocyte cell lines in which WT and mutant loricrin are expressed in an inducible manner using an edysone-inducible promoter system (19).

Here, we demonstrate that overexpression of the mutant loricrin causes the release of vascular endothelial growth factor (VEGF) and transforming growth factor-α (TGF-α) from HaCaT keratinocytes and the subsequent activation of vascular endothelial growth factor receptor 2 (VEGFR 2). We speculate that the activation of VEGFR 2 by an autocrine/paracrine pathway links loricrin gene mutations to rapid cell proliferation in a cellular model of LK.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Genomic DNA containing the entire coding region of WT loricrin and mutant loricrin was subcloned into the pLND/V5-His vector (Invitrogen) (3–5). The most frequent mutation, 730insG, was chosen for this study. The sequence of each of the plasmid constructs was verified by the dideoxynucleotide chain termination method using the 377 DNA sequencing system (Applied Biosystems Inc., Foster City, CA).

Cell Culture, Plasmid Transfection, and Establishment of Inducible Cell Lines—The edysone-inducible mammalian expression system from Invitrogen was used (19). The culture and transfection of HaCaT cells were carried out as described previously with minor modifications (20). Briefly, cells were plated on 35- or 60-mm culture dishes at a density of 4 × 10^5 cells/ml 24 h before the transfection and cultured in Dulbecco’s modified Eagle’s medium (450 mg/dl glucose) supplemented with 10% (v/v) fetal bovine serum. A portion, 2 × 10^5 cells/ml, 0.4 mg/ml Zeocin, and 0.4 mg/ml doxycycline (Dox) for 35-mm dishes were added to each well of a 6-well plate (Falcon) and treated with muristerone A, and cells were counted every day using trypan blue staining.

DNA Synthesis Labeling and Detection—5-Bromo-2′-deoxyuridine (BrdUrd) labeling and detection were conducted with BrdUrd labeling and detection kit (Sigma). Induced cells on glass coverslips were incubated with BrdUrd labeling medium for 30 min at 37 °C and fixed with ethanol. Detection was with anti-BrdUrd mouse monoclonal antibody and fluorescein isothiocyanate-conjugated antibody to mouse IgG. Propidium iodide (Sigma) was used for nuclear detection. Immunofluorescent images were viewed with a confocal laser microscope (Bio-Rad).

Primary Antibodies and Reagents—The primary antibodies and reagents were purchased from the following: anti-V5 antibody from Invitrogen; anti-keratin antibody from DAKO (Glostrup, Denmark); anti-VEGF (sc-507), anti-Stat3 (c-20), and anti-RhoA antibodies (sc-179) from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-phospho-Akt (Ser-473), anti-phospho-ERK Akt (Thr-308), anti-phospho-GSK-3β (Ser-21,9), anti-phospho-EGFR (Tyr-992), anti-phospho-EGFR (Tyr-1068), anti-phospho-VEGFR 2 (Tyr-1175), anti-phospho-ERK1/2, anti-phospho-p38 MAPK, anti-phospho-SAPK/JNK, anti-phospho-Gab1 (Tyr-307), anti-phospho-Gab1 (Tyr-627), anti-phospho-c-Cbl (Tyr-731), anti-phospho-c-Cbl (Tyr-774), anti-phospho-Stat3 (Tyr-705), anti-phospho-Stat3 (Ser-727), anti-Akt, anti-EGFR, anti-VEGFR 2, anti-phospho-c-Cbl, anti-ERK1/2, anti-SAPK/JNK, anti-phospho-pharycine (Tyr(P)) mouse monoclonal, anti-Gab1, anti-P13K p85, anti-SHP-2 and anti-c-cbl antibodies from Cell Signaling Technology (Beverly, MA). An anti-Rho A antibody (Santa Cruz Biotechnology) was used as an irrelevant antibody for the ChIP assay. A peptide with the C-terminal 18 amino acids of mutant loricrin plus cysteine at the N terminus (CPGYHGGEGVGGVFQGHRWA) was synthesized (21). This peptide was covalently coupled to keyhole limpet hemocyanin through the sulfhydryl group of the keyhole limpet hemocyanin and used to immunize a rabbit. Serum with a high enzyme-linked immunosorbent assay titer to the mutant loricrin peptide were obtained from the rabbit. Immunoblotting of HaCaT cells with mutant loricrin showed a band of 39 kDa, but no band was detected in the rabbit. Immunoblotting of HaCaT cells with mutant loricrin showed a band of 39 kDa, but no band was detected in

Immunofluorescence Microscopy—Immunostaining was performed as described previously (24). Induced cells on glass coverslips were fixed with methanol. Cells on glass coverslips were incubated with primary antibodies (anti-V5 (1:300) and anti-phospho-Akt (Ser-473) (1:300)) overnight at 4 °C, and detec-

G418. For induction, mock, WL-1, WL-13, VL-2, and VL-5 cells were treated with 1 mM muristerone A. Cell proliferation and viability were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or trypan blue staining. Aliquots of 1 ml of cell suspensions (15 × 10^6 cells/ml) were added to each well of a 6-well plate (Falcon) and treated with muristerone A, and cells were counted every day using trypan blue staining.

Loricrin Keratoderma and Signal Transduction

Cell Culture and Cell Proliferation—HaCaT cells stably expressing mock, WT loricrin, or mutant loricrin were cultured in Dulbecco’s modified Eagle’s medium (450 mg/dl glucose) with 10% fetal bovine serum, 0.4 mg/ml Zeocin, and 0.4 mg/ml doxycycline (Dox) for 35-mm dishes were added to each well of a 6-well plate (Falcon) and treated with muristerone A, and cells were counted every day using trypan blue staining.
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Growth medium, formaldehyde was added at a final concentration of 1%. The cells were incubated at 37 °C for 10 min, then scraped from the dish into a microcentrifuge tube, and centrifuged. The cell pellet was resuspended in SDS lysis buffer containing protease inhibitor. The cell lysis was then sonicated on wet ice. The cell lysis was preserved by incubation with 60 μl of protein G-agarose for 1 h at 4 °C followed by centrifugation for 1 min at 3,000 × g. The supernatant was incubated with 1 mg/ml anti-RNA polymerase II antibody, anti-RhoA rabbit polyclonal antibody, anti-Stat3 rabbit polyclonal antibody, and normal mouse IgG overnight at 4 °C with rotation. Next, 60 μl of protein G-agarose was added to each microcentrifuge tube, and incubation was further conducted for 1 h at 4 °C with rotation. Protein G-agarose-antibody-protein–chromatin complexes were washed with wash buffer and TE buffer. Protein-DNA complexes were eluted with elution buffer; 8 μl of 5 M NaCl was added to 200 μl of eluate, and incubation was further conducted at 65 °C overnight to reverse the DNA-protein cross-links. RNase A (1 μl) was added to all tubes, and incubation was done for 30 min at 37 °C. Subsequently, 4 μl of 0.5 M EDTA, 8 μl of 1 M Tris-HCl, and 1 μl of proteinase K were added, and incubation was conducted at 45 °C for 2 h. Next, DNA was purified using spin columns in the kit. The purified DNA was subjected to PCR with primers specific for a 130-bp region (−913 to −783) spanning the Stat3-binding site (−848 in the VEGF promoter (24). The sequences of the primers used were as follows: VEGF forward (+), 5′-CTGGCCGAGAGTATTGAG-3′, and VEGF reverse (−), 5′-TTTGAGCCCAAGCAGAGCCCAAAA-3′. PCR was run for 38 cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min), and final products were resolved on a 2.5% agarose gel.

**TGα Measurement**—The amount of TGα in the culture medium was measured with a quantitative sandwich enzyme immunoassay technique using a Quantikine TGα immunoassay kit (R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, 50 μl of culture medium was added to each plate coated with goat polyclonal anti-TGα antibody and incubated for 2 h at room temperature on
a horizontal shaker. After four washes of the plates with the washing buffer supplied, horseradish peroxidase-conjugated goat polyclonal anti-TGF-α antibody was added, and plates were incubated for 2 h at room temperature to allow formation of the polyclonal antibody-TGF-α-polyclonal antibody-horseradish peroxidase complex. After four washes with the washing buffer, the plates were incubated with a chromogenic solution containing tetramethylbenzidine and H₂O₂ for 30 min. The reaction was terminated by the addition of 0.36 N H₂SO₄, and absorbance at A₄₅₀ was measured with a spectrophotometer (ELNX96, TFB, Tokyo, Japan).

**VEGF Measurement**—The amount of VEGF in the culture medium was measured with a quantitative sandwich enzyme immunoassay technique using a Quantikine VEGF immunoassay kit (R & D Systems) according to the manufacturer’s instructions. Briefly, 50 μl of culture medium was added to each plate coated with goat polyclonal anti-VEGF antibody and incubated for 2 h at room temperature on a horizontal shaker. After four washes with the washing buffer supplied, horseradish peroxidase-conjugated goat polyclonal anti-VEGF antibody was added, and plates were incubated for 2 h at room temperature to allow formation of the polyclonal antibody-VEGF-polyclonal antibody-horseradish peroxidase complex. After four washes with the washing buffer, the plates were incubated with a chromogenic solution containing tetramethylbenzidine and H₂O₂ for 30 min. The reaction was terminated by the addition of 0.36 N H₂SO₄, and absorbance at A₄₅₀ was measured with a spectrophotometer (ELNX96, TFB, Tokyo, Japan).

**Statistics**—All experiments were performed at least three times. All values are presented as means ± S.E. One-way ANOVA with Tukey-Kramer multiple comparisons test was used, and the results are presented in Fig. 1D. One-way ANOVA with Dunnett’s multiple comparisons test was used, and the results are shown in Fig. 1E and Fig. 10, A and B. A p value of less than 0.05 was considered statistically significant.

**RESULTS**

**Generation of Keratinocyte Cell Lines That Express WT and Mutant Loricrin**—The clones with the highest levels of expression and tightest regulation were selected and named WL-1, WL-13, VL-2, and VL-5. WL-1 and WL-13 cell lines expressed WT loricrin. VL-2 and VL-5 cell lines expressed mutant loricrin. To examine whether WT and mutant loricrin were indeed expressed in VL-2, VL-5, and VL-5 cells, we performed immunoblot analyses using the anti-V5 antibody and anti-mutant loricrin antibody. Immunoblotting using the anti-V5 antibody showed that the WT (35 kDa) and mutant (42 kDa) were expressed. The analysis using the anti-mutant loricrin antibody showed that the mutant was specifically expressed in VL-2 and VL-5 cells (Fig. 1A). The amounts of WT loricrin and mutant loricrin were dependent on the dose of muristerone A (Fig. 1B). To know proliferative capacity, we treated WL-1 cells, VL-5 cells, and a control (mock-transfected) cell line with 1 mM muristerone A and counted cell numbers daily based on trypan blue staining. The proliferation of VL-5 cells was significantly increased (Fig. 1E).

**Expression of Mutant Loricrin Up-regulates Phosphorylation of Epidermal Growth Factor Receptor and Vascular Endothelial Growth Factor Receptor**—To determine whether the EGFR and VEGFR 2 are phosphorylated in WL-1 and VL-5 cells, we conducted immunoblot analyses with the anti-EGFR (Tyr-992) antibody, anti-phospho-EGFR (Tyr-1068) antibody, and anti-phospho-VEGFR 2 (Tyr-1175) antibody. EGFR and VEGFR 2 were both phosphorylated in the control (mock-transfected) cell line, WL-1 cells, WL-13 cells, VL-2 cells, and VL-5 cells 2 h after the addition of muristerone A. In contrast, EGFR and VEGFR 2 continued to be phosphorylated only in VL-2 and VL-5 cells 20 h after the addition of muristerone A (Fig. 2). We were unable to detect phosphorylation of EGFR or VEGFR 2 on noninduced WL-1, VL-13, VL-2, VL-5, or HaCaT cells (data not shown).

**Phosphorylation of Akt and Effect of Δp85 Transfection on Akt Activity in VL-5 Cells**—To determine whether Akt is phosphorylated in mock-transfected, WL-1, and VL-5 cells, we conducted an immunoblot analysis with the anti-phospho-Akt (Ser-473) antibody. Akt kinase was phosphorylated only in VL-5 cells 20 h after the addition of muristerone A (Fig. 3A). To identify signal transduction pathways relevant to EGFR- or VEGFR 2-dependent VL-5 cell proliferation, we first determined the effect of a transient transfection with SRαΔp85 (Fig. 3B). Phosphoinositide 3-kinase (PI3K) is a heterodimer consisting of regulatory 85 kDa (p85) and catalytic 110 kDa (p110) subunits. Δp85 protein lacks a binding site for p110. Overexpression of Δp85 acts to suppress endogenous PI3K activity. When SRαΔp85 was transfected, Akt kinase activity was reduced, suggesting that PI3K is required for phosphorylation of Akt in VL-5 cells (Fig. 3B).

**Effect of PI3K Inhibitors on Akt Activity and Distribution of Phospho-Akt in VL-5 Cells**—We also examined the effect of PI3K inhibitors on Akt activity. Akt activity is represented by the intensity of bands on the phospho-GSK-3α/β (Ser-21/9) immunoblot. Wortmannin is a specific inhibitor of PI3K. When wortmannin was used at 0.2 μM, phospho-Akt (Thr-308) was not detected. When wortmannin was used at 1 μM, neither phospho-Akt (Ser-473) nor phospho-Akt (Thr-308) was detected. When LY294002 was used at a concentration of 10 μM, both phospho-Akt (Ser-473) and phospho-Akt (Thr-308) were detected. When LY294002 was used at 50 μM, neither phospho-Akt (Ser-473) nor phospho-Akt (Thr-308) was detected (Fig. 4A). The density of immunostaining was measured in five experiments and quantified with NIH Image software. The level of Akt kinase activity was nine times higher in VL-5 cells than the control cell lines (mock-transfected and WL-1 cells). The activity of Akt kinase was suppressed by treatment with wortmannin or LY294002 (Fig. 4B). We double-stained WL-1 and VL-5 cells with anti-phospho-Akt (Ser-473) and anti-V5 antibodies. Phospho-Akt (Ser-473) distributed in the cytoplasm...
and nucleus in WL-1 and VL-5 cells. A substantial portion of phosho-Akt (Ser-473) colocalized with aggregates of the V5-tagged mutant loricrin (arrows) (Fig. 4C, VL-5). These results, when taken together, indicate that the expression of mutant, but not WT, loricrin in keratinocytes leads to PI3K-de-
loricrin expression. The level of phosphorylated ERK was elevated at 20 h in VL-2 and VL-5 cells (Fig. 6A). The levels of phosphorylated JNK and p38 MAPK were almost unchanged during the experimental period in mock-transfected, WL-1, WL-13, VL-2, and VL-5 cells (Fig. 6B, 8 and C).

Stat3 Up-regulates VEGF Expression through the VEGF Gene Promoter—Because the Jak/Stat3 pathway is critical to the leptin-induced activation of VEGF and VEGFR 2 (28, 29), we investigated whether Stat3 protein could directly bind to the Stat3-binding site in the VEGF promoter by conducting ChIP assays (30). This technique allows for the detection of specific genomic DNA sequences that are associated with a particular transcription factor in intact cells. As shown in Fig. 7A, an association of Stat3 with the VEGF promoter in VL-5 cells was detected. Immunoprecipitation with a Stat3 antibody followed by PCR using oligonucleotide primers that amplify a 130-bp region spanning the Stat3-binding site at −848 within the VEGF promoter yielded a 130-bp band, demonstrating the specificity of the interaction between Stat3 and the VEGF promoter. In contrast, PCR amplification of the murine albumin promoter (which does not possess Stat3 sites) from all samples revealed no enrichment for nonspecific DNA sequences in the Stat3 immunoprecipitation reaction. We next investigated whether Stat3 was phosphorylated or not. The immunoblot experiment with anti-phospho-Stat3 (Tyr-705) and anti-phospho-Stat3 (Ser-727) showed that Stat3 is phosphorylated at tyrosine 705 and serine 727 in VL-5 cells (Fig. 7B).

Release of TGF-α from Mock-transfected, WL-1, WL-13, VL-2, and VL-5 Cells—Immunoblotting using the anti-phospho-EGFR (Tyr-992) and anti-phospho-EGFR (Tyr-1068) antibodies showed that EGFR was phosphorylated. Known EGFR ligands are TGF-α, amphiregulin, heparin-binding EGF, and epiregulin. We thus measured the amount of TGF-α in the culture medium with a solid phase enzyme-amplified sensitivity immunoassay (Fig. 8A). The condition of TGF-α was significantly higher in VL-2 cells (23.5 ± 1.8 pg/ml) and VL-5 cells (24.2 ± 1.7 pg/ml) than in mock-transfected HaCaT cells (7.4 ± 0.9 pg/ml), WL-1 cells (4.8 ± 1.3 pg/ml), or WL-13 cells (6.8 ± 1.1 pg/ml). We then assessed the expression of TGF-α protein in keratinocytes by conducting immunoblot analysis and found levels to be significantly higher in VL-2 and VL-5 cells than in mock-transfected, WL-1, or WL-13 cells (Fig. 8B). These results suggest that the expression of mutant loricrin stimulates the synthesis and release of TGF-α in cultured VL-2 and VL-5 cells.

Stimulation of VEGF 2 by an Autocrine/Paracrine Mechanism—The binding of VEGF to VEGFR 2 leads to the tyrosine phosphorylation of the Grb2-bound adapter Gab1. Because VEGFR 2 is expressed in keratinocytes (31), the possible involvement of the VEGF pathway in mock-transfected, WL-1, WL-13, VL-2, and VL-5 cells was examined. We measured the amount of VEGF in the medium with a solid phase enzyme amplified sensitivity immunoassay. The concentration of VEGF was significantly higher in VL-2 cells (1750.0 ± 25.2 pg/ml) and VL-5 cells (1552.2 ± 24.1 pg/ml) than in VL-1 cells (729.4 ± 17.8 pg/ml), WL-13 cells (576.1 ± 23.2 pg/ml), or mock-transfected cells (773.1 ± 22.9 pg/ml) (Fig. 9A). We then assessed the expression of VEGF protein in keratinocytes by conducting an immunoblot analysis and found levels to be significantly higher in VL-2 cells than in mock-transfected or WL-1 cells (Fig. 9B). These results suggest that the expression of mutant loricrin stimulates the synthesis and release of VEGF in cultured VL-2 and VL-5 cells.

Effect of VEGF Inhibitors CBO-P11 and JE-11 plus PD 153035 on VL-5 Cells—Our results suggest that the autocrine and/or paracrine secretion of VEGF and TGF-α stim-
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A

phospho-Akt (Ser473)
phospho-Akt (Thr308)
Akt
phospho-GSK-3α/β (Ser21/9)

Mock   WL-1
0  0  0  0  0  0  Wortmannin (μM)  LY294002 (μM)
0  0  0  10  50

VL-5

B

Akt Kinase Activity

Mock   WL-1
0  0  0  0  0  Wortmannin (μM)  LY294002 (μM)
0  0  0  10  50

VL-5

C

WL-1

VL-5

pAkt  WT Lor

DAPI  Merge

pAkt  Mut Lor

DAPI  Merge

pAkt  WT Lor

DAPI  Merge

pAkt  Mut Lor

DAPI  Merge
Akt (Ser-473) antibody (cells to proliferate, we used two PI3K inhibitors at various concentrations and conducted an immunoblot analysis. Wortmannin and LY294002 were added immediately after the treatment with 1 μM muristerone A for 20 h. The cells were lysed and processed for Gab1 immunoprecipitation (IP) followed by anti-phospho-Gab1 (PY) at 20 h. The cells were lysed and processed for c-Cbl immunoprecipitation (IP) followed by anti-phospho-Gab1 (PY), anti-Grb2, and anti-Shc immunoblotting (left panel) (n = 4). The lysates were also subjected to anti-phospho-Akt (Ser-473) and anti-phospho-c-Cbl (Tyr-774) immunoblotting (right panel) (n = 4).

FIGURE 4. Effect of PI3K inhibitors on Akt activity and distribution of phospho-Akt in VL-5 cells. A, to determine whether PI3K is really required for VL-5 cells to proliferate, we used two PI3K inhibitors at various concentrations and conducted an immunoblot analysis. Wortmannin and LY294002 were added immediately after the treatment with 1 μM muristerone A. We also conducted an Akt kinase activity assay. Akt activity is represented as the intensity of bands phosphorylated at Ser-473. B, density of immunostaining was measured in five experiments and quantified with NIH image software. The level of Akt activity was nine times higher in VL-5 cells than the control cells (mock and WL-1 cells). C, representative double stainings with anti-phospho-Akt (Ser-473) antibody (green) and anti-V5 antibody (red) of VL-5 cells. Scale bars, 80 μm. Mut, mutant; Lor, loricrin.

DISCUSSION
Loricrin is a major component of the cornified cell envelope of terminally differentiated epidermal keratinocytes. Mutations in the loricrin gene have been identified in Vohwinkel syndrome with ichthyosis and progressive symmetric erythrokeratoderma (6–17). A transgenic animal model system expressing a mutant loricrin showed a Vohwinkel syndrome-like phenotype (34). To acquire a causative relationship in signal transduction between loricrin mutations and LK, we developed a stable cell line expressing a mutant loricrin using the edcsyone-inducible system. To our knowledge, this study is the first to show that the expression of a mutant loricrin caused the activation of VEGF 2 slightly (Fig. 10, A and B). Cyclo-VEGF (CBO-P11) at 20 μM inhibited the phosphorylation of VEGFR 2 slightly. JE-11 at 10 μM also inhibited the phosphorylation (Fig. 10C). Cyclo-VEGF (CBO-P11) at 20 μM plus PD 153035 at 1 μM inhibited the phosphorylation of VEGFR 2. JE-11 at 10 μM plus PD 153035 at 1 μM also inhibited the phosphorylation of VEGFR 2 (Fig. 10D).
both phosphorylated in VL-5 cells. Stat3 and ERK1/2 were both phosphorylated as well. VEGF and TGF-α levels were increased in LK model cells. VEGF inhibitors, CBO-P11 and JE-11, hindered LK model cell proliferation.

LK model cells showed significant phosphorylation of EGFR and VEGFR 2 at 20 h after induction. TGF-α along with epidermal growth factor and amphiregulin are ligands for the EGFR (35). EGFR and VEGFR 2 are membrane-bound receptor tyrosine kinases. On binding ligands, these receptors form homo- and heterodimers leading to the autophosphorylation of tyrosine residues in the cytosolic domains of the proteins. The phosphorylated tyrosine residues become docking sites for signaling molecules such as Gab1 and c-Cbl that activate cellular signaling pathways regulating a number of cellular processes, including proliferation and survival. We detected phosphorylated forms of Gab1 and c-Cbl only in LK model cells and observed increased TGF-α secretion and prolonged EGFR phosphorylation. VEGF secretion and prolonged VEGFR 2 phosphorylation were also observed. VEGF is a dimeric glycoprotein and a hypoxia-inducible endothelial cell mitogen (36). Although VEGF had been thought to be highly specific for endothelial cells, it has become increasingly clear that it also elicits responses in nonendothelial cell types such as keratinocytes. In normal human skin, VEGF is expressed and secreted by epidermal keratinocytes (37). Originally, the keratinocyte-derived VEGF was thought to act in a paracrine manner on vascular hyperpermeability and angiogenesis during wound healing. Yang et al. (31) found that HaCaT cells expressed all five known VEGF receptors and coreceptors. Most importantly, neutralizing VEGFR 2 could block the VEGF-induced proliferation and migration of HaCaT cells. In addition, Lichtenerberger et al. (38) found that VEGF could be considered as a potent growth factor for epidermal tumors. They demonstrated that VEGFR signaling is cell-autonomously required in skin tumor cells to stimulate their proliferation in an autocrine- and angiogenesis-independent manner and that VEGFR and EGFR signaling synergize in neoplastic cells to promote tumor growth. We observed that simultaneous use of VEGF and EGFR inhibitors blocked VL-5 cell proliferation significantly. This blockade effect may be attributed to a synergic action of EGFR and VEGF signaling (38).

The differences in cell proliferation between control (mock and WL-1 cells) and cellular model of LK (VL-5 cells) was rather small, and one wonders whether it explained the clinically seen hyperproliferation of LK. Perhaps there may be a signaling loop from keratinocytes to dermal fibroblasts and back in vivo. One candidate of cytokines secreted by keratinocytes may be interleukin-1β. Another candidate of growth factors secreted by fibroblasts may be keratinocyte growth factor.
It is well known that interleukin-1β is the most potent inducer of keratinocyte growth factor in fibroblasts through the c-Jun pathway (39). Such signaling loop may partly explain the hyperproliferation observed in LK.

VEGF stimulation drives the formation of a complex between Gab1 and PI3K, a heterodimer consisting of regulatory 85-kDa (p85) and catalytic 110-kDa (p110) subunits. This interaction takes place via the phosphorylated YMXXM motifs of Gab1 and the Src homology region 2 domains of p85. We conducted immunoprecipitation experiments and found Gab1 to interact with p85 and also phosphorylation within the C terminus at Ser-473. We considered statistically significant at \( p < 0.05 \). Gab1 and the Src homology region 2 domains of p85. We conducted immunoprecipitation experiments and found Gab1 to interact with p85 and also phosphorylation within the C terminus at Ser-473. We considered statistically significant at \( p < 0.05 \). Gab1 and the Src homology region 2 domains of p85. We conducted immunoprecipitation experiments and found Gab1 to interact with p85 and also phosphorylation within the C terminus at Ser-473. We considered statistically significant at \( p < 0.05 \).

Akt is activated by PIP3 phospholipids and is thus sensitive to inhibition by the PI3K inhibitors wortmannin and LY294002. The activation of Akt requires binding to PIP3 via the pleckstrin homology domain, phosphorylation on the activation loop of Thr-308 by 3-phosphoinositide-dependent kinase-1 (PDK1), and also phosphorylation within the C terminus at Ser-473. We detected Akt phosphorylated at serine 473 and threonine 308 in VL-5 cells. It is well known that interleukin-1β is the most potent inducer of keratinocyte growth factor in fibroblasts through the c-Jun pathway (39). Such signaling loop may partly explain the hyperproliferation observed in LK.

The blocking of VEGF is an attractive way to treat LK because it is the most potent inducer of keratinocyte growth factor in fibroblasts. VEGF inhibitors, CBO-P11 and JE-11, plus PD 153035 or PD 153035 plus PD 153035 inhibited the proliferation of VL-5 cells and attenuated the phosphorylation of VEGFR 2. CBO-P11 is also known to inhibit the VEGF-stimulated phosphorylation of MAPK(33). Thus, CBO-P11 plus PD 153035 or JE-11 plus PD 153035 are promising candidates for the development of new inhibitors useful for the treatment of LK.

In conclusion, this study highlights a novel role for the activation of VEGFR 2 and EGFR in the hereditary keratinizing disorder LK. The release of VEGF and TGF-α and subsequent activation of VEGFR 2 and EGFR by an autocrine/paracrine pathway link loricrin gene mutations to the rapid cell proliferation in the HaCaT LK cellular model. It would be of interest to examine the involvement of this mechanism in the pathogenesis of other hereditary keratinizing disorders.

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