βig-h3 Induces Keratinocyte Differentiation via Modulation of Involucrin and Transglutaminase Expression through the Integron α3β1 and the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway*

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βig-h3 is an extracellular matrix protein whose expression is highly induced by transforming growth factor (TGF)-β1. Whereas βig-h3 is known to mediate keratinocyte adhesion and migration, its effects on keratinocyte differentiation remain unclear. In the present study, it was demonstrated that expression of both βig-h3 and TGF-β1 was enhanced during keratinocyte differentiation and that expression of the former was strongly induced by that of the latter. This study also asked whether changes in βig-h3 expression would affect keratinocyte differentiation. Indeed, down-regulation of βig-h3 by transfection with antisense βig-h3 cDNA constructs effectively inhibited keratinocyte differentiation by decreasing the promoter activities and thus expression of involucrin and transglutaminase. The result was a 2-fold increase in mitotic capacity of the cells. Conversely, overexpression of βig-h3, either by transfection with βig-h3 expression plasmids or by exposure to recombinant βig-h3, enhanced keratinocyte differentiation by inhibiting cell proliferation and concomitantly increasing involucrin and transglutaminase expression. Recombinant βig-h3 also promoted keratinocyte adhesion through interaction with integrin α3β1. Changes in βig-h3 expression did not affect intracellular calcium levels. Subsequent analysis revealed not only induction of Akt phosphorylation by recombinant βig-h3 but also blockage of Akt phosphorylation by LY294002, an inhibitor of phosphatidylinositol 3-kinase. Taken together, these findings indicate that enhanced βig-h3, induced by enhanced TGF-β during keratinocyte differentiation, provoked cell differentiation by enhancing involucrin and transglutaminase expression through the integrin α3β1 and phosphatidylinositol 3-kinase/Akt signaling pathway. Lastly, it was observed that βig-h3-mediated keratinocyte differentiation was caused by promotion of cell adhesion and not by calcium regulation.

Transforming growth factor-β (TGF-β1)-inducible gene-h3 (βig-h3) was first cloned from A549 lung adenocarcinoma cells that had been stimulated with TGF-β1 (1, 2). βig-h3 has since been shown to be an extracellular matrix protein that can be highly induced by TGF-β in several cell types, including mammary epithelial cells, keratinocytes, and lung fibroblasts (1, 2). With structural homology to the insect protein fascin, βig-h3 is a 76- to 78-kDa protein containing four repeat regions and 11 cysteine residues, mostly clustered in a distinct amino terminus. The βig-h3 molecule appears to undergo partial processing at the carboxyl terminus to yield a 68–70-kDa isoform (2). Although the βig-h3 transcript has been detected in a variety of human and mouse tissues, including breast, heart, kidney, liver, stomach, and skeletal muscle (2), little information is available regarding the distribution of the protein in such human tissues as the arteries, eye, kidney, lung, and skin (3–6).

It is known that βig-h3 acts as a cell adhesion molecule in several cell types (7) and as a bifunctional linker protein to connect various matrix molecules to each other and to cells (8, 9). βig-h3 contains multiple cell adhesion motifs within its fascin-like domains capable of mediating interactions with a variety of cell types via integrins α3β1 (10, 11), α1β1 (12), and αvβ5 (7). It is known to mediate the migration and proliferation of normal human epidermal keratinocytes (NHEKs) through two integrin α3β1-interacting motifs in the second and fourth fas-1 domains (10). It has also been shown to bind in vitro to a number of other matrix components, including fibronectin, laminin, and several collagen types (13, 14). The precise roles of βig-h3 in cell development are currently unknown, but it has been implicated in cell growth (2, 10), osteoblast differentiation (15, 16), and wound healing (6). During wound healing, for example, βig-h3 is produced by a range of cell types including activated macrophages, neutrophils, fibroblasts, and keratinocytes (18).

βig-h3 has been reported in both the dermis and epidermis, with increased staining intensities in the papillary dermis and granular layer of the epidermis (4). Similarly, TGF-β has been localized in the normal dermis and epidermis. The investigators of the present study previously demonstrated that expression of TGF-β, a potent inducer of differentiation for normal epithelial cells, is increased in and near terminal differentiation of mucosal keratinocytes (19). Moreover, the epidermis was found to display a highly coordinated program of sequential changes in gene expression coincident with the phenotypic


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Evolution from a proliferating basal cell to the mature, nonviable cell (20). Epidermal and mucosal keratinocytes undergo terminal differentiation when they migrate from the basal layer to the surface (21), and both cell types display a limited number of divisions in vivo. Serial subculture-induced keratinocyte differentiation mimics the physiological maturation process observed in the intact epidermis in vivo (19) and is more similar in some ways with this in vivo process than with calcium-induced differentiation (22). The in vitro differentiation system is thus useful for investigating the mechanisms of keratinocyte differentiation. Many proteins are known or suspected to be associated with the overall process of keratinocyte differentiation. In particular, the roles of involucrin and transglutaminase have been identified as the substrate and enzyme, respectively, required for cornified envelope formation.

The role of TGF-β in keratinocyte differentiation is well established. TGF-β is a multifunctional cytokine that regulates the proliferation, differentiation, and function of many cell types. The findings herein demonstrate that enhanced TGF-β signaling contributes to the process of keratinocyte differentiation. The role of TGF-β in skin biology is mediated through the integrin α3β1 and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway. Finally, the present study demonstrates that β3mediated keratinocyte differentiation was caused by promotion of cell adhesion and not by calcium regulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Normal human oral keratinocytes (NHOKs) were prepared and maintained as previously reported (19). Briefly, NHOKs were isolated from human gingival tissue specimens obtained from healthy volunteers (age range 20 to 30 years) who were undergoing oral surgery. Oral keratinocytes were isolated from separated epithelial tissue by trypsinization, and primary cultures were established in keratinocyte growth medium containing 0.15 mM calcium and a supplement of recombinant human epidermal growth factor bullet kit (keratinocyte growth medium; Clonetech, San Diego, CA). Primary NHEKs were prepared in a manner similar to the NHOKs from human foreskins obtained from patients (1 to 3 years of age) undergoing surgery. Approximately 70% confluent primary NHOKs and NHEKs were plated at 1 × 10^6 cells per 60-mm culture dish and cultured until the cells reached 70% confluence. Cells were then subcultured at every 70% confluence until they reached the post-mitotic stage of proliferation, at which time the culture was maintained for 12 days without further passage.

**Determination of NHOK and NHEK Culture Population Doublings**—Individual keratinocytes isolated from gingival epithelial specimens and foreskins were plated in 60-mm culture dishes. Three days after seeding, the number of cells that had been originally plated was determined by colony counting. Cells were cultured until they reached 70% confluence and then the cell numbers in the various dishes were counted in a hemocytometer. This count allowed determination of the number of population doublings (PDs) of the primary cultures. Harvested primary cultures were subcultured until they reached the post-mitotic stage of proliferation. The PD was calculated at the end of each passage according to the formula 2^PD = (Cf/Ci), where N denotes the number of PDs; Cf, the total number of cells harvested at the end of a passage; and Ci, the total number of attached cells at seeding.

**Construction of Vectors Expressing Antisense RNA and Wild-type β3-h3 and Cloning of the Promoter Regions of Involucrin and Transglutaminase**—Antisense β3-h3 constructs were made by inserting 375bp human β3-h3 cDNA fragments (nucleotides 579 to 1253) containing the ATG start codon, in an antisense orientation, into the EcoRI sites of a pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA), which expresses a neomycin (G418) resistance gene. β3-h3 expression plasmids were made by inserting 2204-bp human β3-h3 cDNA fragments (nucleotides 5 to 2199) containing the ATG start codon, in a sense orientation, into the NotI sites of the same pcDNA3.1(+) vector. The human β3-h3 cDNA encompassing nucleotides 5 to 2199 was amplified by reverse transcriptase-PCR using sense (5′-GACCATGGGGCTCTTTCTTCGTGC-3′) and antisense (5′-TGGCAAGAGCTCAATCTCATAA′) primers. Similarly, the human involucrin promoter region (nucleotides 2063 to 1325) was amplified with sense (5′-GAACGTTAGACGGTGTCATCCTGAGTCGTTGAGTGTGTA-3′) and antisense primers (5′-TGTGGACAGCACTGACTGCTGTCAGTTTGTGCAAGAGGGAGG-3′). Amplification was performed with an ABI Prism™ 310 Genetic Analyzer (PerkinElmer Life Sciences) and the ABI Prism Dye Terminater Cycle Sequencing Kit (PerkinElmer Life Sciences). The resulting sequences were compared with published sequences of the promoter regions of involucrin (24) and transglutaminase-1 (25). The amplified 3.4-kb fragment of human involucrin promoter and the 2.3-kb fragment of human transglutaminase-1 promoter were subcloned into a pCAT-basic vector (Promega, Madison, WI), linking them to the chloramphenicol acetyltransferase (CAT) gene. The correct orientation of the inserts with respect to the CAT sequence was verified by restriction enzyme analysis.

**Transfection, Selection, and CAT Assay**—NHOKs were transfected in suspension with antisense β3-h3 or pcDNA3.1(+) vector using a Polybrene/glycerol method (26) and then incubated in keratinocyte growth medium. Beginning 2 days after transfection, cells were incubated for 4 days in 70 μl G418 (Invitrogen). Selected cells were serially subcultured until they reached the post-mitotic stage of proliferation and were harvested. The intracellular levels of β3-h3, involucrin, transglutaminase, Akt, phospho-Akt, and calcium were quantitated by Western blotting and dual-wavelength fluorescence imaging, as described elsewhere. In other experiments of the present study, exponentially proliferating keratinocytes, plated in 35-mm culture dishes, were co-transfected with antisense β3-h3 or pcDNA3.1(+) vector, involucrin- or transglutaminase-1 promoter constructs, and the cDNA of the gene of interest. Cells were transfected with the reporter pGL3basic vector and then assayed for luciferase activity.

**Fluorometric Assay**—Fluorometric analysis of the cell surface integrin expression level was performed as described previously (28). Briefly, NHOKs were detached by gentle treatment with 0.05% trypsin and 0.53 mM EDTA in phosphate-buffered saline (PBS), washed, and incubated with anti-integrin mAbs (anti-α2, α3, α5, β1, and β4) for 45 min at 4°C. After washing, cells were incubated with fluorescein isothiocyanate-labeled secondary antibodies for 45 min at 4°C. Finally, cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**Adhesion Inhibition Assay**—To identify the β3-h3 receptor on NHOKs, 5 μg/ml of mAbs to different types of integrins were incubated with exponentially proliferating (PD 13) or terminally differentiated (PD 20) NHOKs in 0.5 ml of incubation solution (2 × 10^5 cells/ml) for 30 minutes. The mAbs were used at different sites of a 96-well plate coated with 10 μg/ml of recombinant β3-h3 protein (plates had been protein-coated and stored overnight at 4°C) and then incubated for an additional 1 h at 37°C. After incubation, unattached cells were removed by two rinses with PBS. Attached cells were fixed with 10% formalin in PBS for 15 min, rinsed twice with PBS, and stained with 0.005% crystal violet for 1 h. The plates were gently rinsed with double distilled water three times. To ensure a representative count, each plate
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was divided into quarters and two fields per quarter were photographed using an Olympus BX51 microscope at ×100 magnification.

Cell Adhesion Assay—Cell adhesion was assayed as described previously (29). Briefly, 24-well culture plates (Nunc, Roskilde, Denmark) were coated with 10 μg/ml recombinant Big-h3 protein and held overnight at 4 °C before being blocked with PBS containing 1% heat-inactivated bovine serum albumin (BSA, Sigma) for 1 h at 37 °C. Cells were detached by treatment with 0.05% trypsin and 0.53 mM EDTA in PBS, resuspended in the culture media (1 × 10^5 cells/500 μl), added to each plate, and incubated for 1 h at 37 °C. Removal of unattached cells and fixing, staining, and imaging of attached cells was identical as in the adhesion inhibition assay.

Effects of Exogenous TGF-β1 and Recombinant Big-h3 Protein on Keratinocyte Differentiation—To determine the effects of TGF-β1 and recombinant Big-h3 protein on keratinocyte differentiation and the F3K/Akt signaling pathway, the expression of involucrin, transglutaminase, andBig-h3; the promoter activities of involucrin and transglutaminase; the intracellular calcium level; and the phosphorylation status of Akt were determined in exponentially proliferating NHOKs, which were cultured for 4 days in the presence of 10 or 20 ng/ml TGF-β1 or 10 μg/ml recombinant Big-h3 protein.

Measurement of Intracellular Calcium—After cells were transfected with either the antisense Big-h3 cDNA constructs or Big-h3 expression plasmids, the intracellular calcium level was measured by digital video fluorimetry using an intensified CCD camera coupled to a microscope (Olympus IX71SFS-2, Japan) supported by Metafluor software on a Pentium computer (Shutter Instrument, Novato, CA). This procedure has been described previously (23).

Western Blot Analysis—Western blot analysis was performed as in previous reports (19) using anti-human involucrin (SY5) mAb (Sigma), anti-human TGF-β1 (sc-146) polyclonal antibody (Santa Cruz, Santa Cruz, CA), anti-human transglutaminase (Ab-1) polyclonal antibody (Oncogene, Uniondale, NY), polyclonal anti-Big-h3 antiserum against recombinant Big-h3 protein (REGEN Biotech, Korea), and anti-β-actin (20-33) mAb (Sigma). After probing with each antibody, the membrane was stained with 1X Ponceau S stain for 10 min or subjected to immunoblotting using an intensified CCD camera coupled to a microscope (Olympus IX71SFS-2, Japan) supported by Metafluor software on a Pentium computer (Shutter Instrument, Novato, CA). This procedure has been described previously (23).

RESULTS

Expression of Big-h3 and TGF-β1 Is Enhanced during Keratinocyte Differentiation—Primary NHOKs (PD 13.4) were serially subcultured until they reached the post-mitotic stage of cell proliferation, at which time the culture was maintained for 12 days without further passage. The total cell numbers at the beginning and end of each passage were used to determine the number of PDs. A, microscopic features of NHOKs with increasing PDs. B, levels of involucrin and Big-h3 proteins in NHOKs with different PDs C and D, levels of Big-h3 and TGF-β1 in the conditioned medium of exponentially proliferating (PD 13.4) and terminally differentiated (PD 17.3) NHOKs, respectively. Serum-free culture medium was collected from the cultures over 48 h and concentrated. A structural protein stained with Ponceau S served as the internal control to account for loading error.

Because TGF-β is known to induce both Big-h3 expression in keratinocytes (1, 2) and cell differentiation (19), the expression of TGF-β and Big-h3 was determined in serially subcultured NHOKs. Because TGF-β and Big-h3 are secreted proteins, we determined their levels in cell lysates and the NHOK medium with different PDs. Western blotting of culture media showed that the exponentially proliferating NHOKs with PD 13.4 had very low levels of Big-h3 and TGF-β1 proteins. Expression of both of these proteins was notably increased, however, in the terminally differentiated NHOKs with PD 17.3 (Fig. 1, C and D). Similarly, the level of intracellular 68-kDa Big-3 expression was significantly enhanced in terminally differentiated NHOKs (Fig. 1B). These results suggest that variations in Big-h3 expression are closely correlated with keratinocyte differentiation.

TGF-β Induces Big-h3 Expression and Promotes Keratinocyte Differentiation—Big-h3 is known to be induced by TGF-β1 in many but not all cell types. The present study showed that TGF-β induces Big-h3 expression in NHOKs. When exponentially proliferating NHOKs (PD 13.4) were cultured for 6, 12, 24, 48, 72, or 96 h in the presence of 10 ng/ml TGF-β1, the levels of both Big-h3 protein and Big-h3 transcript were increased in a time-dependent manner compared with untreated cells (Fig. 2A). A second investigation into the effects of TGF-β1 on keratinocyte differentiation and Big-h3 expression had NHOKs with PD 13.4, cells that do not normally show a differentiated phenotype, being treated with 10 or 20 ng/ml TGF-β1 for 96 h. TGF-β1 treatment in keratinocytes resulted in loosening of cell-cell contact as well as cell spreading. Characteristics of differentiation were also exhibited (Fig. 2B). Western blotting showed that TGF-β1 treatment induced Big-h3 expression in cell lysates and culture media and led to significantly increased expression of involucrin and transglutaminase (Fig. 2C). Involucrin and transglutaminase are both markers of keratinocyte differentiation (31–33). These results indicate that TGF-β1 treatment induces Big-h3 expression in NHOKs.
promotes their differentiation. This agrees with a previous study by these authors showing TGF-β expression to be significantly enhanced in and near terminally differentiated NHOKs versus counterparts—

ting showed that TGF-β1 treatment induced β-h3 expression in cell lysates and culture media, which led to significantly increased expression of involucrin and transglutaminase (Fig. 3C). Such changes were very similar to that of NHOKs. These results indicate that TGF-β1 treatment induces β-h3 expression in NHEKs and promotes their differentiation.

**Suppression of β-h3 Expression Inhibits Keratinocyte Differentiation and Extends the Mitotic Capacity of Cells**—To test whether β-h3 could mediate keratinocyte differentiation, and to study the underlying mechanisms of β-h3-mediated cell differentiation, expression of β-h3 was down-regulated by transfecting NHOKs with antisense β-h3 cDNA constructs. If β-h3 is required for the induction of keratinocyte differentiation, cells transfected with antisense β-h3 constructs should fail to induce keratinocyte differentiation. NHOK cultures (PD 11.3) were transfected with antisense β-h3 constructs, and transfectants were selected with G418 and further cultured for 12 days. The transfected cells showed a significant reduction in expression of β-h3 versus vector-transfected cells. Furthermore, NHOKs transfected with the antisense β-h3 retained an undifferentiated phenotype, whereas some cells transfected with the vector alone were enlarged and flattened (Fig. 4, A and B). Similarly, the levels of involucrin and transglutaminase in the antisense-β-h3-transfected cells were significantly reduced (Fig. 4B), indicating that suppression of β-h3 inhibited keratinocyte differentiation.

An explanation of how β-h3 expression causes inhibition of keratinocyte differentiation was sought by evaluation of involucrin and transglutaminase promoter activity following co-transfection of NHOKs with an antisense β-h3 construct and involucrin or transglutaminase promoter constructs. As predicted, involucrin and transglutaminase promoter activities were significantly inhibited in the antisense β-h3 construct-transfected cells compared with vector-transfected cells (Fig. 4C).

Because decreased β-h3 expression did inhibit keratinocyte differentiation, it was further investigated whether a decrease of β-h3 would also affect the mitotic ability of cells, in essence extending the in vitro lifespan of NHOKs. NHOK cultures (PD 12.1) were transfected with an antisense β-h3 construct, selected with G418, and subcultured until they reached the post-mitotic stage of proliferation. As shown in Fig. 4D, the in vitro lifespan of cells that were transfected with the antisense β-h3 construct was increased ~2-fold over vector-transfected cells. This increase in mitotic ability was presumably a consequence of decreased β-h3 expression. These results indicate that suppression of β-h3 expression inhibits keratinocyte differentiation by decreasing involucrin and transglutaminase promoter activities and also extends the mitotic capacity of the cells.

**Overexpression of β-h3 and Recombinant β-h3 Protein Promote Keratinocyte Differentiation by Increasing Involucrin and Transglutaminase Promoter Activities**—The role of β-h3 in cell differentiation was evaluated by examining the effect of overexpression of β-h3 in keratinocytes. For this, NHOK cultures (PD 11.8) were transfected with β-h3 expression plasmids to increase β-h3 expression and transfectants were selected with G418, further cultured for 12 days, and assayed for their levels of β-h3 and involucrin. Most cells transfected with vector displayed typical keratinocyte morphology and an undifferentiated phenotype, although some were enlarged and flattened. When NHOK cultures were transfected with the β-h3 expression plasmids, the majority of cells displayed the characteristics of keratinocyte differentiation, mainly a highly enlarged and elongated cytoplasm (Fig. 5A). Furthermore, the levels of involucrin as well as
FIG. 3. Expression of βig-h3 during epidermal differentiation, response of NHEKs to TGF-β1, and the role of TGF-β1 in epidermal differentiation are very similar to that of NHOKs. Primary NHEKs (PD 13.5) were serially subcultured until they reached the post-mitotic stage of cell proliferation, at which time the culture was maintained for 12 days without further passage as described in the legend to Fig. 1. A, levels of involucrin and βig-h3 proteins in NHEKs with different Pds. B, levels of βig-h3 transcript and protein in exponentially proliferating NHEKs treated with 10 ng/ml of TGF-β1. Assay conditions were the same as described in the legend to Fig. 2A, except that NHEKs (PD 13.5) were used. A structural protein stained with Ponceau S served as the internal control to account for loading error. 28 S RNA was used as a control. C, levels of involucrin, transglutaminase, and βig-h3 proteins in cell lysates and/or serum-free culture media from NHEKs cultured for 96 h in the presence of 10 or 20 ng/ml TGF-β1.

Fig. 4. Suppression of βig-h3 expression by transfection with the antisense βig-h3 cDNA construct inhibits keratinocyte differentiation by decreasing involucrin and transglutaminase expression. Phase-contrast micrographs (A) and decreased levels of βig-h3, involucrin, and transglutaminase (B) in NHOKs transfected with the antisense βig-h3 construct. NHOK cultures (PD 11.3) were transfected with the antisense βig-h3 construct (A) or pcDNA3.1(+) vector (V) and selected with G418. The selected cells were further cultured for 12 days and then harvested. C, involucrin and transglutaminase promoter activities in antisense βig-h3 construct-transfected keratinocytes. Either the antisense βig-h3 construct or the pcDNA3.1(+) vector was transfected into NHOKs (PD 14.1) with involucrin or transglutaminase promoter-CAT constructs and a β-galactosidase expression vector. Two days after transfection, cell lysates were prepared. Data are expressed as percentages of the value for vector-transfected cells (mean ± S.D., n = 6). *, p < 0.01 versus vector-transfected cells. D, extension of the in vitro lifespan of keratinocytes as a consequence of decreased βig-h3 expression by transfection with the antisense βig-h3 construct. The assay conditions were the same as in A. Selected cells were serially subcultured until they reached the post-mitotic stage of proliferation, and the number of Pds was determined at the end of each passage after selection.

βig-h3 were significantly increased in the cells transfected with βig-h3 expression plasmids compared with vector-transfected cells (Fig. 5B), indicating that overexpression of βig-h3 induces keratinocyte differentiation.

If βig-h3 protein is required for the induction of involucrin and transglutaminase gene expression, their promoter activities should be increased in cells transfected with the βig-h3 expression plasmids. As expected, transfection of cells with the βig-h3 expression plasmids significantly increased both involucrin and transglutaminase promoter activities compared with vector-transfected cells (Fig. 5C).

A direct causal role for βig-h3 in keratinocyte differentiation was further tested by examining the effect of recombinant βig-h3 protein on NHOKs. When NHOKs (PD 12.4) were cultured for 4 days in the presence of 10 µg/ml recombinant βig-h3, the cells displayed a differentiated phenotype (Fig. 6A) and expressed higher levels of involucrin and transglutaminase than did untreated control cells (Fig. 6B). Generally, cell growth is vital to cell differentiation, so the influence of recombinant βig-h3 on cell growth was also evaluated: treatment with 10 µg/ml recombinant βig-h3 decreased cell growth (Fig. 6C). Taken together, these observations indicate that overexpression of βig-h3 and recombinant βig-h3 promotes keratinocyte differentiation by increasing involucrin and transglutaminase expression and by inhibiting cell growth.

Changes in βig-h3 Expression Do Not Affect Intracellular Calcium Levels—Calcium is well known to induce differentiation of human keratinocytes (34). To study the involvement of βig-h3 in calcium regulation, changes in βig-h3 expression were evaluated for their affects on intracellular calcium levels in NHOKs. Neither down-regulation of βig-h3 (transfection with antisense βig-h3) nor overexpression of βig-h3 (transfection with βig-h3 expression plasmids) changed the intracellular calcium level compared with vector-transfected cells (data not shown). Similarly, stimulation with 10 µg/ml recombinant βig-h3 for 6 min in serum-free medium did not affect the intracellular calcium level (Fig. 6D). In general, these results suggest that calcium is not involved in βig-h3-mediated keratinocyte differentiation.

βig-h3 Mediates Keratinocyte Adhesion Through Integrin α3β1—After establishing that βig-h3 mediates keratinocyte
differentiation, this study turned to whether \( \beta \)-h3 induces said differentiation by promoting cell adhesion. Accordingly, a cell adhesion assay was devised. Exponentially proliferating (PD 13) and terminally differentiated (PD 20) NHOKs were seeded onto 24-well plates coated with recombinant \( \beta \)-h3 or BSA. As shown in Fig. 7A, \( \beta \)-h3 was promotive of cell adhesion of both types of NHOKs. These findings are in accordance with a previous study that showed \( \beta \)-h3 supported adhesion and spreading of endothelial epithelial cells as well as NHOKs (10, 35). To further investigate whether restraint of \( \beta \)-h3 expression modifies cell adhesion, expression of \( \beta \)-h3 was reduced by transfecting NHOKs with antisense \( \beta \)-h3 cDNA constructs. NHOK cultures (PD 12) were transfected with antisense \( \beta \)-h3 constructs, and transfecants were selected with G418, then cultured for 12 days. When the transfected cells were seeded onto 96-well plates coated with or without recombinant \( \beta \)-h3, cell adhesion was significantly inhibited in antisense \( \beta \)-h3 construct-transfected cells compared with vector-transfected cells in both conditions (Fig. 7B). Next, in the absence of \( \beta \)-h3, the effects of TGF-\( \beta \) on keratinocyte adhesion were tested. Exponentially proliferating NHOKs (PD 11) were pretreated with different concentrations of TGF-\( \beta \) for 24 h and then were used for cell adhesion assay. TGF-\( \beta \)-treated cells adhered to \( \beta \)-h3-uncoated 24-well plates in a dose-dependent manner (Fig. 7C). Taken together, these results suggest that a change in cell adhesion properties is required for keratinocyte differentiation to proceed, and that \( \beta \)-h3, in response to TGF-\( \beta \), mediates cell adhesion to allow expression of keratinocyte differentiation-related genes.

In conjunction with its role in cell adhesion, \( \beta \)-h3 has been shown to mitigate several cellular functions through integrins (7, 10–12). A pursuit was therefore set upon to identify the particular integrin or integrins by which adhesion, spreading, and differentiation of NHOKs are mediated by \( \beta \)-h3. The first step was to determine those integrins that are indeed expressed on the surface of NHOKs. This was accomplished by fluorescence-activated cell sorter using mAbs specific to each integrin type. Fig. 7 (D and E) shows that exponentially proliferating and terminally differentiated NHOKs expressed several integrins, including \( \alpha \)3\( \beta \)1 and \( \alpha \)6\( \beta \)4. The terminally differentiated NHOKs showed lower surface expressions of \( \alpha \) and \( \beta \) integrin subunits than exponentially proliferating NHOKs (Fig. 7E). Next, monoclonal integrin function-blocking antibodies were employed with the result that only antibodies to integrin subunits \( \alpha3 \) and \( \beta1 \) significantly inhibited \( \beta \)-h3-mediated adhesion of both types of NHOKs (Fig. 7F and G). These findings suggest that \( \beta \)-h3 mediates adhesion of human oral keratinocytes and might mediate keratinocyte differentiation via integrin \( \alpha3 \beta1 \).

\( \beta \)-h3 Activates Phosphorylation of Akt—Currently, the identities of integrin \( \alpha3 \beta1 \)-associated signaling molecules that are responsible for mediating keratinocyte adhesion and/or differentiation in response to \( \beta \)-h3 are unclear. It has been shown that the activation of integrins upon cell adhesion to extracellular matrix protein leads to an increase in phosphorylation of focal adhesion kinase, which is the major tyrosine-phosphorylated protein (36–38). In fact, the focal adhesion kinase pathway is activated by most integrins. However, the downstream signaling pathways that mediate integrin-focal adhesion kinase survival signaling are diverse, and the factors determining which pathway is utilized remain obscure. To determine the signaling pathways that contribute to NHOK adhesion and differentiation as induced by \( \beta \)-h3, an examination was conducted into the effects of \( \beta \)-h3 on the phosphorylation status of Akt and MAPKs.
Exponentially replicating and terminally differentiated keratinocytes. Cells were incubated with the mAbs specific to the

Therefore, this study examined whether

proliferating NHOKs (PD 11) were pretreated with different concentrations of TGF-

were incubated with the secondary antibody alone ()

were adapted for 12 h, pretreated with 20

blocked by LY294002. For this, NHOK cultures (PD 11.6)

was increased in cells treated with

were incubated with 10

mAbs to the PD 12 were used. Ten μg/ml of recombinant

ig-h3 was used for coating. Data are expressed as mean

were found to induce phos-

were incubated for 1 h on plates precoated with 10 μg/ml recombinant

Negative control cells

were incubated with BSA or recombinant

Fig. 7. Recombinant βg-h3 mediates keratinocyte adhesion through integrin αβ1. A, cell adhesion levels of exponentially proliferating

was enhanced during keratinocyte differentiation and that TGF-β1 exposure induced keratinocyte differentiation as well as βg-h3

mRNA and protein expression. This study thus examined

whether changes in βg-h3 expression would affect keratinocyte differentiation.

DISCUSSION

The authors of the present study previously demonstrated that TGF-β, a potent inducer of differentiation for normal epithelial cells known to localize in the normal epidermis (4), also induces keratinocyte differentiation (19). Here, it was observed that βg-h3 and TGF-β1 expression were significantly enhanced during keratinocyte differentiation and that TGF-β1 exposure induced keratinocyte differentiation as well as βg-h3 mRNA and protein expression. This study thus examined whether changes in βg-h3 expression would affect keratinocyte differentiation.

The role of βg-h3 in mediating the differentiation of keratinocytes was demonstrated as follows: (i) a decrease in βg-h3

(ERK, JNK, and p38). Overexpression of βg-h3 or treatment with 10 μg/ml recombinant βg-h3 was found to induce phosphorylation of Akt (Fig. 8, A and B), although these had no effect on the phosphorylation of ERK, JNK, or p38 (data not shown). To further test whether PI3K/Akt is involved in βg-h3-mediated NHOK adhesion and differentiation, NHOK cultures (PD 12.4) were adapted for 12 h, pretreated with 20 μM LY294002, a synthetic inhibitor of the p110 catalytic subunit of PI3K, for 1 h, and then stimulated with 10 μg/ml recombinant βg-h3 for 5, 10, 20, 30, or 60 min. Whereas expression of total Akt did not appear to change, phosphorylation of Akt (data not shown), the levels of involucrin and transglutaminase expression were notably decreased in recombinant βg-h3-treated cells pretreated with LY294002 compared with recombinant βg-h3-treated cells without pretreatment (Fig. 8E). Taken together, these data suggest that the PI3K/Akt signaling pathway is associated with βg-h3-mediated keratinocyte differentiation.

phosphorylation of Akt. When LY294002 blocked the phos-

expression and the phos-

phorylation status of Akt. When LY294002 blocked the phos-

were incubated with PBS, fixed in 10% formalin in PBS, and stained with crystal violet. Data are expressed as percentages of the value for BSA-coated control (mean

expression and the phos-

were incubated for 1 h on plates precoated with 10 μg/ml recombinant

Unbound cells were washed away and the adherent cells fixed, stained with crystal violet, and counted. Data are expressed as percentages of the value for cells preincubated without integrin antibodies (None; mean

when paired with

and then stained with the fluorescein isothiocyanate-conjugated secondary antibody for flow cytometry. The negative control cells

were used for cell adhesion assay. Data are expressed as percentages of the value for cells preincubated without integrin antibodies (None; mean ± S.D., n = 3).

Adhesion of the exponentially proliferating (F) and terminally differentiated (G) NHOKs to βg-h3 is blocked by antibodies to α3β1 integrin. Cells were preincubated with 5 μg/ml of the function-blocking mAbs to integrin α2, α3, α5, α6, β1, and β4 subunits for 30 min at 37 °C and then incubated for 1 h on plates precoated with 10 μg/ml recombinant βg-h3. Unbound cells were washed away and the adherent cells fixed, stained with crystal violet, and counted. Data are expressed as percentages of the value for cells preincubated without integrin antibodies (None; mean ± S.D., n = 3).

Fig. 7. Recombinant βg-h3 mediates keratinocyte adhesion through integrin αβ1. A, cell adhesion levels of exponentially proliferating (PD 13) and terminally differentiated (PD 20) NHOKs on surfaces coated with BSA or recombinant βg-h3. One percent of BSA or 10 μg/ml of recombinant βg-h3 were used for coating. The cells were incubated with PBS, fixed in 10% formalin in PBS, and stained with crystal violet. Data are expressed as percentages of the value for BSA-coated control (mean ± S.D., n = 4), *p < 0.01 versus BSA-coated control. B, cell adhesion levels of NHOKs transfected with the antisense βg-h3 construct or pcDNA3.1(+) vector on 96-well plates coated with or without recombinant βg-h3 (PS). Assay conditions were the same as described in the legend to Fig. 4A, except that NHOKs with PD 12 were used. Ten μg/ml of recombinant

were incubated with the mAbs specific to the

were incubated for 1 h in plates precoated with 10 μg/ml recombinant βg-h3. Unbound cells were washed away and the adherent cells fixed, stained with crystal violet, and counted. Data are expressed as percentages of the value for cells preincubated without integrin antibodies (None; mean ± S.D., n = 4). *p < 0.01 versus vehicle-treated control. D, flow cytometric analyses of the integrin subunits on exponentially replicating and terminally differentiated keratinocytes. Cells were incubated with the mAbs specific to the α2, α3, α5, α6, β1, or β4 integrin subunits and then stained with the fluorescein isothiocyanate-conjugated secondary antibody for flow cytometry. The negative control cells were incubated with the secondary antibody alone (Con). Data are expressed as the cell number (y axis) as a function of fluorescence intensity (x axis). E, expression of integrin subunits in exponentially replicating and terminally differentiated NHOKs. MFI, mean fluorescence intensity. Adhesion of the exponentially proliferating (F) and terminally differentiated (G) NHOKs to βg-h3-uncoated polystyrene plates in a dose-dependent manner. Exponentially

expression was significantly enhanced during keratinocyte differentiation as well as βg-h3 mRNA and protein expression. This study thus examined whether changes in βg-h3 expression would affect keratinocyte differentiation.

The role of βg-h3 in mediating the differentiation of keratinocytes was demonstrated as follows: (i) a decrease in βg-h3

were incubated with the mAbs specific to the

were incubated with the secondary antibody alone (Con). Data are expressed as the cell number (y axis) as a function of fluorescence intensity (x axis).
expression led to inhibition of keratinocyte differentiation and a ~2-fold increase in mitotic capacity; (ii) down-regulation of βig-h3 expression resulted in decreased promoter activities and thus expression of the involucrin and transglutaminase genes; (iii) overexpression of βig-h3 significantly promoted keratinocyte differentiation and increased promoter activities and thus expression of the involucrin and transglutaminase genes. Together, these results demonstrate that βig-h3 induces keratinocyte differentiation by increasing promoter activities and expression of involucrin and transglutaminase, markers of keratinocyte differentiation (31–33) that serve as substrate and enzyme for cornified envelope formation.

A growing body of reports identifies βig-h3 as a cell adhesion substrate, regulator of cell growth, linker of matrix components, and transducer of TGF-β-mediated signaling (1, 4, 7, 8). Although the precise functions of βig-h3 in cell development are currently unknown, it has been associated with inhibition of osteoblast differentiation (15, 16, 39). Reports show the level of βig-h3 mRNA to be decreased in human bone marrow stromal cells treated with dexamethasone, a promoter of osteogenic cell differentiation (15). βig-h3 is also reported to inhibit bone nodule formation of mouse osteoblasts (16) and human periodontal ligament cells in vitro (39). Nevertheless, the role of βig-h3 on keratinocyte differentiation remains unknown.

Whereas one published study reports in vivo expression of βig-h3 in the granular layer of the epidermis (4), another reports that in situ hybridization could not detect βig-h3 expression in that tissue but rather detected βig-h3 transcripts in basal keratinocytes (10). Numerous studies in various cell types, including keratinocytes, involve TGF-β1 in the induction of βig-h3 (1, 2). TGF-β has been localized in the normal dermis and epidermis and TGF-β1 is constitutively expressed in suprabasal keratinocytes (30). Although there is no direct evidence showing that TGF-β1 produced by suprabasal keratinocytes stimulates basal keratinocytes to synthesize βig-h3 in normal skin and mucosa, expression of βig-h3 might be regulated by TGF-β1 in the epidermis. Participation of βig-h3 in keratinocyte differentiation is supported by our results, namely that overexpression of βig-h3 by TGF-β1 treatment promoted keratinocyte differentiation.

The present findings agree with these authors previous report that expression of TGF-β is significantly enhanced in and near terminally differentiated NHOKs and is associated with keratinocyte differentiation (19). These results were confirmed using recombinant βig-h3 protein: βig-h3 treatment resulted in both an increase of involucrin and transglutaminase expression and a differentiated phenotype in normal human keratinocytes. Here, it was also shown that βig-h3 was capable of decreasing cell growth. As has been reported by others, overexpression of βig-h3 was found to decrease cellular growth rate (2). This further suggests that βig-h3 might be involved in differentiation.

One mechanism of action for βig-h3 in keratinocyte differentiation would be to promote cell adhesion. In fact, βig-h3 has been reported to mediate cell adhesion in several cell types such as skin fibroblasts (4), epidermal keratinocytes (10), corneal epithelial cells (6, 7), and chondrocytes (12). In studies with NHOKs, βig-h3 was also found to promote keratinocyte adhesion. To further investigate whether reduction of βig-h3 expression and TGF-β1 modify cell adhesion, NHOKs transfected with antisense βig-h3 cDNA constructs and NHOKs pretreated with TGF-β1 were examined. Cell adhesion was significantly inhibited in cells with reduced βig-h3 compared with vector-transfected cells, but cell adhesion was significantly enhanced in TGF-β1-pretreated cells. This suggests that βig-h3-mediated keratinocyte differentiation is caused by the promotion of cell adhesion. The results presented here further indicate that βig-h3 might mediate NHOK adhesion and differentiation by interacting with integrin α3β1. Integrin α3β1 is a known receptor for βig-h3 (7, 10). Recently, TGF-β1 has been shown to increase affinity of the integrin α3β1 for βig-h3, resulting in enhanced adhesion and migration of epidermal keratinocytes toward βig-h3 (17).

Calcium action is another possible mechanism for keratinocyte differentiation, as it is well known to induce differentiation of human keratinocytes. Specifically, calcium concentrations higher than 0.1 mM in epidermal keratinocytes or 0.15 mM in oral keratinocytes induce terminal differentiation of human basal keratinocytes in vitro. Furthermore, a gradient of extracellular and intracellular calcium across the epidermis, with a lower concentration of calcium in the basal cell compartment than in the granular cell layer, is recognized as a principle regulator of keratinocyte maturation in vitro (33, 34). These investigators have previously shown that intracellular calcium concentration gradually increases during NHOK differentiation (23) and that treatment with calcium induces said differentiation (22). In terms of intracellular calcium level, changes in βig-h3 expression or exposure to recombinant βig-h3 were
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found to have no effect, suggesting that βig-h3-mediated keratinocyte differentiation is not caused by calcium regulation.

The phosphorylation status of MAPKs (ERK, JNK, p38) and Akt, a downstream target of PI3K, has been examined in cells transfected with the wild-type βig-h3 gene and in cells treated with recombinant βig-h3 protein so as to elucidate mechanisms of the signaling pathway of βig-h3-mediated keratinocyte differentiation. Both types of cells up-regulated for βig-h3 showed a higher level of phosphorylated Akt than did vector-transfected and βig-h3-untreated cells, respectively. The phosphorylation status of MAPKs in the βig-h3 up-regulated cells was similar to that of the control (data not shown). Subsequent analysis revealed blocking of Akt phosphorylation by LY294002, an inhibitor of PI3K. The notion that exogenous calcium administration might induce keratinocyte differentiation by activating PI3K/Akt was tested and found not to be the case. Calcium did not affect Akt phosphorylation (data not shown).

It has been reported that, because the βig-h3 gene is induced in several cell lines whose proliferation was affected by TGF-β, βig-h3 might be involved in mediating some of the signals of TGF-β (1). One recent study reports that TGF-β1 activates focal adhesion kinase and Akt in epidermal keratinocytes and that PI3K could be a common downstream factor that transduces signals from TGF-β1 and leads to activation of the integrin α3β1 by βig-h3 (17). Because overexpression of βig-h3 or treatment with recombinant βig-h3 was found to activate the PI3K/Akt pathway, decrease cell proliferation, and promote keratinocyte differentiation, we suggest that βig-h3 serves as a downstream factor of TGF-β in keratinocyte differentiation. Whether TGF-β1-induced βig-h3 expression completely accounts for the increase in involucrin and transglutaminase levels observed with TGF-β1 remains unknown. Nevertheless, exogenous recombinant βig-h3 administration in exponentially proliferating keratinocytes, which do not normally show the differentiation phenotype, was sufficient to induce differentiation. We propose that βig-h3 affects keratinocyte differentiation by promoting involucrin and transglutaminase expression through a molecular pathway that involves both integrin α3β1 and PI3K/Akt (Fig. 9).

Considering that βig-h3 and TGF-β1 expression are significantly enhanced during keratinocyte differentiation, and that TGF-β1 exposure induces said differentiation in addition to βig-h3 mRNA and protein expression, βig-h3 must be granted an important role in keratinocyte differentiation. This role is at least partially explained by the findings of the present study: (i) enhanced TGF-β during keratinocyte differentiation induced βig-h3 expression and led to keratinocyte differentiation by enhancing involucrin and transglutaminase expression, (ii) involucrin and transglutaminase expression were mediated through the integrin α3β1 and PI3K/Akt signaling pathway, and (iii) βig-h3-mediated keratinocyte differentiation was caused by enhanced cell adhesion, not by calcium regulation.

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