Human lactoferrin stimulates skin keratinocyte function and wound re-epithelialization

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Accepted for publication
19 February 2010

Key words
apoptosis, keratinocyte, migration, proliferation, recombinant human lactoferrin, re-epithelialization

Conflicts of interest
All authors declare no conflict of interest.

DOI 10.1111/j.1365-2133.2010.09748.x

Summary

Background Human lactoferrin (hLF), a member of the transferrin family, is known for its antimicrobial and anti-inflammatory effects. Recent studies on various nonskin cell lines indicate that hLF may have a stimulatory effect on cell proliferation.

Objectives To study the potential role of hLF in wound re-epithelialization.

Materials and methods The effects of hLF on cell growth, migration, attachment and survival were assessed, with a rice-derived recombinant hLF (holo-rhLF), using proliferation analysis, scratch migration assay, calcein-AM/propidium iodide staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method, respectively. The mechanisms of hLF on cell proliferation and migration were explored using specific pathway inhibitors. The involvement of lactoferrin receptor low-density lipoprotein receptor-related protein 1 (LRP1) was examined with RNA interference technique. An in vivo swine second-degree burn wound model was also used to assess wound re-epithelialization.

Results Studies revealed that holo-rhLF significantly stimulated keratinocyte proliferation which could be blocked by mitogen-activated protein kinase (MAPK) kinase 1 inhibitor. Holo-rhLF also showed strong promoting effects on keratinocyte migration, which could be blocked by either inhibition of the MAPK, Src and Rho/ROCK pathways, or downregulation of the LRP1 receptor. With cells under starving or 12-O-tetradecanoylphorbol-13-acetate exposure, the addition of holo-rhLF was found greatly to increase cell viability and inhibit cell apoptosis. Additionally, holo-rhLF significantly increased the rate of wound re-epithelialization in swine second-degree burn wounds.

Conclusions Our studies demonstrate the direct effects of holo-rhLF on wound re-epithelialization including the enhancement of keratinocyte proliferation and migration as well as the protection of cells from apoptosis. The data strongly indicate its potential therapeutic applications in wound healing.

The ability of keratinocytes to migrate and proliferate is essential for wound re-epithelialization and re-establishment of the skin barrier.1–4 Reportedly, keratinocytes on the edges of chronic wounds are unable to migrate properly and therefore it is difficult for wounds to close. One reason for the inability of nonhealing wound keratinocytes to migrate is that they are unresponsive to activation signals.5

Human lactoferrin (hLF), a member of the transferrin family, is expressed in a variety of glandular epithelial cells and skin epidermal keratinocytes.6,7 Low-density lipoprotein receptor-related protein 1 (LRP1), one of the major lactoferrin (LF) receptors in mammalian cells, is found in human skin keratinocytes8,9 and fibroblasts.10–12 LF is widely demonstrated to have antimicrobial effects and to regulate immune system functions.13,14 Recent studies have shown that LF stimulates cell growth in a variety of cell lines15–22 and reduces osteoblast apoptosis.23 In addition, it is reported that LF inhibits ultraviolet B radiation-induced corneal epithelial damage in rats.24 Recent progress with transgenic recombinant hLF has provided us with some new information on hLF in the field of wound healing. Talactoferrin (a recombinant hLF produced in Aspergillus niger var. awamori) was found to enhance the...
healing of wounds in mice in association with the modulation of early inflammatory mediators.  

However, no research has reported the biological function of hLF in human epithelial keratinocytes, which are major cells in the epidermis. In this study, we examined the effects of LF on cell proliferation, migration and survival/apoptosis in vitro as well as on the re-epithelialization in a porcine burn wound model in vivo, using a rice-derived recombinant hLF (holo-hLF).

Materials and methods

Reagents

The lyophilized holo-rhLF (Lacromin™) powder used in this study was provided by InVitria, Ventria Bioscience (Ft Collins, CO, U.S.A.). The stock solution was prepared by dissolving holo-hLF powder in phosphate-buffered saline (PBS) at a concentration of 50 mg mL⁻¹.

The rhLF from transgenic rice was partially (~50%) iron saturated (pis-rhLF), and further modified to generate iron-saturated (> 90%) holo-hLF. 26,27 holo-rhLF exhibits similar biochemical, biophysical and biological properties compared with native hLF from milk. 23,26,28,29

Cells and cell cultures

Normal human epidermal keratinocytes (HEKs) from neonatal foreskin were used in this study. HEKs were maintained in EpiLife™ Medium, a keratinocyte growth medium from (Cascade Biologics, Portland, OR, U.S.A.), supplemented with human keratinocyte growth supplement (HKGS, Cascade Biologics) at concentrations of 0.2% v/v of bovine pituitary extract, 5 µg mL⁻¹ bovine insulin, 0-18 µg mL⁻¹ hydrocortisone, 5 µg mL⁻¹ bovine transferrin, 0-2 mg mL⁻¹ human epidermal growth factor (EGF) plus antibiotic–antimicotic (Mediatech, Manassas, VA, U.S.A.) at 100 µg mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 0-25 µg mL⁻¹ amphotericin B at 37 °C in a 5% CO₂, humidified atmosphere. In the cell proliferation, viability and migration assays, holo-rhLF in PBS was used in treatment groups; the same volume of PBS was used in the control groups. In the cell proliferation and migration inhibition assays, the same volume of Me₂SO (the solvent for inhibitors) was used for negative control groups.

Cell proliferation assay

Cell proliferation analysis was conducted in 12-well cell culture plates (Corning Inc.), 2-0 × 10⁶ HEKs per well were plated and grown in the keratinocyte growth medium without transferrin but supplemented with holo-hLF (200 µg mL⁻¹) in the presence or absence of 50 µmol L⁻¹ PD98059 (Sigma-Aldrich, St Louis, MO, U.S.A.) for 5 days. Each treatment was tested in triplicate and repeated twice. Cell counting was performed using a Vi-Cell analyzer (Beckman Coulter, Fullerton, CA, U.S.A.) after 1, 2, 3, 5, 6 and 7 days of incubation. In the further assay, HEKs were cultured for 5 days in EpiLife™ supplemented with HKGS without human EGF but with the addition of 0, 100 or 200 µg mL⁻¹ holo-hLF. As a positive control, cells were cultured in the same medium without holo-hLF but with 0-2 ng mL⁻¹ human EGF.

Cell proliferation inhibition assay with MEK1 inhibitor PD98059

In 24-well cell culture plates (Corning Inc.), 2-0 × 10⁶ HEKs per well were plated and grown in the keratinocyte growth medium without transferrin but supplemented with holo-hLF (200 µg mL⁻¹) in the presence or absence of 50 µmol L⁻¹ PD98059 (Sigma-Aldrich, St Louis, MO, U.S.A.) for 14 h. For the TPA test, cells were incubated in 1:1 mixture of Medium 154 (Cascade Biologics) and Epi-Life™ supplemented with HKGS 16 h before initial treatments. For the starving test, HEKs were incubated in Medium 154 without HKGS and treated with or without 200 µg mL⁻¹ of holo-hLF for 14 h. For the TPA test, cells were incubated in 1:1 mixture of Medium 154 and Epi-Life™ supplemented with HKGS in the presence of TPA 100 ng mL⁻¹ (in Me₂SO stock solutions) (Sigma-Aldrich) and treated with or without 200 µg mL⁻¹ of holo-hLF at 37 °C for 14 h. For the starving plus TPA study, cells were treated similarly as for the TPA test, but incubated in basal Medium 154 without HKGS. Each treatment was performed in triplicate and repeated three times. Cell viability was determined by the calcein-AM/propidium iodide staining method. 30 Briefly, culture medium was replaced with 1 µmol L⁻¹ calcein-AM (Sigma-Aldrich) and 5 µg mL⁻¹ PI (Sigma-Aldrich) in Medium 154 for 20 min at 37 °C. Each cell was examined immediately using an AxioVert fluorescence microscope (Carl Zeiss, Thornwood, NY, U.S.A.). In each case, at least eight different random fields were examined, and a minimum of 1000 cells were counted to determine the fraction of calcein-AM+ (green, viable) cells.

Cell viability analysis and calcein-AM/propidium iodide staining

Cell death was induced by starving and/or 12-0-tetradecanoylphorbol-13-acetate (TPA) exposure. In 24-well cell culture plates (Corning Inc.), 1-2 × 10⁵ HEKs per well were plated in 1:1 mixture of Medium 154 (Cascade Biologics) and Epi-Life™ supplemented with HKGS 16 h before initial treatments. For the starving test, HEKs were incubated in Medium 154 without HKGS and treated with or without 200 µg mL⁻¹ of holo-hLF for 14 h. For the TPA test, cells were incubated in 1:1 mixture of Medium 154 and Epi-Life™ supplemented with HKGS in the presence of TPA 100 ng mL⁻¹ (in Me₂SO stock solutions) (Sigma-Aldrich) and treated with or without 200 µg mL⁻¹ of holo-hLF at 37 °C for 14 h. For the starving plus TPA study, cells were treated similarly as for the TPA test, but incubated in basal Medium 154 without HKGS. Each treatment was performed in triplicate and repeated three times. Cell viability was determined by the calcein-AM/propidium iodide staining method. 30 Briefly, culture medium was replaced with 1 µmol L⁻¹ calcein-AM (Sigma-Aldrich) and 5 µg mL⁻¹ PI (Sigma-Aldrich) in Medium 154 for 20 min at 37 °C. Each cell was examined immediately using an AxioVert fluorescence microscope (Carl Zeiss, Thornwood, NY, U.S.A.). In each case, at least eight different random fields were examined, and a minimum of 1000 cells were counted to determine the fraction of calcein-AM+ (green, viable) cells.
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Cell scratch migration assay and migration inhibition assay

Scratch migration assay, an in vitro incisional wound model, was performed as previously described. Brieﬂy, cells were grown to confluency in 12-well cultureplates (Corning Inc.) in Epilife® supplemented with HKGS. Then, a cross-shaped wound (no cell zone) was made among the cells with a pipette tip. The remaining cells were continually incubated in growth medium without transferrin but with 0, 10 or 100 µg mL⁻¹ holo-rhLF added in the presence of 10 µg mL⁻¹ mitomycin (Sigma-Aldrich) for 5 days. The complete medium with 5 µg mL⁻¹ bovine transferrin (LF0 + Tf) was used as the positive control. The migration of cells was recorded by phase-contrast microscopy (Carl Zeiss) at time 0 (immediately after the scratch) and every 24 h for 5 days. The area of open gap left was measured using AxioVision 4.7 software (Carl Zeiss). Two independent experiments were performed with each in triplicate. In a further migration inhibition assay, 200 µg mL⁻¹ holo-rhLF was used in the media and with addition of 50 µmol L⁻¹ MEK1 inhibitor PD98059, 2 µmol L⁻¹ Src family tyrosine kinase inhibitor PP1 or 40 µmol L⁻¹ Rho kinase inhibitor Y27632 (PP1 was purchased from BIOMOL Intl, Plymouth Meeting, PA, U.S.A.; all other inhibitors were from Sigma-Aldrich).

Generation of lentiviral low-density lipoprotein receptor-related protein 1 short hairpin RNA construct, normal human epidermal keratinocyte transfection and scratch migration assay

Small interfering RNA of the LRP1 sequence was used to design a short hairpin RNA (shRNA) construct (sense: 5'-Phos-tgaaggagcttgattcatgaagatctcaaatcaattataacctctttcttc-3'; antisense strand: 5'-Phos-cgagaaaaaggagcttgattcatgaagatctcaaatcaattataacctctttcc-3') and cloned into lentiviral delivery vector pLL3.7 (Addgene, Inc., Cambridge, MA, U.S.A.) that contains an enhanced green ﬂuorescent protein (EGFP) expression cassette and was modiﬁed by the addition of a neomycin-resistant gene. Vectors carrying the LRP1 shRNA were transfected into HEKs using Lipofectamine 200 (SigmaGen Laboratories, Gaithersburg, MD, U.S.A.) according to the manufacturer’s protocol. Vectors containing nonspeciﬁc LUC shRNA were used as a control. Cells were selected with G418 sulphate (Mediatach) at 200 µg mL⁻¹ for 5 days. Cells of the second passage were used to conduct the scratch migration assay for 3 days, as described above, in the presence or absence of 200 µg mL⁻¹ holo-rhLF. The expression of human LRP1 mRNA (primers, sense: 5'-tgacctcggtctctcg-3'; antisense: 5'-gcagggacccgctgctg-3', NM_003332.2) was detected by semiquantitative reverse transcription–polymerase chain reaction. Human β-actin was run as an internal control (primers, sense: 5'-tctttaatgt-cacgcacgatttc-3', antisense: 5'-ctccttaatgt-cacgcacgatttc-3', GI: 28251).

In vivo study using porcine second-degree burn wound model

Four young female speciﬁc pathogen-free pigs (Ken-O-Kaw Farms, Windsor, IL, U.S.A.) weighing 25–30 kg were used for the in vivo experiment. Second-degree burn wounds, a total of 60 wounds (10 for each treatment group), were made on the backs of the animals according to a previously described method. Covalently linked gelatin hydrogels were prepared according to the method by Otani et al. Either pis- or holo-rhLF was added to the gelatin to make rhLF/hydrogels, or to PBS buffer for rhLF/PBS solutions, to ﬁnal concentrations of 167 mg mL⁻¹. Each wound was treated daily with 300 µL of rhLF/hydrogel or rhLF/PBS (containing 50 µg of rhLF). Equal volumes of gelatin and PBS were used as controls. Wound biopsies were taken on day 4 post-wounding and processed for haematoxylin and eosin staining for histological analysis of wound re-epithelialization.

Statistical analysis

GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, U.S.A.) was used for statistical analysis. Data were analysed using an ANOVA followed by an unpaired two-tailed Student’s t-test for the cell proliferation, migration and attachment assays and porcine wound study; an unpaired two-tailed Student’s t-test was used for the cell viability and TUNEL assays. P < 0.05 was considered signiﬁcant.

Results

Rice-derived recombinant human lactoferrin stimulates human keratinocyte proliferation

Transferrin is a component of HKGS used in cell culture medium for keratinocyte growth. Because LF is a member of the transferrin family, we ﬁrst examined the effect of holo-rhLF on keratinocyte proliferation in comparison with transferrin. As shown in Figure 1a, holo-rhLF signiﬁcantly promoted keratinocyte proliferation at the lower concentration of 10 µg mL⁻¹ (LF0–Tf) at day 5 and day 6 compared with the group without LF (LF0–Tf) (P < 0.01). Much stronger stimulatory effects of holo-rhLF on keratinocytes were observed at
of LF on keratinocyte proliferation, we interfered with cell proliferation using PD98059, an inhibitor of MEK1 (MAPK/ERK kinase 1). As shown in Figure 1b, compared with cells treated with 200 μg mL⁻¹ holo-rhLF (LF200–Tf), the addition of 50 μmol L⁻¹ PD98059 (LF200–Tf+PD98059) significantly blocked holo-rhLF-enhanced cell proliferation at day 3 and day 5 (both P < 0.001), which indicates that MAPK activation may be involved in LF-induced cell proliferation.

**Rice-derived recombinant human lactoferrin stimulates human keratinocyte proliferation in the absence of epidermal growth factor**

Because EGF is a well-known potent growth factor for keratinocyte proliferation, we tested the effects of holo-rhLF in comparison with EGF. As shown in Figure 2, both 100 (LF100–EGF) and 200 μg mL⁻¹ holo-rhLF (LF200–EGF) significantly promoted keratinocyte proliferation on day 5 (both P < 0.01) compared with the negative control group (LF0–EGF), although less potent than the EGF (LF0+EGF).

**Rice-derived recombinant human lactoferrin protects keratinocytes from starving- and 12-O-tetradecanoylphorbol-13-acetate-induced cell death**

Acute TPA treatment has been shown to induce keratinocyte detachment, growth arrest or apoptosis in vitro. Serum withdrawal or growth factor depletion is also a well-known stimulator of cell apoptosis. We treated keratinocytes with growth factor depletion (starving) and/or TPA exposure and

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**Fig 1.** (a) Holo-rhLF stimulates keratinocyte proliferation. HEKs were plated at 2 × 10⁴ per well in 12-well plates at day 0 and cultured for 7 days. The numbers indicate the total cell number X 10⁴ per well counted. LF0–Tf, LF10–Tf and LF100–Tf refer to holo-rhLF (LF) at concentrations of 0, 10 and 100 μg mL⁻¹, respectively, without transferrin (Tf); LF0+Tf refers to positive control with 5 μg mL⁻¹ transferrin without LF. Data were expressed as mean ± SE of values from two independent experiments, each in triplicate (n = 6); * and ** denote statistically significant change with P < 0.01 and P < 0.001, respectively, compared with control groups of no added holo-rhLF and without transferrin (LF0–Tf) or with transferrin (LF0+Tf).

(b) MEK1 inhibitor PD98059 blocks the stimulatory effects of cell proliferation induced by LF. HEKs were plated at 2 × 10⁴ cell mL⁻¹ in 24-well plates at day 0 and cultured with holo-rhLF (200 μg mL⁻¹) in the presence or the absence of 50 μmol L⁻¹ PD98059 for 5 days. The numbers indicate the total cell number per well counted at day 3 and day 5 after initial treatments. LF0–Tf and LF200–Tf refer to media with 0 or 200 μg mL⁻¹ holo-rhLF, LF200–Tf+PD98059 refers to medium with 200 μg mL⁻¹ holo-rhLF and 50 μmol L⁻¹ PD98059; all three in the absence of transferrin (−Tf). Data were expressed as mean ± SE of values from two independent experiments, each in triplicate (n = 6); * denotes statistically significant change with P < 0.01. holo-rhLF, rice-derived recombinant human lactoferrin; HEKs, normal human epidermal keratinocytes.

**Fig 2.** Holo-rhLF stimulates human keratinocyte proliferation in the absence of EGF. HEKs were plated at 2 × 10⁴ per well in 12-well plates at day 0 and cultured for 5 days. The numbers indicate the total cell number X 10⁴ per well counted at days 1, 3 and 5 after initial treatments. LF0–EGF, LF100–EGF and LF200–EGF refer to 0, 100 and 200 μg mL⁻¹ of holo-rhLF without EGF, respectively; LF0+EGF refers to 0.2 ng mL⁻¹ EGF added without holo-rhLF, used as positive control. Data were expressed as mean ± SE of values from two independent experiments, each in triplicate (n = 6); * denotes statistically significant change with P < 0.01 by unpaired two-tailed Student’s t-test, compared with negative control without holo-rhLF and EGF (LF0–EGF). holo-rhLF, rice-derived recombinant human lactoferrin; EGF, human epidermal growth factor; HEKs, normal human epidermal keratinocytes.
examined the effects of holo-rhLF on cell survival using calcein-AM/PI staining. As shown in Figure 3, the supplementation of 200 μg mL⁻¹ holo-rhLF in culture media of HEKs treated by starving, TPA, or starving plus TPA significantly increased the cell viability from 58.95% to 88.86%, 66.73% to 90.58%, and 47.21% to 77.29%, respectively (all P < 0.001).

To examine further the effect of holo-rhLF on keratinocyte apoptosis a TUNEL assay was performed. As shown in Figure 4, holo-rhLF at 200 μg mL⁻¹ greatly decreased the proportions of TUNEL+ (apoptotic) cells from 33.06% to 5.54% (P < 0.001) under the starving condition, and from 34.78% to 2.82% (P < 0.0001) under the TPA exposure condition.

**Rice-derived recombinant human lactoferrin stimulates keratinocyte migration**

An early event in the process of wound repair is the migration of keratinocytes from wound edges into the wounded area, which is critical for the timely healing. The cell scratch assay was used to study the effects of holo-rhLF on keratinocyte migration. As shown in Figure 5, the keratinocytes

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**Fig 3.** Holo-rhLF increases cell viability and protects keratinocytes from starving- or TPA-induced cell death. HEKs grown in 24-well plates were treated with growth factor depletion (starving, left panel) or exposed to 100 ng mL⁻¹ TPA (middle) or both (right) for 14 h in the absence (−LF, top) or presence (+LF, bottom) of 200 μg mL⁻¹ of holo-rhLF. Cell viability was examined by calcein-AM/PI staining assay. (a) Representative fields for different treatments. Calcein-AM staining positive cells (green, viable), PI staining positive cells (red, dead). Scale bar = 50 μm. (b) Proportions of calcein-AM+ cells (viable) were quantified by examining a minimum of eight independent fields, counting a minimum of 1000 cells. LF0 and LF200 refer to holo-rhLF at 0 and 200 μg mL⁻¹, respectively. Data are expressed as mean ± SE of values from three independent experiments, each in triplicate (n = 9); * denotes statistically significant change with P < 0.001. holo-rhLF, rice-derived recombinant human lactoferrin; TPA, 12-O-tetradecanoylphorbol-13-acetate; HEKs, normal human epidermal keratinocytes; PI, propidium iodide.

**Fig 4.** Holo-rhLF protects keratinocytes from starving- or TPA-induced cell apoptosis. HEKs seeded on eight-well chamber slides were exposed to growth factor depletion medium (starving, left panel) or 100 ng mL⁻¹ TPA (right panel) for 16 h in the absence (−LF) or presence (+LF) of 200 μg mL⁻¹ holo-rhLF. Cell apoptosis was analysed by TUNEL assays. (a) Representative fields for different treatments in TUNEL assay. TUNEL+ (apoptotic or dead) cells are stained dark blue. Scale bar = 50 μm. (b) TUNEL+ cells were quantified by examining at least six independent fields and counting a minimum of 800 cells for each well. Data are expressed as mean ± SE of values from three independent experiments, each in duplicate (n = 6). LF0 and LF200 refer to holo-rhLF at 0 and 200 μg mL⁻¹, respectively; * and ** denote statistically significant changes with P < 0.001 and P < 0.0001, respectively. holo-rhLF, rice-derived recombinant human lactoferrin; TPA, 12-O-tetradecanoylphorbol-13-acetate; HEKs, normal human epidermal keratinocytes; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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Rice-derived recombinant human lactoferrin stimulates wound re-epithelialization

Holo-rhLF stimulates keratinocyte migration in a scratch migration assay. HEKs were grown to confluence in 12-well plates. A cross-shaped ‘wound gap’ (a cell-free zone) was established by scratching with a pipette tip across the monolayer cells. The migrations of the cells (gap filling) were recorded by phase-contrast microscopy connected to a digital camera at time 0 and then every 24 h for a total of 5 days. (a) Representative fields of wound gap filling by keratinocytes, at time 0, day 3 and day 5, under phase-contrast microscopy. –LF and +LF refer to holo-rhLF at 0 and 100 µg mL⁻¹, respectively. Scale bar = 100 µm. (b) Graphic analysis of keratinocyte migration. LF0–Tf, LF10–Tf and LF100–Tf refer to holo-rhLF (LF) at 0, 10 and 100 µg mL⁻¹ without transferrin (Tf), respectively; LF0+Tf refers to the positive control without LF but with 5 µg mL⁻¹ transferrin. Data are expressed as mean ± SE of values from three independent experiments, each in duplicate (n = 6). Cell migrations were quantified and graphed as a function of time elapsed vs. percentage of the wound open gap left; * and ** denote statistically significant changes with P < 0.05 and P < 0.01, respectively. holo-rhLF, rice-derived recombinant human lactoferrin; HEKs, normal human epidermal keratinocytes.

MAPK/ERK, Src and Rho/ROCK pathway inhibitors attenuate rice-derived recombinant human lactoferrin-enhanced cell migration

MAPK/ERK, membrane-associated tyrosine kinase Src and small GTPase Rho have been shown to be involved in the regulation of cell migration. To test whether holo-rhLF-enhanced keratinocyte migration is involved in these signalling pathways, we further examined the effects of holo-rhLF on keratinocyte migration in the presence of specific pathway inhibitors. As shown in Figure 6, the holo-rhLF-enhanced keratinocyte migration was greatly inhibited by PD98059 (MAPK/ERK kinase 1 inhibitor, P < 0.001), PP1 (Src inhibitor, P < 0.001) and Y27632 (Rho inhibitor, P < 0.05), respectively. Our results suggest that the MAPK/ERK, Src and Rho/ROCK signalling pathways may participate in holo-rhLF-enhanced keratinocyte migration.

Downregulation of low-density lipoprotein receptor-related protein 1 suppresses holo-rhLF-promoted cell migration

To test if holo-rhLF functions through the LRP1 receptor, we generated a lentiviral LRP1 shRNA construct to knockdown the expression of LRP1 in HEK. As shown in Figure 7, the addition of holo-rhLF (200 µg mL⁻¹) greatly promoted the migration of cells transfected with control vector; the percentages of remaining open gaps in cells treated with (control shRNA+LF) or without holo-rhLF (control shRNA) were 33±9% and 68±17% (P < 0.001), respectively. In contrast, the addition of holo-rhLF had no significant effect on the migration of cells with LRP1 receptor knockdown (LRP1 shRNA+LF); the percentage of remaining open gaps was 82±39%. The data demonstrated that holo-rhLF-stimulated cell migration was suppressed by downregulation of LRP1. These data indicate that interaction between LF and receptor LRP1 contributes to the positive effects of LF on skin keratinocyte migration.

Rice-derived recombinant human lactoferrin stimulates wound re-epithelialization in vivo

To study further the role of holo-rhLF in wound repair, we conducted in vivo wound re-epithelialization analysis using...
Porcine second-degree burn wound model. Histological examination revealed that the holo-rhLF in either gel or PBS formulation exhibited stimulating effects on wound re-epithelialization. As shown in Figure 8, the average percentages of re-epithelialization were 74% in wounds treated with holo-rhLF⁄hydrogel (B), compared with 53Æ6% in control wounds treated with hydrogel alone (C) (P = 0Æ0125), 77Æ4% in wounds treated with holo-rhLF⁄PBS (E), and 56Æ6% in control wounds with PBS alone (F) (P = 0Æ572). No significant difference was observed in pis-rhLF treatment groups (A and D) compared with control groups (C and F).

Discussion

This study has demonstrated that holo-rhLF stimulates keratinocyte proliferation. More importantly, our results reveal that holo-rhLF stimulates keratinocyte proliferation in the absence of transferrin, a component commonly used in cell culture media as an iron source, or EGF, which is a potent stimulator of keratinocyte proliferation. Our results are consistent with earlier reports of stimulatory effects of native LF on cell growth.15-18,20,21,23,28 There are also studies that fail to observe stimulating effects of LF on cell proliferation.17,18 The inconsistent results might be related to the use of different cell lines, the source of LF, amount of iron saturation and the method of preparation.

In this study, holo-rhLF also showed significant promoting effects on keratinocyte migration. Keratinocyte migration and proliferation are two essential steps for re-epithelialization in wound repair. The positive roles we observed of holo-rhLF on both migration and proliferation of human keratinocytes suggest that rhLF may have significant value in wound re-epithelialization. In our preliminary study of a porcine burn wound model, holo-rhLF was found to stimulate wound re-epithelialization, which is consistent with the in vitro findings. Our results are in agreement with the report by Engelmayr et al.25 Talactoferrin was found to increase migration of human THP-1 monocytes, Jurkat T lymphocytes, normal dermal fibroblasts and mouse granulocytes. It was also found to accelerate wound closure in mice by modulating early inflammatory mediators. However, Bournazou et al.29,30 reported that LF (milk- and neutrophil-derived) selectively inhibited chemoattractant-induced migration of neutrophils and eosinophils but not monocytes and macrophages. These inconsistent results regarding LF in cell migration may be related to the different cell lines and animal models used, and differing methods in the preparation of LF. It is also possibly due to different receptor-mediated activities. Our study suggests the
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Fig 7. Downregulation of LRP1 suppresses holo-rhLF-promoted cell migration. Cells transfected with vector containing LRP1 shRNA were used to study the effects of downregulation of LRP1 by LRP1 shRNA on HEK migration induced by holo-rhLF (200 \( \mu \)g mL\(^{-1} \)) by scratch migration assays. (a) Representative fields of wound gap filling by keratinocytes, at time 0 and day 3. Control shRNA and control shRNA+LF refers to cells transfected with vector carrying control shRNA and cultured in medium supplemented without or with 200 \( \mu \)g mL\(^{-1} \) holo-rhLF, respectively; LRP1 shRNA+LF refers to cells transfected with vector carrying LRP1 shRNA and cultured in medium supplemented with 200 \( \mu \)g mL\(^{-1} \) holo-rhLF. Scale bar = 100 \( \mu \)m. (b) Graphic analysis of HEK migration with different treatments. Data are expressed as mean ± SE of values from three independent experiments, each in duplicate (n = 6). * denotes statistically significant difference with P < 0.05. A, pis-rhLF/hydrogel; B, holo-rhLF/hydrogel; C, hydrogel without LF; D, pis-rhLF/PBS; E, holo-rhLF/PBS; F, PBS without LF. holo-rhLF, rice-derived recombinant human lactoferrin; pis, partially iron saturated; LF, lactoferrin; PBS, phosphate-buffered saline.

Fig 8. Holo-rhLF stimulates wound re-epithelialization in a porcine wound model. Second-degree burn wounds were treated with 50 mg of pis- or holo-rhLF in gelatin–LF hydrogels or LF/PBS solutions for 4 days. The bars represent the mean ± SE of values of percentage of wound re-epithelialized under microscopy (n = 10). * indicates statistically significant difference with P < 0.05. A, pis-rhLF/hydrogel; B, holo-rhLF/hydrogel; C, hydrogel without LF; D, pis-rhLF/PBS; E, holo-rhLF/PBS; F, PBS without LF. holo-rhLF, rice-derived recombinant human lactoferrin; pis, partially iron saturated; LF, lactoferrin; PBS, phosphate-buffered saline.

Involvement of LRP1 receptor in holo-LF-enhanced keratinocyte migration.

Importantly, we found that holo-rhLF greatly enhanced cell viability and decreased cell apoptosis when keratinocytes cell death was induced by growth factor depletion or TPA exposure. This protective effect of holo-rhLF may be another potential beneficial role of LF in wound healing.

As a member of the transferrin family, LF plays a role in iron uptake. The positive effects of holo-rhLF on cell proliferation were found to be related to the extent of iron saturation in modified rhLF. Holo-rhLF (> 90% iron saturation) was reported to induce higher proliferation activity than pis-rhLF (about 50% iron saturation), which likewise had higher effect than apo-rhLF (< 10% iron saturation).08 Besides iron uptake and delivery, the molecular mechanism by which LF modulates cell functions remains unknown. LF has been found to bind to receptors in the cell surface and then enter the cell cytoplasm and the nuclei to interact with cellular DNA.22,41 Recent studies have shown that LF induced mitogenic effects in osteoblast cells via receptor LRP141 while exerting its anti-apoptotic activity through an LRP1-independent pathway.42

In this study, we observed that keratinocyte migration was suppressed by blocking LRP1 expression, which highlights the presence of LRP1 in holo-rhLF-enhanced cell migration and extends our understanding of the mechanism of hLF in modifying skin cellular function. Our study also found that LF-induced cell proliferation was attenuated by MAPK/ERK inhibitor and that the hLF-enhanced cell migration was significantly blocked by inhibitors of MAPK/ERK, Src and Rho/ROCK. Our findings strongly suggest that these signal transduction pathways may be involved in hLF-stimulated cell proliferation and migration.

Decreased cell proliferation, delayed cell migration and increased cell apoptosis during wound healing are common adverse events that impede tissue repair. Delayed wound healing increases the possibility of open wound infection, which can further impair healing. LF is well known for its antimicrobial and anti-inflammatory effects,14 but none of the currently available growth factors such as EGF has similar effects. Our studies demonstrate that holo-rhLF may be beneficial in wound healing. LF might act as a growth factor for keratinocytes, which may complementarily stimulate keratinocyte activities when the function of EGF is impaired due to the nonresponsiveness of keratinocytes or deficiency of EGF receptors, such as in chronic wounds.

In summary, this study revealed that holo-rhLF enhances the biological functions of human skin keratinocytes and stimulates wound re-epithelialization. To our knowledge, this is the first study to document that hLF has potent direct effects...
on the functions of human skin keratinocytes, including stimulating cell proliferation and migration as well as protecting cells from starving- and TPA-induced cell death. Further studies are warranted to determine the mechanisms of LF activities and its potential therapeutic applications in wounds.

What’s already known about this topic?
- Lactoferrin is known for its antimicrobial and immunomodulation effects.

What does this study add?
- This study, using recombinant human lactoferrin, demonstrates direct stimulating effects of lactoferrin on wound re-epithelialization including:
  - enhancing keratinocyte proliferation and migration;
  - protecting keratinocytes from apoptosis;
  - promoting wound re-epithelialization in a porcine wound model;
  - modulating cell function via MAPK, Src and Rho/ROCK pathways and LRP1 receptor.

Acknowledgments
This work was supported partially by NIH SBIR research grant R43 GM079025 (J.L.) and by funding from the Dermatology Foundation of South Florida (J.L.). We thank Mr Ramon Montero for his help in preparing lactoferrin hydrogel.

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