Wool-derived keratin stimulates human keratinocyte migration and types IV and VII collagen expression

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Abstract: Keratinocyte migration is essential for wound repair. Keratin-based products have recently shown stimulatory effects on wound repair. This study was to test the cellular response to wool-derived oxidized keratin in wound healing to further understand the biological mechanisms underlying observed clinical benefits of keratin-based products as wound treatments. *In vitro* scratch migration assays examined the effects of oxidized keratin on the migration of human skin keratinocytes. Western blotting analysis determined the effects on the marker protein expression of type IV and type VII collagens and keratin 17. We found wool-derived oxidized keratin promoted keratinocyte migration and induced protein expression of type IV and type VII collagens, but not keratin 17. The data suggest that the beneficial effects of keratin-based treatment in wounds may be related to its positive effects on re-epithelialization via stimulating keratinocyte migration and production of basement membrane proteins of types IV and VII collagens.

Key words: collgen expression – human skin keratinocyte – migration – oxidized keratin

Background

The ability of keratinocytes to migrate is critical for wound re-epithelialization (1–4). Keratins are the major protein in keratinocytes and are essential for many cellular functions, such as cell migration (1). Up-regulation of keratin expression has been observed in response to wounding (1–3). Keratin powder dressings have been shown to release keratin peptides into the wound, provide physical support to cell attachment, proliferation and survival and promote wound healing. Their non-immunogenic nature allows keratins to be well tolerated and incorporated by host tissues during reparative processes (5,6).

The importance of this fibrous protein in biological processes, and in particular in the healing wound, has been highlighted through work demonstrating keratin plays a crucial role in healing (7), and additionally as an indicator in chronic wounds where down regulation of keratins has been associated with poor healing (8). The use of exogenous keratin protein in the treatment of tissue injuries has been the subject of extensive research with positive healing responses found in the areas of topical wound care (9), orthopaedic implants (10) and nerve regeneration (11). Keratin-based wound care devices have been approved for use in several regions of the world including use as a topical agent for wound care (9). With keratin intermediate filaments as major components, ovine wool shares high similarity with human hair (12). Clinicians using wool-derived oxidized keratin have found improved healing for hard to heal chronic wounds (13). In addition, as part of a wound treatment regime for patients with epidermolysis bullosa with severe blistering and ulceration, regular use of oxidized keratin has led to reduced blister frequency and improved healing rates (14).

Questions addressed

This study was to test the cellular response to wool-derived oxidized keratin (OKHP) (15), in cell migration and marker gene expression, to elucidate the biological mechanisms underlying observed clinical benefits of keratin-based wound treatment.

Experimental design

Oxidized keratin was tested in human skin primary keratinocytes in a scratch migration assay (16). The cell extracts were analysed by Western blot for protein expression of collagen types IV and VII and keratin 17 (see Data S1).

Results

Oxidized keratin increased keratinocyte migration

The significant enhanced migration was observed starting day 1 when cells were grown in keratinocyte basal medium EpiLife without supplement of HKGS and treated with OKHP at concentrations of 20 or 50 μg/ml compared with negative control without OKHP. The keratinocytes migrated inwardly and filled the gap faster in the presence of OKHP compared with cells without OKHP treatment from day 1 to day 4 (Fig. 1a). At day 1 and day 2, the gap filled was 3.38% and 30.29% in negative control cells without OKHP, which was significantly lower than groups treated with 20 μg/ml OKHP (8.79% and 36.27%, $P < 0.01$) or 50 μg/ml OKHP (6.40% and 34.55%, $P < 0.05$). Greater differences were observed at later stages. At days 3 and 4, the gap filled was 59.46% and 67.75% in negative control without OKHP, 80.36% and 88.48% or 77.10% and 88.63% in cells treated with 20 or 50 μg/ml OKHP, respectively (all $P < 0.01$, Fig. 1b). Furthermore, the cells treated with OKHP migrated at a rate similar to that of positive control group (EpiLife supplemented with 12.5% HKGS without OKHP) (Fig. 1a,b).

Oxidized keratin stimulated type IV and type VII collagen expression but not keratin 17

To further explore the potential mechanisms involved, we sought to examine the effects of OKHP on the expression of types IV and VII collagens and keratin 17 during keratinocyte

Experimental Dermatology

(a)

(b)

(a)

(b) Type IV collagen

(c) Type VII collagen
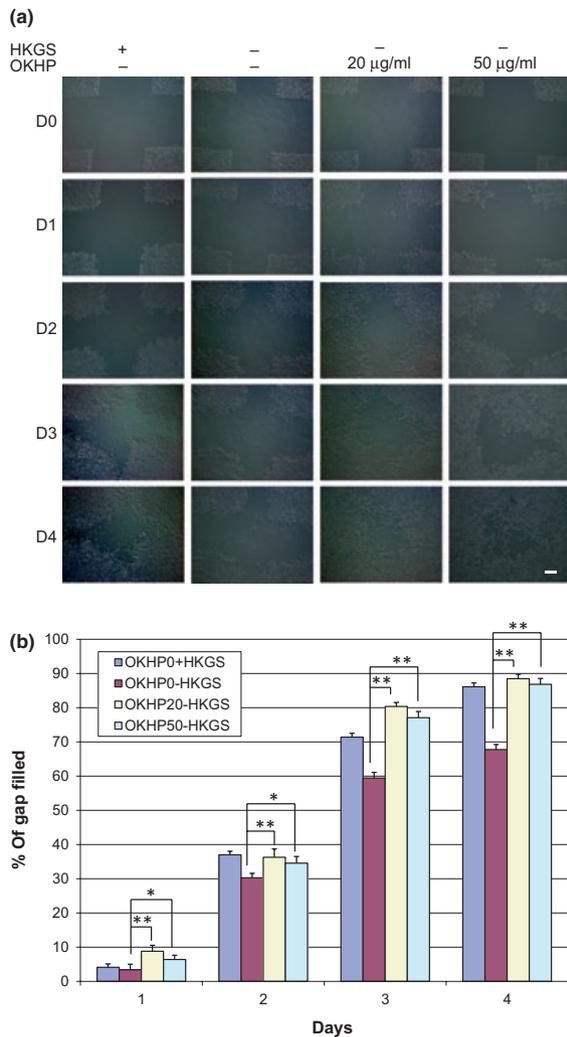
(d) Keratin 17

Figure 1. Oxidized keratin (OKHP) increased human skin keratinocyte migration in a scratch migration assay. Human skin primary keratinocytes 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. Cells were maintained in EpiLife basal medium without growth supplement or with 12.5% human keratinocyte growth supplement (+HKGS) as positive control. The migrations of the cells (gap filling) were recorded by phase-contrast microscopy at time 0 (D0, immediately after scratch) and then every 24 h for a total of 4 days. (a) Representative fields of wound gap filling by keratinocytes, at days 0, 1, 2, 3 and 4. Scale bar = 100 μm. (b) Graphic analysis of keratinocyte migration. OKHP0-HKGS, OKHP20-HKGS and OKHP50-HKGS refer to media with OKHP at 0, 20 and 50 μg/ml, respectively, without HKGS; OKHP0 + HKGS refers to medium without OKHP but supplemented with 12.5% HKGS. Data are expressed as mean ± SD of values from two independent experiments, each in triplicate ($n = 6$). Cell migrations were quantified and graphed as a function of time elapsed versus percentage of the wound open gap filled; * and ** denote statistically significant changes with $P < 0.05$ and $P < 0.01$, respectively.
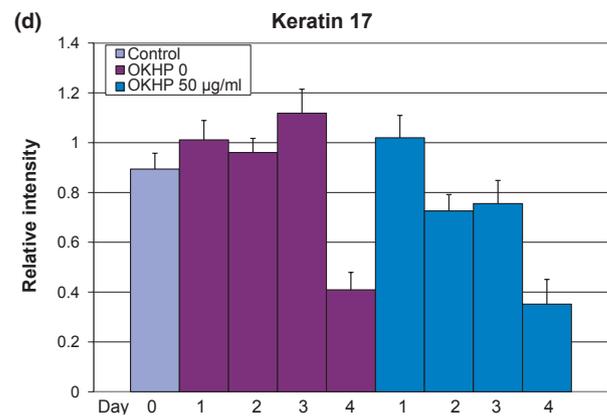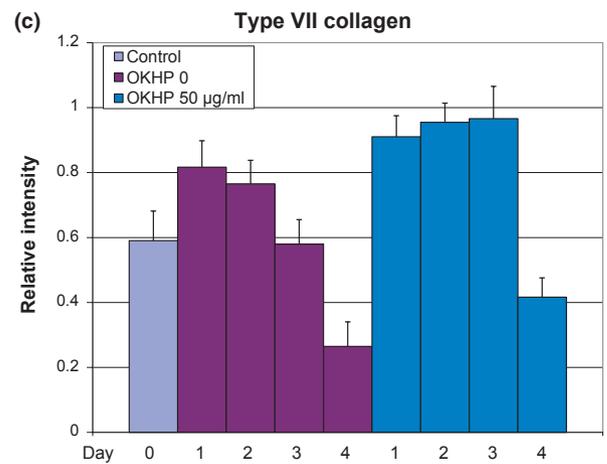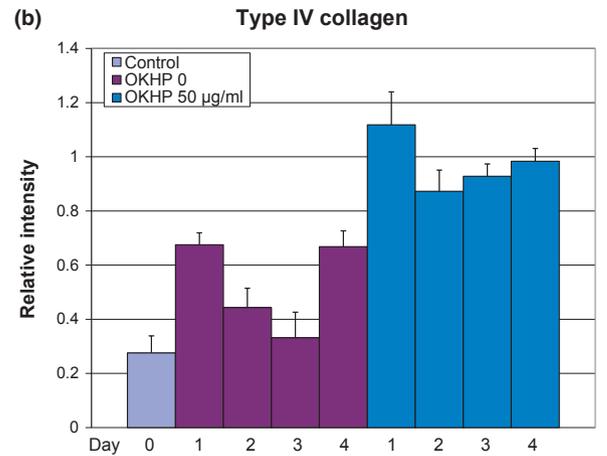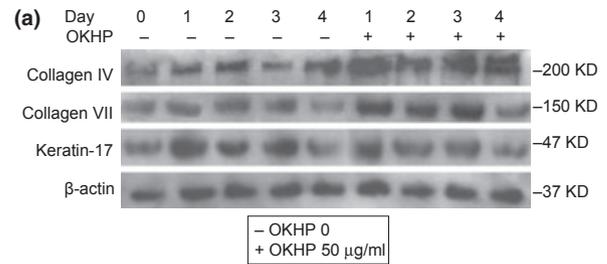
Figure 2. Oxidized keratin (OKHP) stimulated type IV and type VII collagen expression but not keratin 17. Keratinocyte scratch assays were conducted in 12-well plates for 4 days with 0 or 50 μg/ml of OKHP. Cell lysates were collected at days 0, 1, 2, 3 and day 4, and protein was extracted as indicated in the Materials and Methods. (a) Western blot analysis: Equal amount of protein of 20 μg was resolved by SDS–PAGE gels and analysed by Western blotting using antibodies specific to type IV and type VII collagens and keratin 17. Blotting with anti-β-actin was conducted as a protein loading control. Images are representative of results from one of three independent experiments. (b–d) Graphic analysis of protein expressions of type IV collagen (b), type VII collagen (c) and keratin 17 (d). Bar graphs represent the relative intensities of protein expression normalized to β-actin. Data are mean ± standard error of values from three independent experiments.

migration. Results of Western blotting showed that OKHP could markedly induce protein expression of type IV collagen during keratinocyte migration (Fig. 2a,b). The strongest induction was observed at day 1, while significant increased protein inductions were seen during the entire time-course of cell migration compared with the control no-treatment group. Similar to the type IV collagen, OKHP also stimulated protein expression of type VII collagen (Fig. 2a,c). OKHP did not show the stimulating effects on keratin 17 protein production in migrating cells (Fig. 2a,d).

Conclusions

We found faster cell migration and stimulation of collagen expression with OKHP. The positive effects of OKHP on keratinocyte migration and production of types IV and VII collagen suggest that OKHP may have significant value in wound healing, particularly in chronic non-healing wounds where the keratinocytes are defect in migration or matrix production.

Migration of keratinocytes is the first step in and is essential to wound re-epithelialization. The loss of continuity between epidermis and dermis upon injury allows the keratinocytes to come in contact with collagens in the dermis and start the repair process migrating across the wound bed (17,18). In addition to being major cells for re-epithelialization, keratinocytes play an important role in the reconstitution of basement membrane (BM) in the dermal–epidermal junction that holds the dermis and epidermis together and is critical for skin integrity. Types IV and VII collagens are two of major components of the BM (19–22). The increased production of these two collagens in keratinocytes treated with OKHP suggests it may also stimulate the BM formation.

The importance of keratin filaments and collagen matrix in skin integrity has been demonstrated through a group of human hereditary blistering diseases, Epidermolysis Bullosa (EB), characterized by an easily blistering and fragile epithelium (1). EB Simplex and Dystrophic EB are two common types of EB because of mutations in the gene of keratins 5 or 14 (EB Simplex) and type VII collagen (Dystrophic EB) (18,23–25).

Our data are consistent with a recent report that OKHP stimulated the re-epithelialization in a porcine wound model (26). Furthermore, our results support the clinical observation that regular use of OKHP treatments in patients with a severe form of EB led to reduced blister frequency and improved healing rates (14). The healing effects of OKHP may be due to stimulating keratinocyte migration and/or induction of the expression of collagen IV and collagen VII. More studies are needed to validate the hypothesis and to further elucidate the mechanisms.

Acknowledgement

Author contributions

Authors indicate their specific contributions to this manuscript as the following: Ling Tang performed the research experiments; Jie Li, Rob Kelly, Robert S. Kirsner designed the research study; Rob Kelly contributed essential reagent; Ling Tang, Jose Ollague Sierra, Jie Li analyzed the data; Ling Tang, Jose Ollague Sierra wrote the paper; Jie Li, Rob Kelly, Robert S. Kirsner edited the paper.

Conflict of interests

Rob Kelly is an employee of Keraplast Technologies, LLC, whose product was studied in the present work. Robert Kirsner is a consultant for Keraplast Technologies, LLC. All other authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

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SUPPORTING INFORMATION

Materials and Methods

Reagent

Lyophilized powder of wool-derived oxidized keratin protein peptides (OKHP) used in this study was provided by Keraplast Technologies, LLC (San Antonio, Texas). The OKHP stock solution was freshly prepared by dissolving OKHP powder in phosphate-buffered saline (PBS) at a concentration of 50 mg/mL. It was diluted to working solutions with cell culture medium prior to use. OKHP was derived from New Zealand sheep wool and was prepared by strong oxidation of wool, under acidic conditions, to substantially break the disulfide bonds that make keratins insoluble whilst leaving the keratin protein backbone intact. Specifically 100g of undyed chopped, New Zealand Romney wool, was combined with 1565ml of distilled deionized water and 110ml of 30% hydrogen peroxide. The mixture was heated to reflux for 2 hours, cooled to 50°C and filtered using a Buchner funnel and fast filter paper in order to remove hydrogen peroxide. The filtered solids were rinsed with 250ml of distilled deionized water to further remove residual hydrogen peroxide. Subsequent dissolution at alkali pH using 4L of 0.1N ammonium hydroxide created a mixture of keratin proteins and peptides which were filtered and then lyophilized to create a soluble keratin powder (15). The chemical nature of the OKHP is very similar to that of native keratin in two important ways. The molecular weight profile of OKHP by gel permeation chromatography is consistent with the presence of proteins from both of these categories, with material of Mw characteristic of both high sulfur proteins (20-30kD) and low sulfur proteins (40-60kD). Similarly, the sulfur content of OKHP (from amino acid analysis) as well as the rest of the amino acid profile is similar to the wool source, and hence the distribution between high and low sulfur keratins in the OKHP is similar to that in the original material. The method of isolation of the keratin protein is intended to disrupt the cysteine crosslinks in the protein but leave the protein backbone intact, and so preserve the properties of the keratin.

Anti-collagen IV-specific antibody (ab6586) was purchased from Abcam PLC (Cambridge, MA, USA), anti-collagen VII-specific antibody (C6805) and anti-cytokeratin 17-specific antibody (C9179) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA), anti-β-actin-specific antibody (sc-4778) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti rabbit-IgG (sc-2004) and rabbit anti-mouse-IgG (A-9044) were from Santa Cruz Biotechnology and Sigma-Aldrich Co. respectively. All other reagents are purchased from Sigma-Aldrich Co. except those specifically stated.

Cells and cell cultures

Human normal skin primary keratinocytes were isolated from neonatal foreskin from normal circumcision at the University of Miami (approved by the University IRB board) and grown and maintained in a 5% CO_2 humidified tissue culture incubator at 37 °C in EpiLife® Medium, a keratinocyte growth basal medium, supplemented with human keratinocyte growth supplement (HKGS)(Cascade Biologics, Portland, OR, USA) at concentrations of 0.2% v/v of bovine pituitary extract, 5 μg/mL bovine insulin, 0.18 μg/mL hydrocortisone, 5 μg/mL bovine transferrin, 0.2 ng/mL human epidermal growth factor (EGF) plus antibiotic–antimicotic (Mediatech, Manassas, VA, USA) at 100 μg/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. In the cell scratch migration assays, OKHP dissolved in PBS was used in treatment groups; the same volume of PBS was used in the control groups.

Cell scratch migration assay and analysis

Scratch migration assay, an *in vitro* incisional wound model, was performed as previously described (16). Briefly, cells were grown to confluence in 12-well culture plates (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 EpiLife basal medium without HKGS but with 0, 20 or 50 μg/mL OKHP, in the presence of mitomycin C at 10 μg/ml (Sigma-Aldrich), for 4 days. Cell culture supplemented with 12.5% of HKGS was used as positive control. The migration of cells was recorded by phase-contrast microscopy (Carl Zeiss, Thornwood, NY, USA) at time 0 (immediately after the scratch) and every 24 hours for 4 days. The area of gap filled was measured using AxioVision 4.7 software (Carl Zeiss). The center of the cross (where the two scratch lines meet) was used for the positioning. The percentage of gap filled (*PG*) was calculated as $PG = (1 - A_t/A_0) \times 100$, where A_t is the gap area at time t after scratch, A_0 is the gap area at the time 0. Two independent experiments were performed with each in triplicate. Cell migrations were quantified and graphed as a function of time elapsed versus percentage of the gap filled. GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA) was used to statistically analyze cell migration. Data (n=6) was analyzed using an one-way analysis of variance (ANOVA) followed by an unpaired two-tailed Student T-test. A p value of < 0.05 is considered significant.

Western blotting analysis

During cell scratch migration assay, protein samples were isolated daily and proteins from cells before scratch were used as control. Cells were washed with cold PBS twice, then cells were lysed with cell lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1%SDS) with protease

inhibitors on the ice and cell lysates were transferred to microtubes using a cell scraper. The lysates were then cleared by centrifuging for 30 minutes at 14000 rpm at 4°C. After centrifugation, the supernatants were collected and the protein concentration was measured using Protein Assay Dye Reagent (BIO- RAD, Hercules, CA, USA) and quantified with Spectrophotometry (BIO-RAD) by reading at 595 nm. 20 μg of proteins from each treatment were mixed with sample loading buffer (100 mM Tris, 100 mM DTT, 2% SDS, 10% sucrose and 0.006% bromophenol blue) and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a acrylamide gels (6% for collagen IV and 8% for collagen VII and keratin 17) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline, pH 7.4, and containing 0.1% Tween 20 (TBS-T) at room temperature for 1 hour. The membranes were incubated for 2 h at room temperature with each primary antibody (1:1000 dilutions for collagen VII and keratin 17, 1:500 for collagen IV) in TBS-T, and then for 1 h with HRP-conjugated secondary IgG (1:20,000 dilutions, Sigma-Aldrich Co.). After the blots were washed with TBS-T, the immunoreactive proteins were detected with enhanced chemiluminescence detection kit (ECL plus, GE Life Sciences, Piscataway, NJ, USA). The expression of target protein (intensity of the band) was analyzed using Bio-Rad Gel-Doc equipment and Quantity One software and expressed as the relative intensity normalized with β-actin.