Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Cell Research 296 (2004) 123–134
Experimental Cell Research
www.elsevier.com/locate/yexcr

Hyaluronan suppresses epidermal differentiation in organotypic cultures of rat keratinocytes

Alberto Passi,[a] Parrish Sadeghi,[b] Hiroko Kawamura,[c] Sanjay Anand,[c] Nobuyuki Sato,[c] Laura E. White,[b] Vincent C. Hascall,[c] and Edward V. Maytin[b,c,*]

[a] *Department of Biomedical Experimental and Clinical Sciences, University of Insubria, Varese, Italy*
[b] *Department of Dermatology, Cleveland Clinic Foundation, Cleveland, OH 44195, USA*
[c] *Biomedical Engineering, Cleveland Clinic Foundation, Cleveland, OH 44195, USA*

Received 9 October 2003, revised version received 12 January 2004

Available online 19 March 2004

Abstract

Hyaluronan (hyaluronic acid, HA) is an abundant matrix component between keratinocytes of the epidermis in vivo, but its function there remains unclear. We used a lift culture model, in which rat epidermal keratinocytes (REKs) stratify at an air–liquid interface, to ask whether HA may regulate epidermal proliferation and/or differentiation. In this model, early markers of differentiation (keratin 10), and later markers (profilaggrin, keratohyalin granules, cornified layers) are faithfully expressed, both temporally and spatially. HA, measured using two different analytical techniques, accumulated to high levels only in the presence of an intact basement membrane that seals the epidermal compartment. To test whether HA has a functional role in differentiation, *Streptomyces* hyaluronidase (StrepH, 1 U/ml; digests >95% of HA within 4 h) was added daily to lift cultures during stratification time-course experiments over 5 days. In StrepH-treated cultures, the expression of profilaggrin and the number and size of keratohyalin granules were significantly increased relative to controls using semiquantitative histological analyses. The StrepH-related accumulation of K10 protein and profilaggrin/filaggrin were confirmed by Western analyses. Thus, it appears that the presence of intercellular HA in the epidermis acts as a brake upon intracellular events that occur during keratinocyte differentiation.

Keywords: Hyaluronan; Hyaluronic acid; Epidermis; Differentiation; Filaggrin

Introduction

Hyaluronan (HA) is a carbohydrate polymer (glycosaminoglycan) that was long-recognized as an abundant component in the extracellular matrix of connective tissues such as cartilage and the dermis of skin [1]. Because of its very simple structure, consisting of a disaccharide subunit (glucuronic acid and N-acetylglucosamine) repeated thousands of times in a linear fashion, and devoid of sulfates or other modifications [2], HA was thought to be important for passive functions such as tissue hydration and nutrient diffusion [3], but was presumably incapable of active biological functions that might require specificity. That picture has changed dramatically [4]. Recent studies suggest that HA has active tissue-organizing functions mediated through specific HA-binding proteins [5] and cell-surface receptors such as CD44 [6,7] and RHAMM [8]. HA is increasingly implicated in the pathogenesis of diseases that involve the recruitment and activation of inflammatory cells, such as lung inflammation [9] and inflammatory bowel disease [10]. Synthesis and accumulation of HA in pericellular matrices may also be involved in tumor development and metastasis, contributing to the malignant phenotype of melanomas and carcinomas [11,12].

Because HA was first recognized as an abundant component in mesenchymal connective tissues such as the

Abbreviations: BM, basement membrane; bHABP, biotinylated HA-binding protein; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; HA, hyaluronic acid (hyaluronan); hpf, high power field; KHG, keratohyalin granule; MDCK, Maden-Darby canine kidney cell; MW, molecular weight; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; REK, rat epidermal keratinocyte; StrepH, *Streptomyces* hyaluronidase.

* Corresponding author. Biomedical Engineering, Cleveland Clinic Foundation, ND-20, 9500 Euclid Avenue, Cleveland, OH 44195. Fax: +1-216-444-9198.

E-mail address: maytine@ccf.org (E.V. Maytin).

0014-4827/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2004.01.031

dermis [13], the discovery of significant amounts of HA in epithelial tissues such as the epidermis came as rather a surprise when described over a decade ago [14,15]. Although present in small absolute amounts when compared to the dermis, the concentration of HA between keratinocytes of the epidermis actually exceeds that in the dermis [3]. The rapid turnover of epidermal HA ($t_{1/2}$ approximately 1 day in human skin organ culture) [16], further suggests that HA has functional value for the tissue and indeed for the organism, given that the epidermal barrier is essential to life.

HA in the matrix of tissues clearly supports cellular proliferation and migration. Evidence for this includes a strong correlation between high HA levels and embryonic growth of tissues [17], wound healing [18,19], cell locomotion [20], and cancer invasiveness [11]. However, the notion that HA might influence cellular functions in the growth-arrested, terminally differentiating compartments of epithelial tissues, exemplified by the epidermis, is an idea that has not been rigorously tested. A number of compelling lines of evidence suggest that HA in the intercellular space may indeed influence epidermal differentiation as well as proliferation. For example, the anatomical observation that HA in the epidermis of human skin organ cultures is more abundant in spinous than in basal layers [21,22], led investigators to propose that the majority of epidermal HA content and synthesis could be involved in keratinocyte activities other than proliferation, such as maintenance of the extracellular space and cell–cell interactions during differentiation [21]. Correlative evidence for the latter includes the effect of retinoic acid, which stimulates proliferation and inhibits epidermal differentiation; the reduced differentiation is accompanied by an increased HA accumulation in the upper spinous layers [23]. Hydrocortisone, which inhibits epidermal proliferation and enhances terminal differentiation, is accompanied by a decrease in epidermal HA [24]. Blockade of the HA cell-surface receptor, CD44, using an antisense CD44 transgene under the control of a cytokeratin promoter [25] causes an atrophic condition resembling *lichen sclerosus et atrophicus*, a disease that features a thinned, prematurely differentiated epidermis and a decrease in CD44 receptors [26]. All of these correlations suggest that HA may suppress differentiation as well as support proliferation in the epidermis, but up to now there was no convenient way to test this directly.

In the current manuscript, we approach the question of epidermally derived HA and its role in epidermal homeostasis by studying HA in an organotypic model epidermis. We employ an immortalized rat epidermal keratinocyte (REK) line that forms a normal-appearing epithelium when grown on collagen gels in the complete absence of fibroblasts [27,28]. When the collagen layer is covered with an intact basement membrane, the model faithfully preserves the compartmentalization of epidermal HA, confining the HA produced by the keratinocytes to a location above the basement membrane. With this model, HA can be removed from the matrix by an enzymatic technique, and subsequent effects upon epidermal differentiation readily observed. Such experiments would be difficult or impossible to conduct in the skin in vivo because the vast amounts of HA in the dermis would confound measurements of the relatively minor amount of HA in the epidermal compartment. In the REK model, we ask whether the presence of the REK-derived HA in the matrix influences the normal progression of epithelial formation. Our data show that HA serves to coordinate the finely orchestrated sequence of events in epidermal differentiation, with full-length HA acting as a brake upon the progress of terminal differentiation.

Materials and methods

REK cell line

The REK cell line used in this study was isolated by Donald MacCallum from neonatal REKS (a gift from Howard Baden) originally isolated by Baden and Kubilus [29]. These keratinocytes possess the unique ability to stratify and terminally differentiate in organotypic culture without the aid of feeder fibroblasts. The cells were grown in Dulbecco's MEM (1 g glucose/l, 10% fetal bovine serum, 50 μg/ml gentamicin sulfate) at 37°C in a 5% humidified CO_2 incubator. REKs in monolayer culture were passaged after trypsin release (0.05% trypsin in Ca^{2+}, Mg^{2+}-free Earle's balanced salt solution plus 0.02% EDTA, buffered to pH 7.4 with 20 mM HEPES); cells were seeded at a 1:6 dilution.

Preparation of collagen gels

All procedures were carried out on ice, to prevent premature polymerization of the collagen. Commercially available rat tail collagen I (BD Biosciences, Bedford, MA; 3.9 mg/ml) was mixed with Hank's salt solution containing phenol red, and buffered with 20 mM HEPES. The pH was adjusted by adding small (25 μl) aliquots of 1 N sodium hydroxide until a pale orange (approximately pH 7), homogeneous solution was formed, being careful to avoid bubble formation during mixing. The homogeneous solution (800 μl/insert) was added to plastic Transwell inserts (Costar; diameter 2.5 cm; pore size 3.0 μm) housed in 6-well cluster plates. Polymerized collagen fibrils were formed after incubation at 37°C for 2 h in the 5% CO_2 incubator. Collagen gels, once formed, were stored immersed in PBS with 50 μg/ml gentamycin sulfate at 4°C until use. Before use, the gels were soaked twice in DMEM for 10 min, then equilibrated at 37°C in complete DMEM medium in the CO_2 incubator overnight.

Preparation of basement membranes

MDCK cells (gift of Donald MacCallum, University of Michigan) were seeded onto the collagen gels at a high density (200,000 cells/2.5 cm insert) in DMEM/10% FBS.

The cells were allowed to grow for 18–22 days and the media changed thrice weekly. The cells were removed by detergent lysis and washed as described [28]. Briefly, cells were immersed in hypotonic lysis buffer (10 mM Tris–HCl, pH 7.5, with 0.1% bovine serum albumin and 0.1 mM $CaCl_2$) for 10 min at 37°C in a humidified atmosphere. They were then treated with 0.2% deoxycholate in hypotonic lysis buffer, twice for 5 min. Complete removal of adherent MDCK cells was monitored by light microscopy. Collagen gels with denuded basement membranes on their surface were stored at 4°C immersed in PBS with 50 μg/ml gentamycin sulfate until use. Before use, the gels were immersed twice in DMEM for 10 min, then equilibrated overnight at 37°C in complete DMEM medium in the CO_2 incubator.

Establishment of organotypic cultures

REKs growing in subconfluent monolayers without signs of stratification, were trypsinized and seeded onto inserts containing a collagen-basement membrane at a density of 200,000 to 250,000 cells per 2.5 cm insert. The seeded REKs were then immersed for 48 h in DMEM/10% FBS present both in the upper and lower chambers of the Transwell. When the cells reached confluence, the medium was removed from the upper chamber to expose the cells to air. The medium in the lower chamber was changed daily (1.5 ml) for the duration of the experiment. Typically, differentiation time course studies were carried out for 5 days at 37°C in the 5% CO_2 incubator, with Transwells harvested at various time points for morphology, RNA, protein, and FACE analyses.

Histochemical analysis of proteins and HA

Cell cultures on Transwell filters were fixed at 4°C overnight in Histochoice fixative (Amresco, Solon, OH). The tissue was dehydrated in serial alcohols, cut from the plastic frame, rolled like a cigarette, secured temporarily inside a surgical grade stainless steel ring (4 mm diameter), and then embedded in paraffin. Five-micron sections were cut, rehydrated, and stained with hematoxylin and eosin using standard procedures. For immunofluorescent staining of filaggrin, K10, laminin, or PCNA, slides were warmed for 2 h at 56°C before the deparaffinization and rehydration steps to enhance epitope retrieval. For filaggrin and for laminin, additional unmasking steps were needed. For filaggrin, slides were heated in a citrate unmasking buffer (Vector Labs, Burlingame, CA) at approximately 98°C for 30 min. For laminin, slides were heated in Retrieve-All Hier solution (pH 9.5, Signet Laboratories, Dedham, MA) at approximately 98°C for 30 min. All specimens were blocked for 1 h with 3% normal donkey serum before application of primary rabbit antisera. Incubation in rabbit polyclonal antisera against rat filaggrin/profilaggrin (gift of Dr. Beverly Dale-Crunk; 1:2000), murine K10 (Santa Cruz Biotech, 1:50) or murine laminin (Sigma, 1:100) was done overnight at 4°C in a humidified chamber. After PBS rinses, incubation in donkey Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch; 1:1500) was done at room temperature for 4 h. Slides were mounted in 30% glycerol.

For analysis of cell proliferation, a monoclonal antibody against PCNA (DAKO; 1:200) was applied to rehydrated specimens; no unmasking was required. After 1 h incubation, specimens were rinsed several times in PBS, and PCNA-positive cells were visualized using a biotinylated secondary antibody and streptavidin-peroxidase (R.T.U. Vectastain kit, Vector Labs) as instructed by the manufacturer. Slides were mounted in 30% glycerol.

For histological detection of HA, a biotinylated hyaluronan binding protein (bHABP) derived from cartilage (Seikagaku Ltd; Tokyo, Japan) was used. After blocking in serum (3% FBS) for 10 min, REK specimens were overlaid with 10 μg/ml of bHABP in PBS and 3% serum overnight at 4°C in a humidified chamber. Cy3-conjugated streptavidin (Jackson Immunoresearch; 1:500) was used to detect the bHABP. Slides were mounted in 30% glycerol. To check the specificity of bHABP binding, some samples were incubated with hyaluronidase from *Streptococcus dysgalactiae* (from Seikagaku; 250 mU/ml in 0.1 M ammonium acetate buffer, pH 7) for 30 min at 37°C, to demonstrate specific digestion of HA molecules.

For semiquantitative analysis of histological specimens, high-power light-microscopic or immunofluorescence images were digitally captured using a Polaroid DU-DMC2 Camera. Image-processing was done with IPLab Spectrum software (Signal Analytics, Vienna, VA) using a combination of manual tracing and computer-assisted integration of the signal areas. These data are presented in Fig. 6.

HA analysis by fluorophore-assisted carbohydrate electrophoresis

Quantitation of HA, in the culture medium and in the cell layer, was obtained using FACE analysis as described by Calabro et al. [30,31]. After collection, all samples were frozen immediately and stored until use. For analysis of the medium, aliquots (usually 300 μl with serum or without serum) were precipitated with ethanol (1: 9 vol) at minus 20°C overnight to remove glucose still present in the medium after cell incubation. After centrifugation, the pellet was resuspended in ammonium acetate (100 mM, pH 7), and protein was digested with protease K (3 U per sample) for 3 h at 60°C. For analysis of REK cell layers, the entire polyester membrane with the overlying gel was cut from the plastic frame, the cell layers were carefully separated from the collagen gel using micro-surgery tools under a stereomicroscope, and the cell layer and collagen gels were each digested separately using protease K, as described above.

After inactivation of the protease K (100° C for 5 min), samples were precipitated with cold ethanol (1:4 ratio) at −20°C overnight. The resulting pellet was resuspended in

ammonium acetate (100 mM, pH 7), digested with hyaluronidase SD from *S. dysgalactiae* (100 mU/ml, Seikagaku) for 1 h at 37°C, followed by the addition of chondroitinase ABC from *Proteus vulgaris* (100 mU/ml, Seikagaku) and incubation of 3 h at 37°C. Samples were then frozen on dry ice and lyophilized.

End-labeling at the reducing end of the HA digestion products with the fluorescent probe, 2 aminoacridone (AMAC), was done as follows. To each freeze-dried sample, 40 μl of AMAC (500 nmol) in 85% DMSO/15% acetic acid was added, and the sample incubated for 15 min at room temperature. Then, 40 μl of sodium cyanoborohydride (1.25 M) in ultrapure water was added, and the mixture incubated for 16 h at 37°C. At completion of this derivatization reaction, 20 μl of glycerol (20% final conc.) was added to each sample before electrophoresis. For storage, samples were kept in the dark at minus 80°C. If samples formed a precipitate during storage, they were heated to 60°C for 5–10 min. For electrophoretic analysis, samples were run on high-percentage acrylamide gels (Monogel, from GLYKO; 1 h at 500 V, cooled at 4°C), and the resulting bands were illuminated under UV light (365 nm) for image-capture and analysis via a Quantix CCD camera, as described [30].

HA fragmentation analysis by SDS-PAGE

To generate an HA-digestion ladder as an assay of *StrepH* activity, reagent-grade HA (Sigma Co., 1 mg/ml in DMEM) was incubated in the presence of *StrepH* (1 U/ml) at different times, in media containing different amounts of FBS (see Fig. 5). After enzyme inactivation by boiling, each reaction product was mixed with Laemmli sample buffer and run on a 5% SDS-polyacrylamide gel (BioRad minigel). The gels were stained with 0.5% Alcian blue in 10% acetic acid.

HA "knock-out" time course experiments

To completely remove HA from the extracellular spaces in REK organotypic cultures during differentiation experiments, a "functional knockout" was created using *Streptomyces* hyaluronidase (StrepH; from Seikagaku Ltd.). Small scale experiments showed that 1 U/ml of StrepH was able to completely digest full-length HA (1 mg/ml) after 4 h at 37°C, as assessed by FACE analysis and SDS-PAGE. StrepH was not inhibited by 10% FBS (see Fig. 5) and maintained more than 70% of activity after 24-h incubation with cells. To perform time-course experiments in which HA was effectively removed from the intercellular spaces, confluent monolayer REK cultures were lifted to the air–liquid interface in a reverse-serial fashion, as follows. Day 5 cultures were lifted on the first day of the experiment and Day 4 cultures were lifted on the second day, until all cultures (including the Day 0 time point) were harvested together on the 5th day. Lifted digested inserts received 1.5 ml of medium (DMEM/10% FBS) containing 1 U/ml of StrepH daily, while all other inserts (controls) received medium alone. Media changes were performed at exactly 24 h, to avoid any effects from nutrient depletion. In early experiments, we also added 200 μl of StrepH/media on the top of the cells, but because the newly formed stratum corneum was effective at repelling liquids (forming droplets by 48 h), we did not add StrepH to the upper chamber in later experiments.

Western blot analysis

The REK cell layers from different time courses and conditions were peeled off and treated with 0.05% trypsin for 15 min at 37°C. Cells were centrifuged at 5000 rpm at 4°C for 1 min followed by a wash with ice-cold PBS. Cells in lysis buffer (7 M urea, 2% IGEPAL, 5% β-mercaptoethanol) were lysed with three 4-s bursts using an ultrasonic probe. Equal quantities of protein, along with prestained molecular size markers (BioRad), were resolved on a 12% SDS-PAGE gel and transferred to PVDF membranes (Immobilon, Millipore Corp). Western blotting with rabbit polyclonal antisera specific for K10 (1:10,000) and filaggrin (1:10,000) was done as described [32]. Blots were incubated with peroxidase-conjugated goat anti-rabbit IgG and developed using enhanced chemiluminescence reagents (ECL kit, Amersham) followed by exposure to X-ray film (Biomax, Kodak).

Results

The REK epidermal model faithfully reproduces the expression pattern of differentiation markers normally seen in the epidermis

To adequately address the question of a role for extracellular HA in the physiological processes of epidermal differentiation and stratification, we sought an organotypic model that (i) replicates the program of epidermal differentiation normally seen in vivo, (ii) accumulates HA in the correct intercellular location (i.e., in the matrix between the keratinocytes), and (iii) does not obscure the detection of epidermal HA by the overwhelming presence of HA produced by dermal fibroblasts. Since nearly all organotypic (skin equivalent) models in current use employ dermal substrate-containing fibroblasts, we used an in vitro system described by Lillie et al. [27] that forms a nearly perfect epidermis without the need for fibroblast coculture. The basis of this system is an immortalized rat keratinocyte (REK) line originally derived by Baden and Kubilus [29], which differentiates and stratifies independently of fibroblast-derived factors when grown at the air–liquid interface on a renatured collagen gel. To optimize the tissue morphology and functional behavior of the stratified REK epithelium, a basement membrane is deposited by culturing MDCK cells upon the collagen gel and then removing them

before seeding the REK cells [28], see Fig. 1A. MDCK cells synthesize components (e.g., laminin and collagen IV) found in normal epidermal basement membranes [28,33], and when grown on a collagen gel, can assemble these molecules into a physiologically well-organized structure that will then support attachment and adhesion of REK cells. Examination of the BM zone of these cultures by electron microscopy reveals architectural features characteristic of a normal epidermal basement membrane, that is, presence of a lamina lucida, lamina densa, and hemidesmosomes [3,28].

Adaptation of the REK lift culture model for the study of epidermal HA metabolism was recently described [28], but more analysis is needed to determine the expression of keratinocyte differentiation characteristics in the system. Fig. 1 illustrates the spatial and temporal patterns of several morphological differentiation markers in the REK model. After REKs were seeded and grown to confluence on the basement membrane (Figs. 1B–D, at 0 d), the cultures were lifted to the air–medium interface. By 2 days, the cultures began to stratify, and by 5 days, several features associated with terminal differentiation became fully evident. These

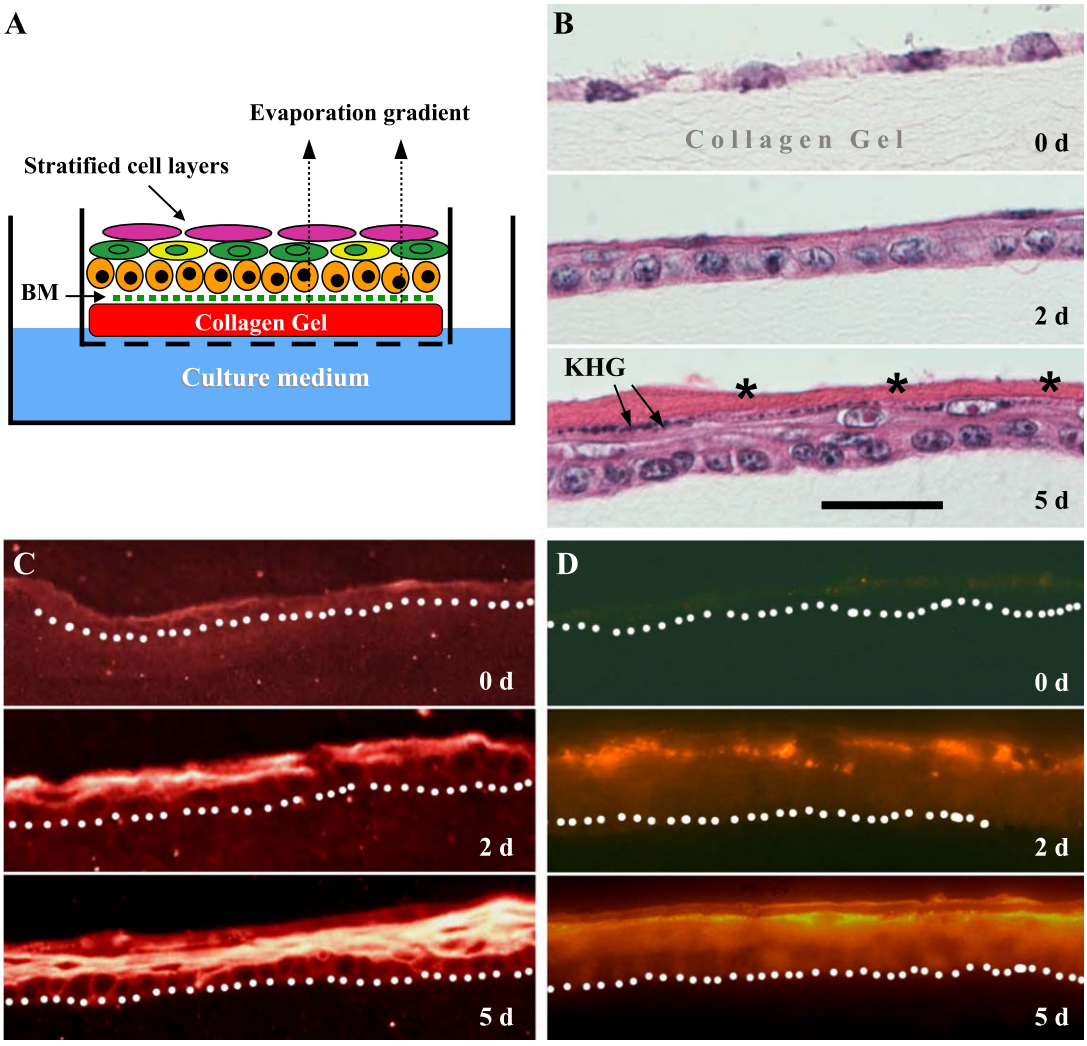

Fig. 1. The REK organotypic system reproduces normal epidermal morphology and tissue-specific differentiation markers. (A) Schematic diagram of the REK lift-culture epidermal model. A collagen gel is polymerized onto a porous polyester membrane (3.0 μm pores) in a Transwell tissue culture insert. Subsequently, a preformed basement membrane (BM) is created by culturing MDCK renal epithelial cells on the collagen for 3 weeks, then removing the MDCK cells (not shown). Subsequently, keratinocytes (REK) are plated and grown submerged on the BM. When the keratinocytes reach confluence, the cultures are exposed to air by reducing the volume of culture medium to the level of the gel. This initiates an epidermal differentiation program that involves the sequential expression of differentiation-related gene markers, and the formation of several stratified layers. (B, C, D) Normal tissue architecture and a normal expression pattern of differentiation markers develop in the REK model epidermis during stratification. REKs seeded on a collagen/BM substrate (as in this figure) were allowed to grow to confluence submerged for 2 days, and then were raised and maintained at the air–liquid interface for 0, 2, or 5 days before harvest, fixation, paraffin-embedding, and microtome sectioning. (B) Analysis by H and E staining; *KHG*, keratohyalin granule; asterisk, stratum corneum. (C) Immunofluorescent stains using an anti-K10 primary antibody and Cy3-tagged secondary antiserum. Dotted lines denote the substrata-epidermal junction. (D) Immunofluorescent stains using an anti-filaggrin antibody (gift of Beverly Dale-Crunk; 1:2000) and the Cy3-tagged secondary antiserum. Scale bar, 25 μm.

included the presence of two or three layers of suprabasal cells, abundant *keratohyalin granules* (KHG; Fig 1B, arrows), and a well-developed *stratum corneum* (Fig. 1B, asterisks). Immunostaining revealed expression of *keratin 10* (K10) in the suprabasal layers, starting at 2 days and increasing at 5 days (Fig. 1C). Increases in K10 were independently confirmed on Western blots (Fig. 2A). Filaggrin, a late marker of differentiation, is expressed by the REKs most prominently at 5 days, as shown by immunostaining (Fig. 1D). Filaggrin can be considered the molecular analog of the KHG, since it is stored in keratohyalin granules as a large precursor polypeptide (*profilaggrin*), that is eventually cleaved into filaggrin subunits as the keratinocytes begin to cornify [34,35]. In our model, the processed subunits appear as a ladder on Western blots from lift cultures at day 2, and increase substantially as the cultures mature over the ensuing days (see Fig. 2B).

The REK model elaborates and retains intercellular HA within a closed epidermal compartment

To show that the REK organotypic model is adequate for these studies, it was important to demonstrate that HA is produced by REK cells and retained within the epidermis. For these experiments, REK cultures grown directly on bare collagen (Figs. 3A–C) were compared with REK cultures in which a preformed MDCK membrane had been applied (Figs. 3E–G), as confirmed by staining with an antibody to laminin (Figs. 3D, H). To visualize HA, tissue sections were incubated with biotinylated HA-binding protein (bHABP), a fragment of aggrecan/link protein that binds to HA with high affinity [14]. Incubation with bHABP was followed by addition of avidin-Cy3. Bright intercellular staining was observed in fully mature REK lift cultures seeded onto basement membrane (Fig. 3G). However, this was not the case for REK cultures seeded onto bare collagen (Fig. 3C). It appeared that in the absence of an intact BM, HA produced by the keratinocytes was lost into the collagen gel. To confirm this, we used FACE analysis to measure the amounts of HA quantitatively (Fig. 4A). The fluorophore-assisted carbohydrate electrophoresis (FACE) technology measures the HA content of very small amounts of tissue, while circumventing problems inherent in previous detection methods that relied upon radiolabeling [36]. Total glycosaminoglycans are extracted from a tissue and enzymaticaly cleaved to the component disaccharides, which are then end-labeled with a fluorescent tag, separated on a gel, and detected fluorometricaly. Fig. 4A shows a FACE analysis from REK cultures in which the cellular layer and the collagen gel were examined separately. In the absence of a basement membrane, the amount of HA was more abundant in the collagen gel than in the cell layer, whereas in the presence of the basement membrane, the ratio was inverted, favoring retention of HA in the cell layer. Interestingly, the total amount of HA produced by REKs did not change markedly in the presence or absence of a basal membrane. Rather, the notable difference was the HA distribution between different compartments. By FACE analysis, >70% of total HA was present in the epidermal cell layer when a basement membrane was present, compared to less than 15% when the basement membrane was absent, allowing leakage into the collagen below.

FACE was also used to quantify the rate at which HA accumulates in the epidermal compartment during differentiation and stratification (Fig. 4B). Over a 5-day time course, the amount of HA in the epidermal cell layer rose 10-fold, increasing from 215 to 2040 pmol disaccharide/culture (Fig. 4B, lower). Epidermal HA also increased in the absence of a basement membrane but to a lesser extent, from 95 to 460 pmol disaccharide/culture (not shown). The greatest incremental increases occurred in the first 24 h, during which HA more than doubled. A more detailed examination of earlier times showed that increases in epidermal HA could be detected at between 12 and 18 h after lifting to the air–liquid interface (Fig. 4B, upper).

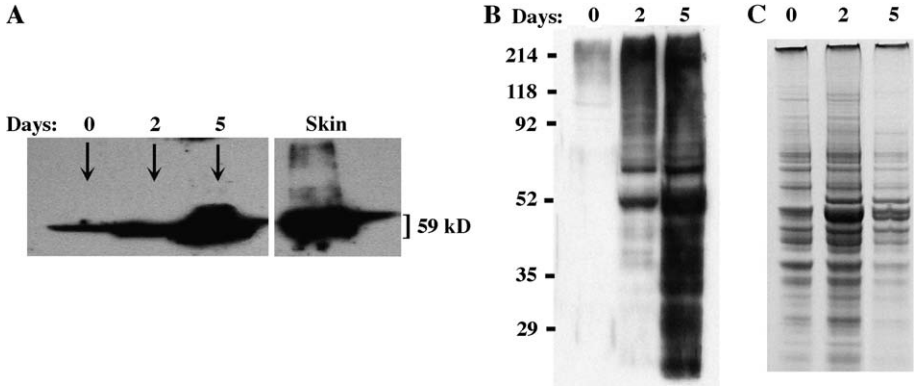

Fig. 2. Differentiation-specific proteins, analyzed by Western blotting, are upregulated in REK cultures exposed to air. REK lift cultures were grown at the air–medium interface for the times indicated (in days), lysed, and equivalent amounts of protein separated on immunoblots and probed with antisera specific to: (A) the early marker, K10; and (B) the late marker, profilaggrin/filaggrin. In the rightmost lane of panel A, an aliquot of protein from mouse skin was run as a positive control. (C) A gel loaded with the same volumes of lysate as in the immunoblots of B and C, and stained with Coomassie Blue dye. Locations of MW markers are shown (kDa).

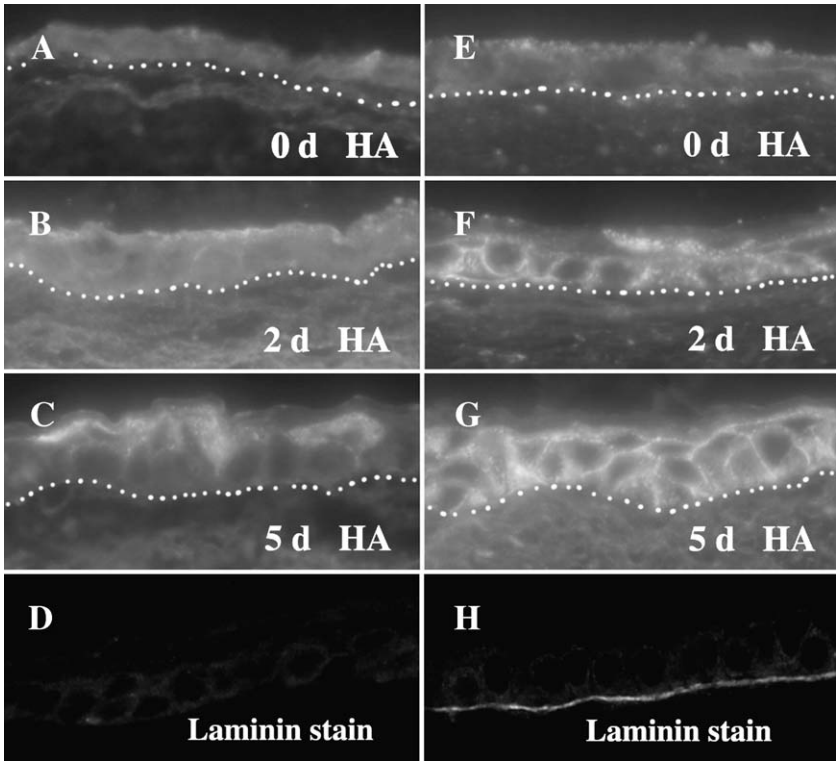

Fig. 3. Analysis of HA, by in situ staining with biotinylated HA binding protein (bHABP), detects intercellular accumulation of HA in the REK lift cultures in the absence (A–D) or presence (E–H) of a basement membrane. Cultures were harvested after maintenance at the air–liquid interface for 0, 2, or 5 days. Paraffin sections were incubated with bHABP followed by Cy3-avidin, which yields a bright signal, (A–C) and (E–G). Dotted lines indicate the interface between the epidermal and collagen compartments. Note that HA accumulation in the epidermis is highly dependent upon the presence of an intact basement membrane, confirmed in cultures with REK cells on a preformed MDCK-derived membrane (H) but not in cultures on bare collagen (D); immunostaining was performed with anti-laminin followed by a Cy3-labeled secondary antibody.

An exogenous hyaluronidase enzyme from Streptomyces can be used experimentally to remove intact HA from the intercellular matrix of the REK epidermis

To directly address the role of HA in the keratinocyte differentiation program, we needed a method to directly manipulate intercellular HA levels in the living epidermal cultures. A hyaluronidase enzyme derived from the micro-organism *Streptomyces*, and known to be highly specific for HA, was initially tested for its ability to degrade HA in the presence of potential inhibitors in the cell culture medium, principally FBS (Fig. 5). Our results show that even in the presence of 10% FBS, StrepH is capable of degrading HA, causing significant breakdown within the first 15 min and nearly complete degradation of the polymer by 4 h (Fig. 5A). HA is reduced from its initial size of >1 million kDa, to small oligosaccharides that can be detected by end-labeling with a fluorescent probe (AMAC) and separation on Glyko FACE

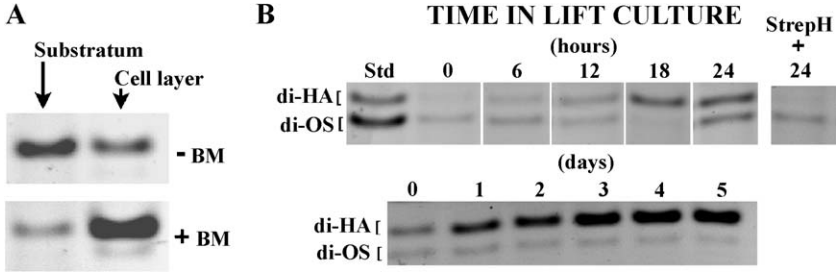

Fig. 4. Measurement of HA accumulation in the REK lift cultures using FACE analysis. (A) Accumulation of HA from 5-day lifted REK cultures that were grown either on bare collagen (−BM) or with the preformed basement membrane (+BM). HA in each compartment (substratum, the collagen gel; and the cell layer, the tissue peeled away from the collagen) was analyzed separately. (B) FACE analysis of total cellular material from REK lift culture model, illustrating the increase in total HA during differentiation over the first 24 h after lift (upper panel), or over the ensuing 5 days (lower panel). Disaccharide standards (Std) derived from HA (di-HA) or from a nonsulfated form of chondroitin (di-0S) are indicated. In the last lane, labeled "StrepH+24," a 24-h sample was digested with *Streptomyces* hyaluronidase before gel analysis to demonstrate specificity of the HA-derived band.

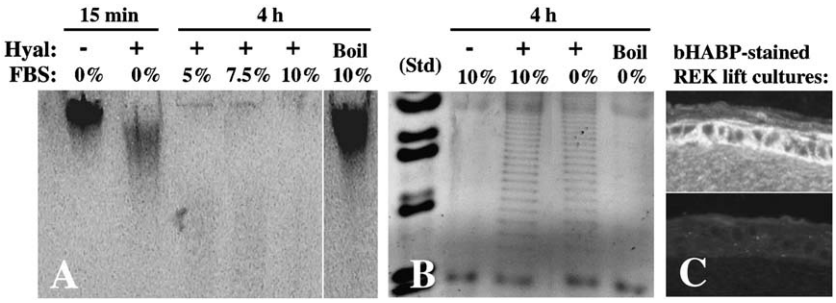

Fig. 5. Demonstration and validation of the StrepH digestion technique. (A) Acrylamide gel of purified HA (1 mg/ml, from Sigma) incubated with StrepH (1 U/ml) for 15 min or 4 h in the presence of various concentrations of fetal bovine serum (FBS). The gel was stained with Alcian Blue. *Boil*, the Strep hyaluronidase was destroyed by boiling as a negative control. (B) Similar experiment, but now run on a high-concentration acrylamide gel (Glyko) after end-labeling the digested HA fragments with AMAC to tag them for fluorometric visualization. (C) REK cells on collagen, after 5 days in lift culture in the absence (top) or presence (bottom) of StrepH added daily; cultures were fixed and stained with bHABP. Note the complete absence of HA after digestion with StrepH (lower panel).

high-concentration acrylamide gels (Fig. 5B). In Fig. 5C, the StrepH enzyme was added to the REK culture medium for 24 h, and after paraffin-embedding and sectioning, the HA content of the tissue was assessed using the fluorescent-bHABP technique. The results show that StrepH effectively digests intercellular HA, confirming the utility of StrepH as a reagent to remove hyaluronan from the extracellular matrix during epidermal differentiation experiments.

Removal of intact HA from the REK epidermis accelerates the keratinocyte differentiation program

Having established that StrepH in the culture medium can effectively digest and remove HA from between keratinocytes in the organotypic epidermis, we conducted time course experiments during REK stratification in the presence or absence of StrepH. Cultures were harvested at 0, 2,

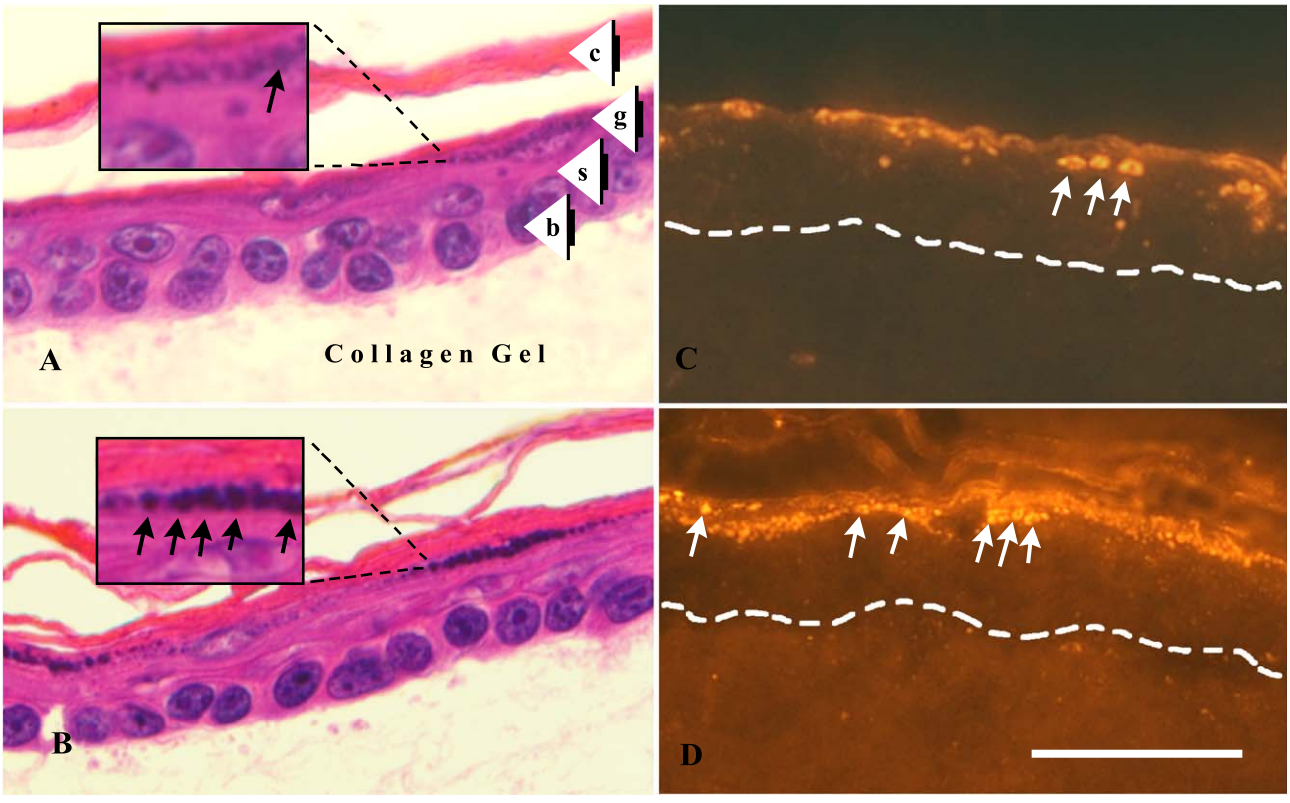

Fig. 6. Illustrative changes in keratohyalin granules and filaggrin expression in organotypic REK cultures differentiating at the air–liquid interface for 5 days with (+*StrepH*; B, D) or without (Control; A, C) *Streptomyces* hyaluronidase added to the culture medium. Paraffin-embedded 5 μm sections were stained with hematoxylin–eosin (A, B) or with antibody to filaggrin followed by Cy3-labeled immunofluorescent secondary antisera (C, D). For orientation purposes, the locations of the basal (b), spinous (s), granular (g), and cornified (c) layers are indicated in panel A. Dashed lines in panels C and D denote the basement membrane. INSETS: Enlargements to show keratohyalin granules. Arrows indicate very large ("giant") KHGs, which are more numerous in StrepH-treated cultures than in control cultures. Scale bar, 25 μm.

or 5 days after the initiation of air exposure, and then were fixed and processed for the histochemical analyses (Fig. 6) summarized in Fig. 7. Loss of intercellular HA did not prevent formation of the REK epithelium, which appeared grossly normal in the presence of StrepH. However, our survey of markers of proliferation and differentiation (Fig. 7) revealed significant abnormalities in differentiation at the molecular level in the StrepH-treated cultures. Proliferation was suppressed in all lifted cultures with respect to the initial submerged cultures (Fig. 7A), but no differential effect could be attributed to HA since an equivalent number of PCNA-stained nuclei were seen in StrepH-treated cultures as compared to time-matched, untreated controls (Fig. 7A). However, the differentiation markers K10 and filaggrin were significantly affected. Expression of K10, an early differentiation marker, was not reproducibly altered at 2 days (not shown), but at 5 days was increased in StrepH-treated cultures compared to controls (Fig. 8A). More dramatically, the expression of filaggrin and KHGs, two late markers of differentiation, was strongly increased in StrepH-treated cultures. Tissue sections immunostained to detect filaggrin revealed strongly positive staining in the granular layer (Figs. 6C, D); the signal corresponds to the expected location of profilaggrin/filaggrin, known to reside within KHGs [35] as seen on hematoxylin–eosin-stained sections (Figs. 6A, B). Interestingly, the number of these filaggrin-positive granules was higher in StrepH-treated cultures (Figs. 6B, D) compared to controls (Figs. 6A, C); computer-assisted counting revealed that the difference was statistically significant (Fig. 7B). In addition to the higher number of filaggrin-positive KHGs, a shift in the average

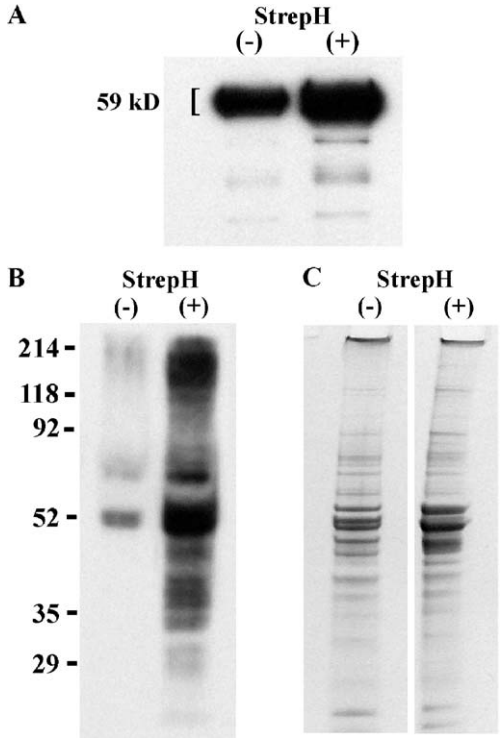

Fig. 8. Digestion of intercellular HA leads to enhanced expression of differentiation markers in organotypic REK cultures. REK lift cultures were maintained at the air–medium interface for 5 days in the absence (−) or presence (+) of StrepH, then harvested for Western blotting. (A) Immunoblot for keratin 10; (B) Immunoblot for filaggrin/profilaggrin; (C) A gel, loaded with the same volumes of lysate as in the immunoblots of B and C, and stained with Coomassie Blue to evaluate total protein. Locations of MW markers are shown in kDa (on the left).

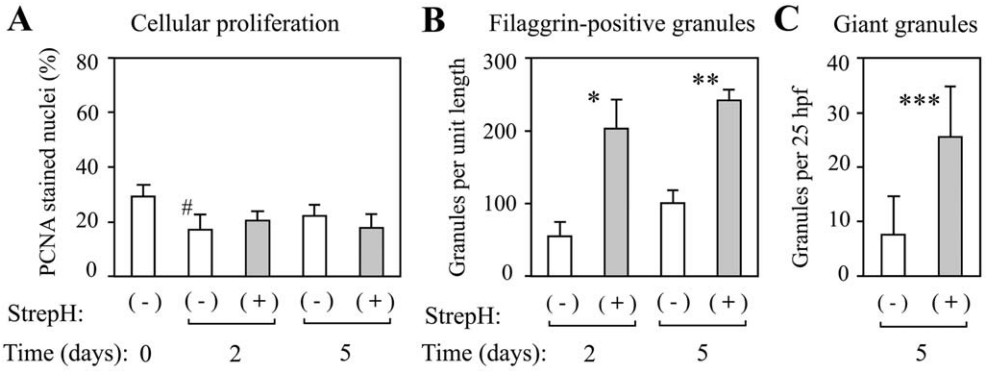

Fig. 7. Quantitation of changes in histologic markers of proliferation and differentiation in organotypic REK cultures, in the absence (open bars) or presence (gray bars) of StrepH. (A) PCNA-labeled nuclei. PCNA-positive nuclei, expressed as a percentage of total nuclei per high-power field. Data from two experiments, 20 sections per time point; mean±SD. A slight decrease in proliferation in the lifted cultures is noted, but is significant only at the point indicated (#), $P < 0.05$ by Student's t test. (B) Filaggrin immunopositive granules. Discrete granules in the stratum granulosum, specifically stained with anti-filaggrin antiserum, were segmented and scored using the IPLab Spectrum program and expressed as number of granules per unit length of epidermis (arbitrary units). Data from two experiments, three fields per condition, mean±SD. (*) and (**), differences relative to controls lacking StrepH are statistically significant by Student's t test, at the $P < 0.001$ level. (C) Giant keratohyalin granules. To measure the relative increase in average sizes of KHGs observed in StrepH-treated cultures, hematoxylin–eosin-stained sections were scored for the presence of very large ("giant") KHGs, defined as a granule equal or larger in size than a nucleolus in the nuclei of basal keratinocytes. One of the authors (H.K.), unaware of the identity of each slide, counted 25 high power fields per condition in each experiment in three separate experiments. Data are expressed as percentage of high power fields (hpf) that contain one or more large granules, mean±SEM. (***), Difference between StrepH-treated vs. untreated controls is statistically significant by Student's t test, at the $P < 0.01$ level.

size of the granules was noted, characterized by the appearance of unusually large (“giant”) KHGs in the StrepH-digested cultures. To quantify the latter change, a size threshold was established to define the giant KHGs (see legend to Fig. 7), which were then counted in three experiments. As shown in Fig. 7C, a statistically significant increase (approximately 3-fold) in the number of giant KHGs was evident in the StrepH-treated specimens. To begin to investigate the mechanism for this enlargement of KHGs, we tested the hypothesis that processing of profilaggrin to filaggrin (a prerequisite for the filaggrin peptides to leave the KHG and travel to the cytoplasm) might be selectively altered. On Western blots of cellular proteins from 5-day REK lift cultures, the total amount of immunoreactive filaggrin material appears greatly increased in the StrepH-treated cultures (Fig. 8B), yet the overall pattern of high molecular weight profilaggrin combined with smaller processed fragments (filaggrin multimers) looks relatively unchanged, as compared to control cultures (see Fig. 2B). Thus, the increase in the KHGs appears to be due to a robust increase in profilaggrin, which is then proteolytically processed to filaggrin in roughly the usual proportions.

In summary, it is apparent that StrepH-mediated digestion of intercellular HA leads to increased expression of terminal differentiation markers in the REK system. Accumulation of K10, a very early marker of differentiation [37], accumulates at 2 days of lift to a similar extent in both StrepH-digested and control cultures, but at 5 days accumulates to a higher level in the StrepH-digested cultures. More strikingly, the expression of filaggrin and the accumulation of KHGs (markers of late terminal differentiation) become significantly elevated as early as 2 days after the initiation of lift, in the presence of the StrepH enzyme, an effect that increases even more at 5 days. Overall, the data are consistent with the notion that loss of HA in the epidermis shifts the differentiation program of keratinocytes toward a more terminally differentiated state.

Discussion

While a growing body of circumstantial evidence implicates the intercellular matrix molecule, HA, as a modulator of keratinocyte migration, proliferation, and/or differentiation in various settings (see Introduction), the role of the HA observed between keratinocytes in the three-dimensional epithelial tissue comprising the epidermis had not been previously ascertained. In this study, we employed an organotypic model that features rat epidermal keratinocytes (REK cells) stratifying on collagen gels at the air–liquid interface [27,28] to experimentally evaluate the requirement of HA for proliferation and differentiation in epidermal tissue. This particular model was chosen for three reasons: (i) because the model tissue faithfully displays known markers of the keratinocyte differentiation program; (ii) because the REKs have a unique ability to stratify in the absence of any cocultured fibroblasts; and (iii) the model includes a functionally intact basement membrane to confine HA within the epidermal compartment. Regarding the last feature, the use of an MDCK-derived basement membrane (as opposed to growing keratinocytes on bare collagen) largely accounts for the unique ability of the REK organotypic model to simulate the in vivo environment of epidermal HA. The effect of adding this exogenously produced BM to the basic REK model was described in recent work from Tammi et al. [28], which showed that REK cells alone fail to produce a basal lamina capable of retaining HA in the epidermal space, whereas MDCK-derived membranes (a highly organized structure containing laminin and collagen IV) successfully prevent HA leakage into the collagen substrate. By electron microscopy, REK basal keratinocytes lack hemidesmosomal structures when grown directly on bare collagen gels, but do form normal hemidesmosomes when grown upon the MDCK-derived BM [3]. Our results confirm the advantages of the MDCK-membrane and its HA-retaining ability, using two techniques not applied in the previous studies. First, we used Cy3-fluorescent immunolabeling that gives a signal more proportional to target concentration, as compared to the previously used enzymatic amplification by horseradish peroxidase. Secondly, the quantitative FACE technique [36] was used to measure small amounts of HA in the epidermis, without the complexities associated with uptake of radiolabeled glucosamine precursors.

In the current study, we designed experiments to test the hypothesis that HA is a functional participant in epidermal growth and differentiation. To do this, we used a commercially available and well-characterized enzyme, the hyaluronidase (hyaluronan lyase) from the microbe *Streptomyces* as a means to degrade HA. StrepH is remarkably specific for HA, compared to other commercially available microbial lyases. At its usual acidic pH optimum, StrepH does not appreciably degrade other glycosaminoglycans, including chondroitin, chondroitin 4/6 sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin [38,39]. Some have asserted that StrepH, at the neutral pH used in this study, might also digest chondroitin or chondroitin sulfate (CS). Even if true (a much less likely scenario for the sulfated molecule compared to chondroitin itself), we have found that chondroitin is absent and CS is seen in only trace amounts in our model tissue as measured by FACE (data not shown). Interestingly, Tammi et al. [28], using radiolabeling techniques, also reported that some chondroitin sulfate is made by REK cells in very small amounts relative to the production of HA. Overall then, the observed effects of StrepH upon keratinocyte differentiation are most likely due to specific degradation of HA in the model epidermis rather than to degradation of other glycosaminoglycans. Whether the effects observed with StrepH bear any relationship to the actions of mammalian hyaluronidases (see Ref. [40], for review), a class of enzymes quite different from the microbial lyases in localization (membrane-an-

chored) and mechanism of action (hydrolases rather than lyases), is a difficult question that will have to await the results of ongoing studies in this emerging field.

Although intentional removal of HA from between the keratinocytes using StrepH did not grossly disrupt epithelial formation nor affect basal cell proliferation to any measurable extent, significant changes in the epidermal differentiation program did occur at both the light-microscopic and molecular levels. In cultures continuously exposed to StrepH over a 5-day period, early markers (K10) and late markers of differentiation (KHG, filaggrin) were significantly increased. This finding is consistent with a concept suggested by correlative types of data in the literature, that is, the notion that HA inhibits cellular differentiation (see Introduction).

Judging from our data, the overall effect of HA may be to act as a modulator (or "brake") to slow the progress of keratinocyte differentiation. The fact that several groups [21,22] in addition to ours have made the anatomic observation that HA appears to be more abundant in the upper epidermis than in the basal layer, now appears to have some functional relevance. That is, the greatest effect of HA removal in our experiments is exerted within cells residing in the upper epidermis, as reflected by the apparent derepression of the synthesis and/or accumulation of profilaggrin in the granular layer. Keratin 10, a differentiation-regulated protein expressed lower down in the spinous layer, also seems to be affected by the removal of HA, but to a lesser extent. Cell proliferation, occurring in the basal layer, does not appear to be affected at all.

Inevitably, the mechanism of how HA exerts its differentiation-suppressive effects is the main question for future study. One possibility is that engagement of the long-chain HA polymer to specific HA-receptors (such as CD44) on the keratinocyte surface acts to trigger intercellular signals, whose effects are to slow the expression of differentiation-specific genes within the cell. Disengagement of full-length HA from the CD44 receptor, because the HA has been degraded by StrepH, might release the block upon differentiation that is normally afforded by ligand-bound CD44. Another possibility is that small oligosaccharide fragments (oligos) of HA, generated during StrepH digestion, are biologically active and work through interactions with either the extracellular matrix, or the keratinocyte itself, to accelerate differentiation. In the matrix, small oligos might compete with the binding of one or more specific HA-binding proteins known to be present in the matrix of other tissues; examples include aggrecan in cartilage [41] and tumor necrosis factor-stimulated gene 6 (TSG6) in the ovarian follicle [42]. At the cell surface, small oligos could competitively displace full-length HA from its binding sites on the CD44 receptor, a displacement that would probably require an oligosaccharide length of at least 10 sugars, based upon previous studies in REKs [43]. A third possibility is that small oligos might act intracellularly. HA is known to be actively taken up into keratinocytes, via a mechanism involving HA-containing vesicles that pinch off from the cell membrane and proceed through an unconventional endosomal pathway that terminates in lysosomes [44]. These HA fragments might elicit as-yet-unknown effects upon regulatory pathways for proliferation and differentiation, but as yet almost no data are available on this topic. Experiments to distinguish among the possibilities mentioned above are underway.

In this paper, we have shown that hyaluronan in the extracellular matrix surrounding keratinocytes is clearly important as a functional participant in the regulation of epidermal differentiation. Now more than ever, HA should attract renewed interest as an important regulator in epidermal physiology, both during normal homeostasis and during various responses to injury (e.g., reepithelialization after wounding).

Acknowledgments

We would like to thank Dr. Donald MacCallum for generous help in establishing the REK organotypic cultures, Dr. Beverly Dale-Crunk for a gift of anti-rat filaggrin antisera, Dr. Judith Mack for help in reviewing the manuscript, and Dr. Philip Bailin for his unwavering support and encouragement.

References

[1] J. McDonald, V.C. Hascall, Hyaluronan minireview series, J. Biol. Chem. 277 (2002) 4575–4579.
[2] V.C. Hascall, T.C. Laurent, Hyaluronan: structure and physical properties, in: V.C. Hascall, M. Yanagishita (Eds.), Glycoforum/Science of Hyaluronan Review Series, 1997, http://www.glycoforum.gr.jp/science/hyaluronan/HA01/HA01E.html.
[3] V.C. Hascall, R. Tammi, M. Tammi, E. Hunziker, D.K. MacCallum, Does keratinocyte hyaluronan determine the volume of extracellular space in the epidermis? in: G. Abatangelo, P.H. Weigel (Eds.), New Frontiers in Medical Sciences: Redefining Hyaluronan, Amsterdam, Elsevier Science B.V., 2000, pp. 289–296.
[4] M.I. Tammi, A.J. Day, E.A. Turley, Hyaluronan and homeostasis: a balancing act, J. Biol. Chem. 277 (2002) 4581–4584.
[5] A.J. Day, G.D. Prestwich, Hyaluronan-binding proteins: tying up the giant, J. Biol. Chem. 277 (2002) 4585–4588.
[6] W. Knudson, C.B. Knudson, The hyaluronan receptor CD44, in: V.C. Hascall, M. Yanagishita (Eds.), Glycoforum/Science of Hyaluronan Review Series, 1999, http://www.glycoforum.gr.jp/science/hyaluronan/HA10/HA10E.html.
[7] W. Knudson, G. Chow, C.B. Knudson, CD44-mediated uptake and degradation of hyaluronan, Matrix Biol. 21 (2002) 15–23.
[8] E.A. Turley, P.W. Noble, L.Y. Bourguignon, Signaling properties of hyaluronan receptors, J. Biol. Chem. 277 (2002) 4589–4592.
[9] P. Teder, R.W. Vandivier, D. Jiang, J. Liang, L. Cohn, E. Pure, P.M. Henson, P.W. Noble, Resolution of lung inflammation by CD44, Science 296 (2002) 155–158.
[10] C.A. de La Motte, V.C. Hascall, A. Calabro, B. Yen-Lieberman, S.A. Strong, Mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human intestinal mucosal smooth muscle cells after virus infection or treatment with poly(I.C.), J. Biol. Chem. 274 (1999) 30747–30755.
[11] B.P. Toole, Hyaluronan promotes the malignant phenotype, Glycobiology 12 (2002) 37R–42R.

[12] B.P. Toole, T.N. Wight, M.I. Tammi, Hyaluronan–cell interactions in cancer and vascular disease, J. Biol. Chem. 277 (2002) 4593–4596.
[13] V.C. Hascall, D. Heinegard, Aggregation of cartilage proteoglycans: I. The role of hyaluronic acid, J. Biol. Chem. 249 (1974) 4232–4241.
[14] R. Tammi, J.A. Ripellino, R.U. Margolis, M. Tammi, Localization of epidermal hyaluronic acid using the hyaluronate binding region of cartilage proteoglycan as a specific probe, J. Invest. Dermatol. 90 (1988) 412–414.
[15] C. Wang, M. Tammi, R. Tammi, Distribution of hyaluronan and its CD44 receptor in the epithelia of human skin appendages, Histochemistry 98 (1992) 105–112.
[16] R. Tammi, A.M. Saamanen, H.I. Maibach, M. Tammi, Degradation of newly synthesized high molecular mass hyaluronan in the epidermal and dermal compartments of human skin in organ culture, J. Invest. Dermatol. 97 (1991) 126–130.
[17] B.P. Toole, Hyaluronan in morphogenesis, J. Intern. Med. 242 (1997) 35–40.
[18] M.T. Longaker, N.S. Adzick, J.L. Hall, S.E. Stair, T.M. Crombleholme,, B.W. Duncan, S.M. Bradley, M.R. Harrison, R. Stern, Studies in fetal wound healing: VII. Fetal wound healing may be modulated by hyaluronic acid stimulating activity in amniotic fluid, J. Pediatr. Surg. 25 (1990) 430–433.
[19] W.Y. Chen, G. Abatangelo, Functions of hyaluronan in wound repair, Wound Repair Regen. 7 (1999) 79–89.
[20] K. Rilla, M.J. Lammi, R. Sironen, K. Torronen, M. Luukkonen, V.C. Hascall, R.J. Midura, M. Hyttinen, J. Pelkonen, M. Tammi, R. Tammi, Changed lamellipodial extension, adhesion plaques and migration in epidermal keratinocytes containing constitutively expressed sense and antisense hyaluronan synthase 2 (Has2) genes, J. Cell Sci. 115 (2002) 3633–3643.
[21] R. Tammi, M. Tammi, Correlations between hyaluronan and epidermal proliferation as studied by [3H]glucosamine and [3H]thymidine incorporations and staining of hyaluronan on mitotic keratinocytes, Exp. Cell Res. 195 (1991) 524–527.
[22] A.F. Wells, A. Lundin, G. Michaelsson, Histochemical localization of hyaluronan in psoriasis, allergic contact dermatitis and normal skin, Acta Derm.-Venereol. 71 (1991) 232–238.
[23] R. Tammi, J.A. Ripellino, R.U. Margolis, H.I. Maibach, M. Tammi, Hyaluronate accumulation in human epidermis treated with retinoic acid in skin organ culture, J. Invest. Dermatol. 92 (1989) 326–332.
[24] U.M. Agren, M. Tammi, R. Tammi, Hydrocortisone regulation of hyaluronan metabolism in human skin organ culture, J. Cell. Physiol. 164 (1995) 240–248.
[25] G. Kaya, I. Rodriguez, J.L. Jorcano, P. Vassalli, I. Stamenkovic, Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation, Genes Dev. 11 (1997) 996–1007.
[26] G. Kaya, E. Augsburger, I. Stamenkovic, J.H. Saurat, Decrease in epidermal CD44 expression as a potential mechanism for abnormal hyaluronate accumulation in superficial dermis in lichen sclerosus et atrophicus, J. Invest. Dermatol. 115 (2000) 1054–1058.
[27] D.K. MacCallum, J.H. Lillie, Evidence for autoregulation of cell division and cell transit in keratinocytes grown on collagen at an air–liquid interface, Skin Pharmacol. 3 (1990) 86–96.
[28] R.H. Tammi, M.I. Tammi, V.C. Hascall, M. Hogg, S. Pasonen, D.K. MacCallum, A preformed basal lamina alters the metabolism and distribution of hyaluronan in epidermal keratinocyte "organotypic" cultures grown on collagen matrices, Histochem. Cell Biol. 113 (2000) 265–277.
[29] H. Baden, J. Kubilus, J. Invest. Dermatol. 80 (1983) 124–130.
[30] A. Calabro, M. Benavides, M. Tammi, V.C. Hascall, R.J. Midura, Microanalysis of enzyme digests of hyaluronan and chondroitin/dermatan sulfate by fluorophore-assisted carbohydrate electrophoresis (FACE), Glycobiology 10 (2000) 273–281.
[31] A. Calabro, V.C. Hascall, R.J. Midura, Adaptation of FACE methodology for microanalysis of total hyaluronan and chondroitin sulfate composition from cartilage, Glycobiology 10 (2000) 283–293.
[32] E.V. Maytin, J.F. Habener, Transcription factors C/EBPα, C/EBPβ, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo, J. Invest. Dermatol. 110 (1998) 238–246.
[33] M. Taub, Retinoic acid inhibits basement membrane biosynthesis while stimulating dome formation by Madin Darby canine kidney cells in hormonally defined serum-free medium, J. Cell. Physiol. 148 (1991) 211–219.
[34] P.V. Haydock, B.A. Dale, Filaggrin, an intermediate filament-associated protein: structural and functional implications from the sequence of a cDNA from rat, DNA Cell Biol. 9 (1990) 251–261.
[35] M.K. Kuechle, C.D. Thulin, R.B. Presland, B.A. Dale, Profilaggrin requires both linker and filaggrin peptide sequences to form granules: implications for profilaggrin processing in vivo, J. Invest. Dermatol. 112 (1999) 843–852.
[36] A. Calabro, R. Midura, A. Wang, L. West, A. Plaas, V.C. Hascall, Fluorophore-assisted carbohydrate electrophoresis (FACE) of glycosaminoglycans, Osteoarthr. Cartil. 9 (2001) S16–S22.
[37] E.V. Maytin, J.C. Lin, R. Krishnamurthy, N. Batchvarova, D. Ron, P.J. Mitchell, J.F. Habener, Keratin 10 gene expression during differentiation of mouse epidermis requires transcription factors C/EBP and AP-2, Dev. Biol. 216 (1999) 164–181.
[38] S. Suzuki, Microbial hyaluronan lyases, in: V.C. Hascall, M. Yanagishita (Eds.), Glycoforum/Science of Hyaluronan review series, 2000, http://www.glycoforum.gr.jp/science/hyaluronan/HA14/HA14E.html.
[39] T. Ohya, Y. Kaneko, Novel hyaluronidase from *Streptomyces*, Biochim. Biophys. Acta 198 (1970) 607–609.
[40] A.B. Csoka, G.I. Frost, R. Stern, The six hyaluronidase-like genes in the human and mouse genomes, Matrix Biol. 20 (2001) 499–508.
[41] L.L. Faltz, C.B. Caputo, J.H. Kimura, J. Schrode, V.C. Hascall, Structure of the complex between hyaluronic acid, the hyaluronic acid-binding region, and the link protein of proteoglycan aggregates from the swarm rat chondrosarcoma, J. Biol. Chem. 254 (1979) 1381–1387.
[42] D. Mukhopadhyay, V.C. Hascall, A.J. Day, A. Salustri, C. Fulop, Two distinct populations of tumor necrosis factor-stimulated gene-6 protein in the extracellular matrix of expanded mouse cumulus cell–oocyte complexes, Arch. Biochem. Biophys. 394 (2001) 173–181.
[43] R. Tammi, D. MacCallum, V.C. Hascall, J.P. Pienimaki, M. Hyttinen, M. Tammi, Hyaluronan bound to CD44 on keratinocytes is displaced by hyaluronan decasaccharides and not hexasaccharides, J. Biol. Chem. 273 (1998) 28878–28888.
[44] R. Tammi, K. Rilla, J.P. Pienimaki, D.K. MacCallum, M. Hogg, M. Luukkonen, V.C. Hascall, M. Tammi, Hyaluronan enters keratinocytes by a novel endocytic route for catabolism, J. Biol. Chem. 276 (2001) 35111–35122.