Hyaluronan metabolism enhanced during epidermal differentiation is suppressed by vitamin C

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Hyaluronan metabolism enhanced during epidermal differentiation is suppressed by vitamin C

Running head: Epidermal maturation, vitamin C and hyaluronan metabolism

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The authors state no conflict of interest.
• What’s already known about this topic?

The extracellular matrix polysaccharide hyaluronan is abundant in the human epidermis and plays an important role in skin homeostasis and wound healing. Epidermis contains high levels of ROS which can induce hyaluronan degradation.

• What does this study add?

Hyaluronan metabolism is activated during epidermal stratification but stabilized again upon maturation. A large part of the hyaluronan exists as fragments which may have signaling functions. Vitamin C further stabilized epidermal hyaluronan by suppressing both synthesis and degradation, which is suggested to contribute to normal differentiation.

• What is the translational message?

Insults to epidermal homeostasis, such as trauma, inflammation and UV radiation activates hyaluronan turnover, which maintains an inflammatory state. The present data show that by slowing down hyaluronan turnover, vitamin C has potential as a suppressor of epidermal inflammation.

ABSTRACT

Background: Hyaluronan is a large, linear glycosaminoglycan present throughout the narrow extracellular space of the vital epidermis. Increased hyaluronan metabolism takes place in epidermal hypertrophy, wound healing and cancer. Hyaluronan is produced by hyaluronan synthases (HAS1-3) and catabolized by hyaluronidases (HYAL1 and -2), reactive oxygen species (ROS), and the KIAA1199 protein.
**Objectives:** To investigate the changes in hyaluronan metabolism during epidermal stratification and maturation, and the impact of vitamin C.

**Methods:** Hyaluronan synthesis and expression of the hyaluronan-related genes were analyzed during epidermal maturation from a simple epithelium to a fully differentiated epidermis in organotypic cultures of rat epidermal keratinocytes (REK) using qRT-PCR, immunostainings, and western blotting, in the presence and absence of vitamin C.

**Results:** With epidermal stratification, both the production and the degradation of hyaluronan were enhanced, resulting in an increase of hyaluronan fragments of various sizes. While the mRNA levels of Has3 and KIAA1199 remained stable during the maturation, Has1, Has2, and Hyal2 showed a transient upregulation during stratification, Hyal1 remained permanently increased, and the hyaluronan receptor Cd44 decreased. At maturation, Vitamin C downregulated Has2, Hyal2 and Cd44, while it increased high molecular mass hyaluronan in the epidermis, and reduced small fragments in the medium, suggesting stabilization of epidermal hyaluronan.

**Conclusions:** Epidermal stratification and maturation is associated with enhanced hyaluronan turnover, and release of large amounts of hyaluronan fragments. The high turnover is suppressed by vitamin C, which is suggested to enhance normal epidermal differentiation in part through its effect on hyaluronan.
INTRODUCTION

Epidermis is formed of continuously proliferating and differentiating keratinocytes\(^1\). Hyaluronan is a very hydrophilic, high molecular mass polysaccharide distributed in the extracellular space throughout the human epidermis up to the granular layer\(^2\). During fetal development the loss of hyaluronan staining precedes the formation of the granular and cornified layers\(^3,4\). Enzymatic removal of hyaluronan facilitates the expression of late differentiation markers\(^5\), suggesting a suppressive effect on terminal differentiation. Accordingly, hyaluronan accumulation associates with delayed differentiation and epidermal hyperplasia\(^4,6-9\). Hyaluronan forms a gel, facilitating the diffusion of nutrients, waste products and signaling molecules, which is of particular importance in tissues lacking blood and lymphatic vessels. Hyaluronan contributes to physiological processes including cell proliferation, migration and differentiation (reviewed in\(^10\)).

Hyaluronan is synthesized at the plasma membrane by hyaluronan synthases (HAS1-3). All three are expressed in keratinocytes, but reports of their relative contributions to epidermal hyaluronan production and roles in epidermal differentiation have varied considerably\(^11-15\).

Epidermal hyaluronan turnover is rapid, (half-life less than 24 h in human skin\(^16\) and organotypic REK cultures\(^17\)), while in monolayer REKs the catabolic activity is limited (R Tammi, unpublished data). The epidermal staining pattern of the hyaluronan receptor CD44 correlates with that of hyaluronan, suggesting their co-regulation and a role for CD44 in hyaluronan turnover\(^18,19\). The hyaluronidases Hyal1 and Hyal2 are expressed in the epidermis\(^9,13,20\). HYAL1 probably degrades hyaluronan in the granular cells\(^20\), while the role of HYAL2 is poorly understood. There are no reports concerning the epidermal expression of KIAA1199 (CEMIP), which is involved in hyaluronan degradation by fibroblasts\(^21\). Hyaluronan is also fragmented by reactive oxygen species (ROS)\(^22,23\). ROS are abundant in the epidermis\(^24,25\), and ROS scavengers inhibit epidermal hyaluronan degradation\(^26\).
We explored the influence of epidermal maturation on hyaluronan content, size, and metabolism using rat epidermal keratinocytes (REK) which form a fully differentiated epidermis without the help of dermal feeder cells\textsuperscript{27}. We also studied the influence of vitamin C, reported to facilitate normal epidermal differentiation\textsuperscript{28}, on hyaluronan metabolism. During the maturation from a simple epithelium into a multilayered, stratified epidermis, both the production and degradation of hyaluronan increased through upregulation of Has1, Has2, Hyal1 and Hyal2. Vitamin C tended to restrict the expression of Has2, Hyal1-2 and CD44, and stabilize hyaluronan by suppressing both its synthesis and degradation.

MATERIALS AND METHODS

Cell culture

REKs\textsuperscript{29} were maintained as monolayers in MEM (Gibco/Thermo Fisher Scientific, Waltham, MA), 10% FBS (HyClone, Thermo), 4 mM L-glutamine (Euroclone, Milan, Italy), 50 µg/ml streptomycin (Euroclone) and 50 U/ml penicillin (Euroclone). Organotypic cultures were seeded with 100,000 REK on polycarbonate inserts (0.4 µm pores, 12-well dishes, Thermo; modified from\textsuperscript{17}). After 2 days, the upper well medium was removed, and lower well medium was replaced with DMEM (Gibco/Thermo Fisher Scientific) containing the same supplements as above ± 50 µg/ml L-ascorbic acid (Sigma-Aldrich, Saint Louis, MO).

Histology

Organotypic cultures were either fixed in 2% paraformaldehyde (Electron Microscopy sciences, Hatfield, PA) or Histochoice® MB (Amresco, Solon, OH), embedded in paraffin, sectioned and stained with hematoxylin-eosin. For hyaluronan staining\textsuperscript{2}, overnight incubation with biotinylated hyaluronan binding complex (bHABC\textsuperscript{30}) was followed by avidin-biotin.
peroxidase (Vector laboratories, Burlingame, CA), diaminobenzidine (DAB, Sigma-Aldrich) plus \( \text{H}_2\text{O}_2 \) and Mayer’s hematoxylin.

For CD44\textsuperscript{\theta}, antigen retrieval was performed (Dako, Glostrup, Denmark) before sequential incubations with the anti-CD44 antibody (OX-50; Chemicon/Millipore, Billerica, MA, 1:200) and biotinylated anti-mouse antibody (1:200, Vector), followed by avidin-biotin peroxidase.

**siRNA transfections**

Cells grown overnight on 6-well plates were transfected with siRNA (final concentration 30 nM) against rHas3 (Eurogentech, Liège, Belgium), rHas2 and rHyal2 (Ambion, Austin, TX), or control siRNA (Origene, Rockville, MD). siRNA and RNAiMAX reagent (Thermo) in \( \alpha \)-MEM (Euroclone) were added to antibiotic-free MEM. After 4-6 h, transfection medium was replaced with normal medium. The cultures were harvested 2 days later.

**RNA isolation, cDNA synthesis and qRT-PCR**

Epidermis was lysed and RNA extracted with TRI Reagent\textsuperscript{\textregistered} (Molecular Research Center, Cincinnati, OH). The purity and the quantity of total RNA were measured by NanoDrop ND-1000 spectrophotometer (Thermo).

1 µg of total RNA was used for cDNA synthesis with Verso™ cDNA Synthesis Kit (Thermo). qRT-PCR was performed with MX3000P (Agilent technologies/Stratagene, Santa Clara, CA) using FastStart Universal SYBR Green Master with Rox (Roche, Mannheim, Germany). Supplemental Table 1 lists the gene-specific primers (Rplp0 used for normalization) and the cycling conditions. Fold changes were calculated as described\textsuperscript{31}.
Quantification of hyaluronan

Epidermis was digested with 0.05% (w/v) proteinase K (Fermentas/Thermo) in 0.1 M Na-phosphate pH 6.5, 10 mM EDTA at 60 °C for 1 h. After boiling for 10 min, hyaluronan was measured by a competitive ELISA-like kit (Echelon Biosciences, Salt Lake City, UT) or by a sandwich-type ELISA-like assay.32

For normalization, DNA was measured using bisbenzimidazol (Hoechst 33258, Sigma-Aldrich),33 with Calf thymus DNA standard (Sigma-Aldrich), in a fluorescence reader (Tecan Group Ltd., Männedorf, Switzerland).

Hyaluronan molecular mass analyses

Hyaluronan molecular size distribution was determined using a Sephacryl S-1000 1x30 cm column.17 Hyaluronan (2500 kDa, Hyalose, Oklahoma City, OK and GV-Healon, Abbott Laboratories, USA), hyaluronan oligosaccharides (HA14, ~2.8 kDa, Seikagaku, Tokyo, Japan) and glucuronic acid (Sigma) were used for calibration. Oligosaccharides and glucuronic acid were assayed as described.34 Hyaluronan in the fractions was measured after concentration (10:1) by both competitive and sandwich type ELISA-like assays. The fractions in the low molecular mass peak were pooled, divided in two equal portions, digested with proteinase K (200 µg/ml at 60°C for 2h), boiled, glycosaminoglycans precipitated with ethanol, and dissolved in 150 mM Na-acetate, pH 6.8. One of the portions was subjected to Streptomyces hyaluronidase (5TRU/ml, 2h at 60°C), and boiled and analyzed with competitive ELISA.
Protein isolation and Western blotting

Epidermis extracted in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% Nonidet, 0.5% Na-deoxycholate, 0.1% SDS, pH 7.5) was sonicated on ice. 10-15 µg of protein was separated with 10% SDS-PAGE, was transferred by semidry blotting (Biometra GmbH, Göttingen, Germany) to Protran® nitrocellulose (GE Healthcare). Blocking with 5% milk powder or 1% BSA (for hyaluronidases 1 and 2, respectively), was followed by incubation with anti-Hyal1 (1:500, LifeSpan BioSciences, Seattle, WA), anti-Hyal2 (1:100, Abcam, Cambridge, UK) or anti-β-actin (1:4000, Sigma-Aldrich), and fluorescent anti-rabbit or anti-mouse secondary antibodies (1:4000, Thermo). The blots were quantified with Odyssey® Infrared System (LI-COR Biosciences, Lincoln, NE).

Analyses of UDP-sugars

8- and 12-day specimens were homogenized in ice-cold PBS in Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA) using a FastPrep® homogenizer (Savant, Thermo), while 4-day cultures were suspended in ice-cold PBS and sonicated. After centrifugation, supernatants were purified by EnviCarb columns (Supelco, Sigma-Aldrich) before HPLC. Total protein was quantified (Pierce BCA kit, Thermo) for normalization.

Statistics

Data were analyzed with IBM SPSS Statistics, version 19 (SPSS, Chicago, IL). Log-transformation or Friedman test was used if the data were not normally distributed or the variances were unequal. Mixed model ANOVA and pairwise comparisons between the treatments were performed using the estimated marginal means (LSD). The controls (set as 1) and treatments were compared using the cumulative distribution function p-norm in R for
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RESULTS

Hyaluronan fragments accumulate in the epidermis during maturation

REK layers on polycarbonate inserts were 1-2 cells thick on day 4, i.e. one day after lifting to the air-liquid interface (Fig. 1a). By day 8, the epidermis consisted of 3-5 vital layers and a thin cornified layer (Fig. 1b). By day 12, a fully matured epidermis with 5-6 vital cell layers and a thick stratum corneum was observed (Fig. 1c).

Initially, all cell layers exhibited equal hyaluronan staining (Fig. 1d), while the signal was depleted in the uppermost vital layer during stratum corneum formation (Fig. 1f). Hyaluronan staining resided on cell surfaces with some intracellular signal detectable (Fig. 1e,f).

The concentration of hyaluronan in the unused medium (derived from serum) was 157 ng/ml (mean from three separate assays). This value, comprising approximately 25% and 7% of the hyaluronan in the culture media collected on days 4 and 12, respectively, has been subtracted from the values presented in Fig. 1h-i.

Culture media were changed 24 h before collecting the samples for the hyaluronan assays. The content of hyaluronan that diffused into medium during the 24 h incubation exceeded that present in the epidermis (Fig. 1g-i). Hyaluronan contents in the epidermis and medium were stable until day 6, then doubled by day 8-10, with little further changes on day 12 (Fig. 1g-i).

The size distribution of hyaluronan in the culture medium and epidermis could be divided into three major pools: one corresponding to high molecular mass HA, one representing small fragments close to the inclusion volume of the column (indicated by HA14; Fig. 1j), and one
between them. In the 4-day-old epidermis they comprised 38%, 41% and 21% of total hyaluronan, respectively (Fig. 1j), and 17%, 44% and 39% in the mature (12-day-old) epidermis, indicating a shift towards low molecular mass species. In the medium, there was no obvious difference in the hyaluronan size distribution between the immature and mature cultures (Fig. 1k). The high molecular mass HA comprised the minority of total hyaluronan in the medium (range 8-22%) (Fig. 1k). The medium sized fragments were the dominant species in one of the 3 experiments, while the small fragments dominated in two experiments. On the average, the small species constituted 54% of the hyaluronan in medium (Fig. 1k).

The expressions of Has1 and Has2 transiently increase during epidermal maturation

Using 3-day-old cultures as a reference point, Has3 and Has2 expression levels were higher in the organotypic cultures than the monolayers (qRT-PCR ΔCt-values for Has3: 12 vs. 16, for Has2: 9 vs. 11, respectively), whereas Has1 expression was approximately the same (ΔCt-value: 13 vs. 14). The level of Has3 mRNA did not significantly change during epidermal maturation, while Has1 and Has2 increased approximately two-fold on days 6 and 4, respectively, and returned back to the reference level in fully differentiated cultures (Fig. 2a-c).

The contributions of the different Has isoenzymes to hyaluronan accumulation were also probed with siRNAs. Attempts to consistently silence Has1 failed, but ~60% knockdown of Has2 and Has3 each reduced hyaluronan by 44% in the culture medium (Supplemental Figure 2a), as previously reported9. Cell-associated hyaluronan at cell-cell contacts and apical cell surfaces was also depleted, especially when combining the Has2 and Has3 siRNAs (Supplemental Figure 2c-e).
The supply of precursor sugars also strongly influences hyaluronan synthesis\(^{38-40}\). We therefore analyzed the concentrations of UDP-GlcNAc, UDP-GlcUA, and UDP-Glc, which showed a progressive decline with maturation (Fig. 2d-f), thus excluding elevated substrate levels as the cause for the increased hyaluronan content in mature epidermis.

**The expressions of Hyal1 and Hyal2 increase during epidermal maturation**

Among the proteins involved in hyaluronan metabolism, the expression of (based on day 3 qRT-PCR \(\Delta Ct\)-values), Hyal2 and Cd44 mRNA levels were relatively high (\(\Delta Ct\)-values: 9 and 8, respectively), whereas Hyall and KIAA1199 (CEMIP) were lower (\(\Delta Ct\)-values: 14 and 12, respectively). Hyall mRNA increased from day 3 to day 6 (2.5-fold), remaining elevated after that, while Hyal2 peaked on day 4 and returned close to the initial level by day 6 (Fig. 3a,b). Cd44 mRNA was significantly decreased on day 10 (0.7-fold; Fig. 3c), whereas KIAA1199 mRNA did not significantly change during maturation (Fig. 3d). With the anti-HYAL1 antibody, two faint bands (~55 and 48 kDa) emerged in the mature cultures (Fig. 3e) as reported previously\(^{41}\), while samples from early cultures were blank (Fig. 3e). This is in line with the initially low Hyal1 mRNA, and its increase with epidermal differentiation (Fig. 3a,e).

The anti-HYAL2 antibody also detected two bands (~56 and 52 kDa) in western blots. Both were quenched by the Hyal2 siRNA, suggesting that they represent HYAL2 (Fig. 3f). Band intensities were ~10-fold higher in the fully differentiated cultures (Fig. 3f,g).

CD44 in the single-layered epidermis was mainly localized on apical and lateral cell surfaces (Fig. 3i). Its staining intensity increased in the basal and spinous layers between culture days 8 (Fig. 3j) and 12 (Fig. 3k), but was always absent in the uppermost vital cells in fully differentiated cultures (Fig. 3k).
Vitamin C slows down epidermal hyaluronan metabolism

As vitamin C exerts a powerful effect on epidermal differentiation, we probed its influence on hyaluronan metabolism. In day 8 samples, only the expression of Has1 of the hyaluronan related genes was consistently influenced by vitamin C, showing a significant upregulation (Fig. 4). Has3 expression was strongly downregulated in 2 of the 3 experiments analyzed, but it did not reach statistical significance (Fig. 4c). In the fully differentiated cultures a marked decrease in the expressions of Has2, Cd44, and Hyal2 was observed, with a similar trend in Hyal1, but no changes in Has1, Has3 and KIAA1199 (Fig. 4). The decrease of HYAL2 was also detected by western blotting (Fig. 3f,g). These changes did not influence the spatial distribution of hyaluronan (Fig. 5a,b) or CD44 (Fig. 5c,d). However, they were accompanied with a reduced leakage of hyaluronan into the medium on day 8 (Fig. 5f), and followed by increased retention in the epidermis on day 12 (Fig. 5e). Hyaluronan size distribution was shifted slightly towards larger species in both the epidermis (from medium-sized to high molecular mass hyaluronan; Fig. 5h), and in the medium (from small fragments to medium-sized hyaluronan; Fig. 5i).

DISCUSSION

This work showed that during the active tissue reorganization from a single layer of keratinocytes into a multilayered epidermis, the metabolism of hyaluronan was stimulated, eventually leading to an elevated hyaluronan content. Interestingly, most of the hyaluronan comprised of medium-sized and small fragments. At maturation, the hyaluronan content remained high even though the Has-enzymes declined back to their pre-stratification level, and their UDP-sugar substrates were depleted. Importantly, vitamin C further enhanced the
differentiated state by suppressing both synthesis and catabolism, facilitating epidermal retention of high molecular mass hyaluronan.

The increased hyaluronan content during maturation of the organotypic epidermal cultures resembles the findings in human reconstituted skin$^{42}$. HAS2 and HAS3 were the main hyaluronan producers in REK, and their expression levels were higher in the 3D model than in conventional monolayers. The high but relatively stable Has3 expression during stratification suggests that it produced hyaluronan at an unchanged rate, not related to the exact maturation status, in line with the results of Sayo and colleagues$^{11}$. In contrast, the expressions of Has1 and Has2 increased transiently during stratification. Of these, Has2 was probably the main isoenzyme responsible for hyaluronan production, as suggested by the Has2 and Has3 siRNA silencing experiments, and the relatively low expression and low enzymatic activity of Has1$^{43}$.

The increased hyaluronan production and Has2 expression during the stratification phase in our model resembles those observed in epidermal hyperplasia during wound healing$^{4}$ and growth factor treatment$^{7,12}$. Considerable amounts of hyaluronan are detected in the hyperplastic epidermis of wounded Has1/3 KO mice, confirming the importance of Has2 in activated epidermis$^{44}$. While the epidermal hyaluronan synthesis does not directly correlate with cell proliferation$^{45}$, terminal differentiation is delayed by hyaluronan synthesis$^{5}$, thereby contributing to epidermal hypertrophy by increasing the number of vital cell layers.

Previous contradictory findings concerning Has2 expression in keratinocytes$^{13,14,46}$ may reflect specific factors in the experimental conditions: growth factors present in serum are known to upregulate Has2$^{7,12,47}$, whereas hydrocortisone$^{48-50}$ and vitamin C (present findings) considerably decrease it. Serum-free media for keratinocytes often include both hydrocortisone and vitamin C, resulting in suppressed Has2 expression.
Hyaluronan turnover is considerably higher in organotypic REK cultures\textsuperscript{17}, as compared to the slow degradation in monolayers (~20\% per day, R. Tammi, unpublished). This is reflected in the large amounts of short hyaluronan fragments found in the organotypic culture medium, previously underestimated because of the types of hyaluronan assays used\textsuperscript{9,16,19}. For example, the sandwich type HA-ELSA virtually ignored fragments smaller than ~50 kDa. The presence of the small fragments indicates that the final steps in the degradation process lag behind the initial extracellular fragmentation. How abundant these fragments are in vivo, and whether they are produced for a specific biological purpose, is unknown. Interestingly, ~50 kDa hyaluronan fragments appear to promote the expression of genes related to epidermal differentiation and tight junction complexes\textsuperscript{51}. The small fragments may also have signaling functions for dermal cells, or they could accumulate in the stratum corneum\textsuperscript{52}, affecting barrier characteristics\textsuperscript{52}, and enhance bacterial defense mechanisms in skin by inducing the secretion of $\beta$-defensin via activation of Toll-like receptors\textsuperscript{53}. Hyaluronan fragments may also act as a self-sustaining regulatory loop to propagate further hyaluronan production. They were also reported to increase hyaluronan and CD44 staining in the mouse epidermis\textsuperscript{54}, together with increased expression of Has2, Has3 and Hyal\textsuperscript{54,55}. The signaling route involved was independent of CD44, but remained otherwise unrevealed. Engagement of Toll-like receptors by hyaluronan fragments activates NFkB (reviewed in\textsuperscript{56,57}), which in turn can control Has2 expression\textsuperscript{58,59}.

During epidermal stratification, hyaluronan fragments increased concomitantly with the upregulation of Hyal2 and Hyal1. The increase in medium-sized and short hyaluronan fragments match the properties of HYAL2, with a reported maximal degradation down to ~20 kDa fragments (reviewed in\textsuperscript{56}). KIAA1199 (CEMIP) is another protein that could account for the hyaluronan fragments\textsuperscript{21}, but its mRNA expression was unaffected by epidermal differentiation, and was actually highest in the monolayers (data not shown). While both

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HYAL1 and HYAL2 are most active at lysosomal pH, the presence of HYAL2 on the plasma membrane suggests a contribution to the extracellular degradation of hyaluronan\textsuperscript{60}. Thus, HYAL2 and ROS (see below) are good candidates for producing the hyaluronan fragments observed.

The low levels of \textit{Hyal1} mRNA and protein in the organotypic REK cultures, and appearance of the HYAL1 protein late during maturation, are in line with its strict localization in the granular layer\textsuperscript{20} where it has been suggested to act as a gatekeeper, restricting high molecular mass hyaluronan entering the stratum corneum\textsuperscript{20}.

CD44 is an abundant cell surface protein in keratinocytes, important for binding and organizing pericellular hyaluronan, and contributing to its endocytosis\textsuperscript{18,61,62}. The mRNA level of \textit{Cd44} was reduced during epidermal maturation. However, no such trend was found in CD44 content in immunohistochemical examination of the basal and spinous layers. Like in the fetal epidermal maturation\textsuperscript{4,63}, CD44 disappeared from the superficial layers upon formation of the granular layer and the diffusion barrier. Disappearance of CD44 and increased HYAL1 activity both probably contribute to the lack of hyaluronan in the granular layer.

Vitamin C has a dual-sided effect on free radicals. While at low doses it acts as an antioxidant, protecting cells from the harmful effects of ROS (reviewed in\textsuperscript{64}), at high (mM) doses it can be a pro-oxidant, leading to apoptosis\textsuperscript{65}. The topical formulations protecting against UV-radiation\textsuperscript{64,66} contain relatively high concentrations of vitamin C (15-20\%\textsuperscript{64}), but the biologically effective dose may be considerably lower due to the poor penetration of vitamin C through stratum corneum\textsuperscript{67}.
In the present study, the relatively low, close to the physiological concentration of vitamin C \(^{68}\) appeared to slow down hyaluronan metabolism in the reconstituted epidermis. The mechanism remains open at present. Although ROS can directly fragment hyaluronan chains non-enzymatically \(^{69,70}\) and scavenging of free radicals by the use of catalase or superoxide dismutase delayed degradation of epidermal hyaluronan in human skin organ cultures \(^{26}\), the fact that vitamin C in REK cultures strongly influenced the expression levels of the enzymes involved in hyaluronan degradation as well as synthesis suggests that other mechanisms were involved. Vitamin C is known to regulate intracellular signaling pathways like NFkB both via ROS dependent and independent mechanisms \(^{71}\).

Since hyaluronan metabolism is stimulated in activated keratinocytes, for example due to wounding, irritation, UV-light, growth factors, malignant transformation, or inflammation \(^{72}\), the finding that vitamin C can normalize hyaluronan turnover fits well with previous reports of the beneficial effects of vitamin C on ceramide synthesis \(^{73}\), formation of cornified envelopes \(^{74}\), and barrier properties of the stratum corneum \(^{28}\). The contribution of hyaluronan metabolism to these effects appears an interesting topic of further research.

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References


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64 Telang PS. Vitamin C in dermatology. *Indian Dermatol Online J* 2013; **4**:143-6.


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FIGURE LEGENDS

Figure 1: Hyaluronan production and distribution in organotypic keratinocyte cultures during differentiation. Organotypic REK cultures were harvested after 4, 8 and 12 days of growth, fixed, and processed for histology. Samples were stained with hematoxylin & eosin (a-c) for morphology and with bHABC for hyaluronan (d-f) using DAB as a chromogen. White arrowheads indicate stratum corneum and black arrowhead loss of hyaluronan staining in granular layer. Magnification bars: 50 µm. Epidermal (g), medium (h) and total (i) hyaluronan were quantified from organotypic keratinocyte cultures collected on the indicated days using a commercial, competitive ELISA-kit. The concentration of hyaluronan in the unused medium, 157 ng/ml, was subtracted from the hyaluronan detected in the used culture media. The data represent means ± SEM from five individual experiments with two replicates in each. Statistical significances for log-transformed values were tested using a mixed model analysis comparing the values to day 4 (* p < 0.05; ** p < 0.01; *** p < 0.001).

The molecular mass of hyaluronan was determined using S1000 size exclusion chromatography, and competitive HA-ELISA from epidermis (j) and culture media (k) of 4- and 12-day-old organotypic keratinocyte cultures (means ± SEM from 3 independent experiments). The positions of hyaluronan standards (Hyalose) and hyaluronan oligosaccharides (HA14) are indicated by arrows, and the 3 different size pools by bars.

Figure 2: Has1, Has2 and Has3 exhibit distinct mRNA expression patterns during epidermal maturation. Relative mRNA expression was determined using qRT-PCR from organotypic keratinocyte cultures during cultivation days 3-12. All results were normalized to Rplp0 with day 3 as a calibrator (=1). Data represent means ± SEM from 4 individual experiments with two replicates in each. Statistical significance was analyzed using mixed
model ANOVA, comparing the values from days 4-12 to day 3 (set as 1) using the \textit{pnorm}-function and correcting for multiple comparisons. For \textit{Has2} the non-parametric Friedman test was used due to unequal variances. Epidermal sheets from REK organotypic cultures on days 4, 8 and 12 were harvested and UDP-GlcNAc (d), UDP-GlcUA (e) and UDP-Glc (f) analysed as described in Materials and Methods. The data represent means ± SEM from 5 independent experiments (4 experiments for day 12) with 1 to 2 replicates in each. Statistical significance was analyzed using mixed model ANOVA, comparing the values from days 8 and 12 to day 4. ** \( p < 0.01 \) and *** \( p < 0.001 \).

\textbf{Figure 3. Hyaluronidase expression is altered during epidermal maturation.} Relative mRNA expression of \textit{Hyal1} (a), \textit{Hyal2} (b), \textit{Cd44} (c) and \textit{KIAA1199} (d) were determined using quantitative real-time PCR. All mRNA levels were normalized to \textit{Rplp0} and day 3 was used as a calibrator (\( = 1 \)). The data represent means ± SEM of 4 (\textit{Hyal1} and \textit{Hyal2}) and 3 (\textit{Cd44} and \textit{KIAA1199}) individual experiments with two biological replicates in each. Statistical differences comparing to day 3 were calculated as explained in Figure 2. HYAL1 and HYAL2 protein expressions were determined from 4, 8 and 12-day-old organotypic cultures using western blot analysis (e, f). HYAL2 was quantified after normalisation (g, h) with \( \beta \)-actin. The data represent means ± SEM from 4 independent experiments. Statistical differences at 8 and 12 day time points compared to day 4 and between control and vitamin C treated (12+C) samples at day 12 were calculated using mixed model ANOVA correcting for multiple comparisons. The distribution of CD44 (i-k) was analysed by immunostaining with the OX50 antibody as described in Materials and Methods. Arrowheads indicate stratum corneum. Magnification bar: 50 µm. * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \).
Figure 4. Influence of vitamin C on hyaluronan related genes in organotypic cultures.
50 µg/ml of vitamin C was added every other day to REK organotypic culture medium from day 4 onwards. mRNA expression of hyaluronan related genes (a-g) was quantified by qRT-PCR, and normalized to Rplp0. Controls at each day were set as 1. Data represent means ± SEM from 3 individual experiments. Statistical difference at each time point was calculated using mixed model ANOVA correcting for multiple comparisons. The treatments were compared to the controls (set as 1) using the pnorm-function (* p < 0.05, ** p < 0.01 and *** p < 0.001; compared to day 3). The data represent means ± SEM.

Figure 5. Influence of vitamin C on CD44 and hyaluronan content and hyaluronan molecular mass distribution in organotypic cultures. 12-day-old REK organotypic cultures with (b, d) or without (a, c) vitamin C treatment were stained for hyaluronan (a, b) and CD44 (c, d) as described in Materials and methods. Arrowheads indicate stratum corneum. Magnification bar represents 50 µm. Hyaluronan in the epithelium and in the medium in control and vitamin C-treated cultures was quantified with competitive hyaluronan ELISA (e-g). Data represent means ± SEM from 4 individual experiments with two replicates in each. The statistical difference at each time point compared to the corresponding control was calculated using mixed model ANOVA. (* p < 0.05, ** p < 0.01 and *** p < 0.001). The molecular mass distribution of hyaluronan was determined from epidermis (h) and culture media (i) from 12-day-old organotypic cultures, with or without vitamin C treatment, using S1000 gel filtration analysis. Means ± SEM from 3 independent experiments are shown. The arrows indicate the elution positions of hyaluronan standards and hyaluronan oligosaccharides (HA14), and the bars the different size pools.
Supplemental Figure 1

Molecular mass of fetal bovine serum and molecular mass dependence of the different types of ELISA-like hyaluronan assays. The molecular mass distributions were analyzed on an S1000 gel filtration column, and the hyaluronan content in the fractions was measured using the sandwich and competitive type ELISA-like assays. (a) Hyaluronan in fetal bovine serum. (b) Pooled culture media of 12-day-old organotypic keratinocyte cultures. The percentage of total detected in each of the fractions with the two assay types are shown (means and ranges of two independent experiments). The low molecular mass fractions of the culture medium were pooled and subjected to *Streptomyces* hyaluronidase digestion, which removed most of the signal detectable with the competitive ELISA-like assay.

Supplemental Figure 2

Effect of *Has2* and *Has3* siRNA suppression on hyaluronan synthesis. Monolayer REK cultures were transfected with control siRNAs and siRNAs targeted against rat *Has2* and *Has3*. (a) Hyaluronan in the media of the siRNA-treated cultures, assayed with the sandwich type assay (means ± SEM of 6 independent cultures). (b-e) REK cultures treated with indicated siRNAs stained for hyaluronan as described in Materials and Methods. The magnification bar represents 50 µm.

Supplemental table 1. Cycling conditions and primer sequences used in qRT-PCR

Supplemental table 2. Sequences of the siRNA-oligos used