CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Basement Membrane Helps Maintain Epidermal Hyaluronan Content

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Hyaluronan (HA) is the major glycosaminoglycan in the extracellular matrix of most mammalian tissues, including the epidermis. It is synthesized in epidermis, and mainly metabolized after transfer to the liver via lymphatic vessels in the dermis following its passage through the basement membrane (BM) at the dermal-epidermal junction. The aim of the present study was to investigate the influence of BM integrity on the level of HA in the epidermis. Epidermal HA content was decreased in sun-exposed skin of older subjects, whose BM structure was impaired, compared with sun-exposed young skin and sun-protected skin, in which BM integrity was well maintained. In an organotypic culture model of sun-exposed facial skin, epidermal HA was increased in the presence of inhibitors of BM-degrading matrix metalloproteinases and heparanase. In a skin equivalent model treated with these inhibitors, HA content was increased in the epidermis, but decreased in conditioned medium. These findings suggest that the BM at the dermal-epidermal junction plays an important role in maintaining epidermal HA levels. (*Am J Pathol* 2021, 191: 1010–1019; <https://doi.org/10.1016/j.ajpath.2021.03.007>)

Hyaluronan (HA) contains numerous negatively charged carboxyl groups, which contribute to the maintenance of skin tissue water content and permeability barrier homeostasis.¹ HA and its receptor CD44 also play roles in many other physiological functions, including cell proliferation, adhesion, migration, differentiation, inflammatory response, and wound healing.^{2,3}

The aging of human skin can be explained in terms of the combination of intrinsic aging (ie, the natural aging process) and photoaging, which is premature skin aging resulting from chronic sunlight exposure. There have been several studies on intrinsic aging- and photoaging-dependent changes of HA in human skin *in vivo*,^{4–6} but the mechanism of age-dependent changes of epidermal HA content remains unclear.

HA is mainly localized around the cells of the basal and spinous layers,^{7,8} and is synthesized directly in the extracellular matrix by three hyaluronan synthases (*HAS1*, *HAS2*, and *HAS3*).⁹ These HASs differ in their temporal expression pattern during development, their specific activity, and the size of the HA polymers they produce.¹⁰ The expression levels of *HAS1-3* in sun-exposed skin are lower than those in sun-protected skin.¹¹ Other studies have found that expression of *HAS1-3* in cultured human keratinocytes increases after UVB irradiation.^{12,13}

The current model of HA turnover in the whole body is as follows. Large negatively charged molecules (1000 to 10,000 kDa) of HA are partially degraded by hyaluronidase-1 (*HYAL1*) in the epidermis,¹⁴ and by *HYAL1*, *HYAL2*, and hyaluronan binding protein involved in hyaluronan depolymerization (*HYBID*) in the dermis,¹⁵ into intermediate size fragments (10 to 100 kDa). The HA fragments in the dermis are transferred into the lymphatic vessels via interaction with lymphatic vessel endothelial hyaluronan receptor 1¹⁶ and are degraded in the lymph nodes.^{17,18} HA that escapes degradation in the lymph nodes ultimately flows into the blood at the thoracic duct, and is completely digested in the liver.^{19,20} Although HA synthesized in the epidermis is mainly digested in the lymph nodes and the liver after passing through the basement membrane (BM), a part of it is degraded in the stratum corneum and eliminated by desquamation.^{21–23} Studies using radiolabeled HA indicated that turnover of HA is rapid, especially in skin, the key organ for HA replacement.^{20,24,25} HA's metabolic half-life is 1 to 1.5 days. Expression of the *HYAL1-3* and *HYBID* genes is up-regulated in sun-exposed skin compared with sun-protected skin.^{11,26}

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Moreover, the lymphatic drainage function is also impaired by UVB irradiation.²⁷ Thus, HA content in the epidermis is temporarily increased from days 1 to 3 after UVB irradiation, and then declines during days 3 to 5.²⁸

The BM at the dermal-epidermal junction (DEJ) binds tightly to the epidermis²⁹ and enables asymmetric cell division for epidermal differentiation and homeostasis.³⁰ In sun-exposed skin, the BM is disrupted at the DEJ and forms a reduplicated lamina densa.^{31,32} Matrix metalloproteinase (MMP)-9 and heparanase in the epidermis are activated by UV irradiation in skin equivalent (SE) models and organotypic human skin models,^{32,33} and UV-damaged BM structure is reconstituted in the presence of MMP and heparanase inhibitors.^{34,35} It is hypothesized that the improvement of BM integrity in sun-exposed skin by MMP and heparanase inhibitors would lead to increased retention of hyaluronan in the epidermis. In the present study, SE models and organotypic human skin are used to test this idea.

Materials and Methods

Subjects

Skin samples were obtained from Biopredic International (Rennes, France). The principles of the Declaration of Helsinki were adhered to, and all participants had given written, informed consent to provide samples for research. Human skin samples were obtained from subjects between the ages of 32 and 68 years. The characteristics of the samples are summarized in [Supplemental Table S1](#). Skin samples were fixed in cold acetone (acetone, methyl benzoate, and xylene; AMeX procedure) and embedded in paraffin for immunohistochemical observation using specific antibodies.

Materials

Heparanase inhibitor 1-[4-(1H-benzimidazol-2-yl)phenyl]-3-[4-(1H-benzimidazol-2-yl)phenyl]urea (BIPBIPU), MMP inhibitor N-hydroxy-2(R)-[(4-methoxyphenyl)sulfonyl](3-picolyl)-amino-3-methylbutanamide hydrochloride (CGS27023A), and bifunctional inhibitor hydroxyethyl-imidazolidinone were synthesized in our laboratories, according to the literature methods.^{35–37}

SE Model

The SE model (EFT-400) and medium (EFT-400-ASY) were purchased from MatTek Corp. (Ashland, MA). The SE model was subsequently cultured in the presence or absence of BIPBIPU (10^{-5} mol/L) or CGS27023A (10^{-5} mol/L) or both. The culture medium was changed every day and used for quantification of HA. At 4 days after the start of culture, samples were fixed in cold acetone (AMeX procedure) and embedded in paraffin for immunohistochemical analysis. At the same time, samples were separated into epidermis and

dermis, and RNA was extracted for quantitative PCR analysis.

Organotypic Human Skin Model

Fresh human abdominal skin (from four females aged 22 to 32 years; the characteristics of the samples are summarized in [Supplemental Table S2](#)) was cut into 1.5×1.5 -cm pieces, which were cultured in the presence or absence of 0.1 mg/mL hydroxyethyl-imidazolidinone, BIPBIPU (10^{-5} mol/L), and CGS27023A (10^{-5} mol/L) after UVB irradiation (50 mJ/cm^2). At 5 days after the start of culture, samples were fixed in cold acetone (AMeX procedure) and embedded in paraffin for immunostaining. Fresh human eyelid skin (from four females aged 60 to 69 years; the characteristics of the samples are summarized in [Supplemental Table S2](#)) was cut into 1.5×1.0 -cm pieces, and cultured in the presence or absence of 0.1 mg/mL hydroxyethyl-imidazolidinone, BIPBIPU (10^{-5} mol/L), and CGS27023A (10^{-5} mol/L). At 3 days after the start of culture, samples were fixed in cold acetone (AMeX procedure) and embedded in paraffin for immunostaining.

Immunohistochemistry

Each section was incubated overnight at 4°C with primary antibodies targeting heparan sulfate (HS; clone 10E4; Seikagaku Kogyo, Tokyo, Japan), CD44 (F10-44-2; Abcam, Tokyo, Japan), or biotin-conjugated HA binding protein (HABP; Hokudo, Hokkaido, Japan). Primary antibodies were detected using Alexa488- or Alexa594-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA). Bound biotin-HABP was detected using streptavidin–horseradish peroxidase (Thermo Fisher Scientific) with a 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific). Sections were examined with an Olympus BX51 microscope (Olympus, Tokyo, Japan), and images were captured with a DP72 controller digital camera (Olympus).

Alcian Blue Staining

Each section was deparaffinized in xylene and acetone. After washing with distilled water, sections were incubated with 3% acetic acid for 3 minutes, stained with Alcian Blue staining solution (pH 2.5; Nacalai, Tokyo, Japan) for 2 hours,³⁸ and examined with an Olympus BX51 microscope. Images were captured with a DP72 controller digital camera.

Enzyme-Linked Immunosorbent Assay for HA and HS

The epidermis and dermis were separated from fresh human skin and the SE model. Each tissue was cut into small pieces and digested with radioimmunoprecipitation assay buffer (Nacalai). The protein concentration of these tissue lysates was normalized on the basis of the result of bicinchoninic acid protein assay (Nakarai, Tokyo, Japan). The contents of HA and

HS in the tissue lysate and culture media were measured with a QnE HA enzyme-linked immunosorbent assay kit (Funakoshi, Tokyo, Japan) and an HS enzyme-linked immunosorbent assay kit (Seikagaku Kogyo, Tokyo, Japan), respectively.

Cell Culture

Human normal epidermal keratinocytes obtained from several aged donors (for details, see [Supplemental Table S3](#)) were cultured with Humedia-KG2 (KURABO, Osaka, Japan). Culture flasks were incubated at 37°C in a humidified atmosphere with 5% CO₂. A total of 2.5×10^5 keratinocytes were seeded into a 6-well plate and incubated for 24 hours, then irradiated with 20 or 50 mJ/cm² UVB. After 24 hours, RNA was extracted from each sample for quantitative PCR analysis, and the culture medium was collected for HA quantification.

Quantitative Real-Time RT-PCR

The total epidermal RNA from the cultured keratinocytes was isolated using a Qiagen Rneasy mini kit (Qiagen, Tokyo, Japan), and cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). Expression of *B2M*, *HAS2*, *HAS3*, *HYAL1*, and *HYAL2* genes in the epidermis was analyzed by quantitative PCR using Platinum SYBR Green qPCR superMix-UDG (Invitrogen Japan, Tokyo, Japan), as described previously.³⁹ The primer sequences used were as follows: *B2M*, 5'-GTGGGATCGA-GACATGTAAGCA-3' (forward) and 5'-CAATCCA-AATGCGGCATCT-3' (reverse); *HAS2*, 5'-TCAGAGCAC-TGGGACGAAG-3' (forward) and 5'-CCCAA-CACCTCCAACC-AT-3' (reverse); *HAS3*, CAGCAACTTC-CATGAGGC-3' (forward) and CACAGTGTGAGA-GTCGCA-3' (reverse); *HYAL1*, 5'-GCACAGGGAAGTCA-CAGATGTGC-3' (forward) and CCACT-GGTCACGTT-CAGGATGAAG-3' (reverse); and *HYAL2*, 5'-CCATGCACTCCCAGTCTACGTC-3' (forward) and 5'-TCACCCCAGAG-GATGACACCAG-3' (reverse).

Statistical Analysis

The data are presented as the mean values $\pm$ SD. Statistical significance was determined by analysis of variance with Bonferroni corrections.

Results

Epidermal HA in Sun-Exposed Facial Skin Decreases with Aging

Because the site dependence of changes of HA content in skin have not been fully elucidated, immunohistochemistry for HABP was used to detect hyaluronic acid in cheek, eyelid, and buttock skin from the same donors. The HA staining intensity was markedly lower in eyelid skin than in cheek skin or buttock skin ([Figure 1](#), A–F). Next, to examine the age-dependent

change of HA content in sun-exposed facial skin, immunohistochemistry for HABP was used to detect hyaluronic acid in samples of aged skin from sun-protected and sun-exposed areas. HA staining intensity was more markedly reduced with aging in sun-exposed eyelid skin than in sun-protected abdominal skin ([Figure 1](#), G–J). Furthermore, the decrease of HA content with aging in sun-exposed eyelid skin was greatest in the epidermis ([Figure 1](#), K and L). Because HA binds to CD44 in the epidermis, immunostaining for CD44 was performed next. The CD44 level in the epidermis was reduced in skin from older subjects compared with that from younger subjects ([Figure 2](#), A–D). *CD44* gene expression also decreased with aging ([Figure 2E](#)). The CD44 level in the epidermis was more markedly reduced in eyelid skin than in cheek skin or buttock skin ([Supplemental Figure S1](#)). Because CD44 is known to bind to large HS chains in core proteins, immunostaining for HS was performed next. The results indicate that HS in the epidermis and at the BM decreases with aging, in agreement with previous findings ([Supplemental Figure S2](#), A–D). The HS content of whole skin was significantly reduced in older subjects compared with younger subjects ([Supplemental Figure S2E](#)). Thus, these findings indicate that both HA and HS are decreased in sun-exposed eyelid skin compared with sun-protected abdominal skin.

Changes of HA-Related Gene Expression with Aging and UV Exposure

Because HA is synthesized by *HAS2-3* and degraded by *HYAL1-2* in the epidermis, gene expression levels were investigated in cultured keratinocytes from donors of different ages. *HAS2* mRNA was significantly reduced, whereas *HAS3* and *HYAL1* mRNAs were increased with age, in agreement with previous findings (Ref ??) ([Supplemental Figure S3](#), A–C), and *HYAL2* mRNA showed no change ([Supplemental Figure S3D](#)). The HA content of the culture medium significantly increased with aging ([Supplemental Figure S3E](#)). Because HA content was reduced in sun-exposed epidermis ([Figure 1F](#)), the effects of UV irradiation were examined on the expression of genes related to HA synthesis and degradation. *HAS2* mRNA was decreased, and *HAS3* mRNA was increased, after UVB irradiation, compared with nonirradiated keratinocytes ([Supplemental Figure S3](#), A and B), whereas *HYAL1-2* mRNAs showed no change ([Supplemental Figure S3](#), C and D). The HA content of the culture medium did not change after UVB irradiation ([Supplemental Figure S3E](#)). Together, these data indicate that the induction of HA synthesis in keratinocytes after UVB irradiation was not associated with the reduction of HA content in the epidermis of sun-exposed facial skin of older subjects.

HA Content Is Increased in Epidermis of the Organotypic Human Eyelid and Abdominal Skin Model Treated with MMP and Heparanase Inhibitors

Epidermal HA content was reduced in photo-damaged skin of older subjects compared with that in younger subjects

Figure 1 Age-dependent change of hyaluronan (HA) contents in epidermis and dermis from donors of different ages. **A–F:** HA binding protein (HBP) staining of buttock (**A** and **D**), cheek (**B** and **E**), and eyelid (**C** and **F**) skin of a 68-year-old subject, detected using diaminobenzidine substrate (**A–C**) and immunofluorescence with Alexa-488 (**D–F**). **G–J:** Immunostaining of biotinylated HBP in 32-year-old sun-protected abdominal skin (**G**), 63-year-old sun-protected abdominal skin (**H**), 36-year-old sun-exposed eyelid skin (**I**), and 66-year-old sun-exposed eyelid skin (**J**). **K** and **L:** The HA content of whole sun-exposed eyelid skin was evaluated using enzyme-linked immunosorbent assay (ELISA; **K**), and the HA contents of the separated epidermis and dermis were also evaluated using ELISA (**L**). **K** and **L:** Young refers to subjects in their 30s, and old refers to subjects in their 60s. Data are the means $\pm$ SD of six independent experiments from six donors (**K** and **L**). * $P < 0.05$, ** $P < 0.01$. Scale bar = 100 μ m (**A–J**).

(Figure 1L), whereas HA secretion from cultured keratinocytes increased with aging, but not with UVB exposure (Supplemental Figure S3E). Because the basement membrane at the DEJ is impaired by UVB exposure, it was hypothesized that the basement membrane acts as a physical barrier to retain HA in the epidermis. The deterioration of the BM at the DEJ of organotypic human eyelid skin and abdominal skin after UVB exposure is blocked by treatment with both the MMP inhibitor CGS27023A and the heparanase inhibitor BIPBIPU. Therefore, the HA content in the epidermis and the ultrastructure of the basement membrane of organotypic human skin were examined after UVB exposure following treatment with MMP and heparanase

inhibitors. Representative HBP staining images of the control before culture are shown in Figure 3A. Corresponding images of the vehicle- and inhibitor-treated human skin explant tissues after 3 days of culture are shown in Figure 3, B–D. The HBP staining intensity was increased after treatment with the inhibitors, compared with the control (Figure 3, B–D).

The intensity of HBP staining was reduced in cultured abdominal skin after UVB irradiation (Figure 3, E and F), and was increased in the presence of both inhibitors (Figure 3, F–H). Alcian Blue staining showed that epidermal GAG content was also increased by the inhibitors (Figure 3, I–L). Transmission electron microscopy revealed

Figure 2 Age-dependent change of CD44 in epidermis from donors of different ages. **A–D:** Immunostaining of CD44 in 32-year-old sun-protected abdominal skin (**A**), 63-year-old sun-protected abdominal skin (**B**), 36-year-old sun-exposed eyelid skin (**C**), and 66-year-old sun-exposed eyelid skin (**D**). **E:** CD44 mRNA in cultured keratinocytes from sun-protected abdominal skin of subjects in their 10s to 30s and subjects in their 40s to 60s was analyzed by real-time quantitative PCR. Data are the means $\pm$ SD of six independent experiments from six donors (**E**). $**P < 0.01$. Scale bar = 100 μ m (**A–D**).

improved continuity of the lamina densa in the presence of both inhibitors, compared with the control (Figure 3, M–O). The HS staining intensity was also increased in the presence of both inhibitors, in agreement with previous findings (Supplemental Figure S4). These findings in *ex vivo* cultured eyelid and abdominal skin are consistent with the idea that the basement membrane acts as a physical barrier to retain HA in the epidermis.

HA Content Is Increased in Epidermis of the SE Model Treated with MMP and Heparanase Inhibitors

BM integrity in the SE model is improved by treatment with MMP and heparanase inhibitors (Ref#42). To investigate the effect of BM integrity at the DEJ on the distribution of HA in the epidermis, immunohistochemical staining for biotinylated HABP and heparan sulfate and Alcian Blue staining were conducted. HABP was increased in the epidermis after the inhibitor treatment (Figure 4, A–D). To investigate the distribution of HA in the SE model, HA contents in the epidermis, dermis, and cultured media of the SE model were evaluated in the presence of MMP and heparanase inhibitors. HA was increased in the epidermis and unchanged in the dermis following treatment with the MMP and heparanase inhibitors (Figure 4, E and F). The inhibitors significantly reduced the HA level in the culture medium (Figure 4G). In addition, Alcian Blue staining showed that the epidermal GAG content was increased in the presence of the two inhibitors (Supplemental Figure S5). Heparan sulfate was

increased in the presence of heparanase inhibitor, as determined by enzyme-linked immunosorbent assay and histologic staining for heparan sulfate, in agreement with previous findings (Supplemental Figure S6, A–F). In addition, transmission electron microscopy revealed that the continuity of lamina densa was improved in the presence of both inhibitors, compared with the control, in agreement with previous findings (Figure 4, H–K). In the epidermis of the SE model, expression of HA-related genes, including *HAS2*, *HAS3*, *HYAL1*, and *HYAL2*, was unchanged in the presence of these inhibitors (Supplemental Figure S7). These results suggest that the increase of HA in the epidermis was due to suppression of HA efflux from the epidermis, rather than induction of HAS gene expression in the epidermis. This, in turn, indicates that the BM at the DEJ regulates transfer of HA from the epidermis into the dermis and culture medium.

Discussion

MMPs and heparanase are activated in the epidermis of sun-exposed skin,^{32,33,40} leading to disruption of the BM with the formation of reduplicated lamina densa.³¹ In particular, MMP-9 is highly expressed in eyelid skin.⁴¹ The present findings show that inhibitors of MMPs and heparanase promote BM reconstruction and the formation of lamina densa in the organotypic human skin model (Figure 3, M–O),^{34,35} as well as in the SE model (Supplemental Figure S7).⁴² The HA content in the epidermis was increased by blocking degradation of the

Figure 3 Epidermal hyaluronan (HA) content was increased by promoting basement membrane formation in organotypic human eyelid and abdominal skin models. Immunostaining of biotinylated HA binding protein in the organotypic human eyelid skin model (A–D) and abdominal skin model (E–H) in cultures with matrix metalloproteinase (MMP) inhibitor and heparanase inhibitor (C and G), with a bifunctional inhibitor (D and H), and without these inhibitors (B and F), compared with the day 0 control (A and E). Alcian Blue staining in the organotypic human abdominal skin model after UVB irradiation (I–L) of cultures with the MMP inhibitor and heparanase inhibitor (K), with a bifunctional inhibitor (L), and without these inhibitors (J), compared with the day 0 control (I). Transmission electron microscopic observation of organotypic human skin: control (M), and treated with both MMP and heparanase inhibitors (N) or the bifunctional inhibitor (O). White arrowheads indicate lamina densa. Scale bars: 100 μm (A–L); 500 nm (M–O).

BM in the SE model (Figure 4) and the organotypic human skin model (Figure 3), whereas the HA content in the epidermis was reduced in sun-exposed eyelid skin from older subjects (Figure 1), in whom the BM is known to be degraded.³² Yoshida et al²⁶ reported that the HA content of whole skin was reduced in sun-exposed skin compared with that in sun-protected skin. Meyer and Stern⁶ found that the HA staining intensity in the epidermis decreased with aging, and no HA was detectable in the epidermis of senescent skin from an 81-year-old subject. Oh et al⁴ reported that the HA content in the epidermis was unaffected by aging in sun-exposed forearm skin, just as in sun-protected buttock skin. Duplication of the lamina densa has been seen in forearm skin from 68- to 84-

year-old donors.³¹ On the other hand, severe disruption and multilayered reduplication of the lamina densa have been observed in the cheek skin of 60-year-old donors.³² Tammi et al⁴³ reported that the BM at the DEJ acts as a barrier that enables accumulation of HA in the epidermis. Thus, the level of HA in the epidermis may be correlated to the structural condition of the BM at the DEJ. In this context, tretinoin is known to induce the accumulation of HA in the epidermis.⁴⁴ Tretinoin not only induces HA production in keratinocytes by the up-regulation of *HAS2-3* gene expression,⁴⁵ but also promotes the formation of the anchoring complex at the DEJ.⁴⁶ Topical application of the bifunctional inhibitor hydroxyethyl-imidazolidinone has previously been found to promote the formation of

Figure 4 Epidermal hyaluronan (HA) content was increased by promoting basement membrane formation in the skin equivalent model. **A–D:** Immunostaining of biotinylated HA binding protein in the skin equivalent model cultured with matrix metalloproteinase (MMP) inhibitor (**B**), heparanase inhibitor (**C**), and both MMP and heparanase inhibitors (**D**), compared with the control (**A**). **E and F:** HA contents in the epidermis (**E**) and dermis (**F**) cultured with or without MMP and heparanase inhibitors were evaluated by enzyme-linked immunosorbent assay (ELISA). **G:** HA content in conditioned media from the skin equivalent model cultured with or without MMP inhibitor and heparanase inhibitor was evaluated using ELISA. **H–K:** Transmission electron microscopy images of the skin equivalent model: untreated control (**H**), treated with either inhibitor alone (**I** and **J**), and treated with both the MMP and heparanase inhibitors (**K**). **K:** The lamina densa is more continuous in the presence of both the MMP and heparanase inhibitors. **Black arrowheads** indicate lamina densa. Data are the means $\pm$ SD of six independent experiments (**E–G**). * $P < 0.05$, ** $P < 0.01$. Scale bars: 100 μ m (**A–D**); 400 nm (**H–K**). BIPBIPU, 1-[4-(1H-benzimidazol-2-yl)phenyl]-3-[4-(1H-benzoimidazol-2-yl)phenyl]urea; CGS27023A, N-hydroxy-2(R)-[[[(4-methoxyphenyl)sulfonyl](3-picolyl)-amino]-3-methylbutanamide hydrochloride.

BM structure at the DEJ, and increased the water content in the stratum corneum.³⁵ All these findings are consistent with the idea that the BM at the DEJ acts as a physical barrier to suppress the loss of newly synthesized HA from the epidermis, thus enabling accumulation of HA in the epidermis.

In this study, the effect of epidermal HA content was evaluated by using three different skin models. In an artificial SE model prepared by seeding keratinocytes on collagen gel containing fibroblasts,⁴⁷ basement membrane appeared after seeding the keratinocytes,⁴⁸ and its formation was promoted by treatment with MMP and heparanase inhibitors.⁴² In human abdominal skin organotypic culture, the basement membrane was well organized, but was degraded after UVB exposure.³⁴ Previous studies have shown that degradation of the BM in UVB-exposed organotypic abdominal skin is

blocked by treatment with MMP and heparanase inhibitors.^{34,35} An organotypic eyelid skin model was also used as a representative of chronically sun-exposed skin. Epidermal HA staining intensity was reduced more in the eyelid area than in the cheek area (Figure 1, A–F). The staining intensity of HABP in epidermis was increased by treatment with MMP and heparanase inhibitors in all three skin models. The ultrastructure of the BM was also improved by treatment with MMP and heparanase inhibitors in the SE model and the organotypic human abdominal skin model. Therefore, these data suggest that the BM has a role in maintaining the epidermal HA level in human skin.

In the kidneys, HS in the glomerular BM is reduced in human glomerular diseases, such as diabetic nephropathy and membranous glomerulopathy.^{49,50} The presence of negatively charged HS in the glomerular BM is crucial for the charge-

selective permeability of the glomerular capillary filter.⁵¹ In this study, not only HS content (Supplemental Figure S6G) but also HA content (Figure 4G) in the culture medium was reduced in the presence of heparanase inhibitor. These data suggest that HS in the BM at the DEJ promotes both the assembly of anchoring complex for the physiological barrier and the functionality of negative charge filtration, favoring the accumulation of HA in the epidermis. In addition, heparanase or MMP inhibitors help prevent degradation of the HS of perlecan in the BM and CD44 at the cell surface of keratinocytes in the epidermis (Supplemental Figure S6, B and C). HABP staining was seen at the cell surface of keratinocytes in the epidermis in the presence of MMP inhibitor or heparanase inhibitor (Figure 4, B and C). CD44 staining was reduced in sun-exposed eyelid skin compared with sun-exposed cheek skin and sun-protected buttock skin, in accordance with the results of HABP staining (Supplemental Figure S1). These results suggest that inhibitors block the degradation of CD44 and HS in the epidermis, thereby promoting retention of HA in the epidermis.

In this study, MMP and heparanase inhibitors were used to prevent HA degradation in organotypic human skin after UVB irradiation,³⁴ thus promoting the formation of the basement membrane.⁴² The effects of these inhibitors were also evaluated on the expression of genes related to HA synthesis in the epidermis, and *HAS2-3* and *HYAL1-2* mRNAs were found to be unchanged in the epidermis, whereas the total HA content was reduced after inhibitor treatment (Figure 3, E–G). Thus, the amount of newly synthesized HA in the epidermis is not increased by the inhibitors.

HAS3 expression was significantly induced in cultured human keratinocytes after UVB irradiation and with aging (Supplemental Figure S3B). *HAS3* gene expression increases after UVB exposure of cultured human keratinocytes¹³ and in mouse epidermis.²⁸ On the other hand, *HAS2* expression was reduced after UVB irradiation (Supplemental Figure S3A), in agreement with a previous report.⁵² The average molecular weight of HA is temporarily reduced to 100 kDa from 1200 kDa at days 3 to 5 after UVB exposure in mouse epidermis.²⁸ In human skin, the molecular weight of HA is 1840 kDa in sun-protected human skin, whereas it is 980 kDa in sun-exposed human skin.²⁶ Furthermore, the level of high-molecular-weight HA is reduced in the skin of older subjects compared with that of younger subjects.^{53,54} Tammi et al⁴³ reported that the BM at the DEJ acts as a barrier that enables accumulation of high-molecular-weight HA in the epidermis. Therefore, here, the molecular weight distribution of HA is hypothesized to shift to a lower value concomitantly with deterioration of the BM at the DEJ in sun-exposed facial skin, leading to a reduced HA content in the epidermis due to increased transfer into the dermis through the BM, in addition to the loss via desquamation.^{21–23} Further work to examine the distribution and metabolism of HA in the epidermis is in progress.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2021.03.007>.

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