

# Hyaluronan Exists in the Normal Stratum Corneum

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Hyaluronan is well known to exist as a water-sorbed macromolecule in the extracellular matrix. We here examined whether hyaluronan exists in the normal stratum corneum. High performance liquid chromatography was used to quantify hyaluronan content in the stratum corneum, epidermis (including stratum corneum), and dermis of mice, with the resulting dry weights being  $22.3 \pm 2.9$ ,  $15.1 \pm 1.5$ , and  $738.6 \pm 31.6 \mu\text{g}$  per g, respectively. Normal mouse skin was then labeled with [ $^3\text{H}$ ]-glucosamine in an organ culture, and accumulation of [ $^3\text{H}$ ]-labeled hyaluronan and its molecular mass were determined separately for the stratum corneum, epidermis, and dermis. In the stratum corneum, [ $^3\text{H}$ ]-labeled hyaluronan was accumulated linearly over the 3-d culture period. After the 3-d culture period, the

epidermis synthesized twice the amount (expressed as dpm per mg dry weight) of [ $^3\text{H}$ ]-labeled hyaluronan as the dermis, whereas the stratum corneum and dermis showed nearly the same content of [ $^3\text{H}$ ]-labeled hyaluronan. The molecular mass of [ $^3\text{H}$ ]-labeled hyaluronan was highest ( $>1.0 \times 10^6$ ) in the dermis and clearly lower ( $<6.0 \times 10^4$ ) in the stratum corneum. Based on these results, we here confirm that hyaluronan is supplied from keratinocytes beneath the stratum corneum layer, and is present in the normal stratum corneum. We speculate that hyaluronan may play a role in moisturizing the stratum corneum and/or regulating its mechanical properties. **Key words:** *horney layer/hyaluronic acid. J Invest Dermatol 114:1184–1187, 2000*

**H**yaluronan (HA), a nonsulfated glycosaminoglycan composed of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid, is one of the major extracellular matrix components in skin. Skin contains 0.5–1 mg per g wet tissue weight HA, or about 50% of the total HA in a given organism (Laurent and Fraser, 1992). HA is produced mainly by fibroblasts and keratinocytes in the skin, has an estimated turnover rate of 2–4.5 d in mammals (Tammi *et al*, 1991), and is well known to hold water, maintain the extracellular space, and facilitate the transport of ion solutes and nutrients.

Histochemical analyses using a biotinylated HA-binding protein have shown that HA is localized not only in the dermis but also in the epidermis (Tammi *et al*, 1988; Alho and Underhill, 1989; Wang *et al*, 1992; Meyer and Stern, 1994; Agren *et al*, 1997a). In these reports, HA was observed in the epidermal intercellular spaces, especially the middle spinous layer, but not in the stratum corneum (SC) or stratum granulosum. Generally, HA was considered to be absent in the normal SC. On the other hand, the parakeratotic SC of a psoriasis lesion (Wells *et al*, 1991) and SC in a skin organ culture (Tammi *et al*, 1989) treated with retinoic acid have been shown to contain abnormal accumulations of HA in the intercellular space of corneocytes.

Recently, several groups have identified three mammalian genes encoding putative HA synthases (Itano and Kimata, 1996a; 1996b; Shyjan *et al*, 1996; Spicer *et al*, 1996, 1997; Watanabe and

Yamaguchi, 1996). We have examined the expression of hyaluronan synthase genes (*Has1* and *Has2*) mRNA in mouse skin using *in situ* mRNA hybridization, and found that both mRNA were abundantly expressed in epidermis. Furthermore, some strong expression of both mRNA were seen in stratum granulosum (Sugiyama *et al*, 1998). These results suggested that the stratum granulosum might supply HA to SC. Accordingly, we examined the existence of HA in the normal SC. In order to investigate a possible role of SC in the metabolism of epidermal HA, we quantified the HA content of SC from normal skin, and examined the incorporation of [ $^3\text{H}$ ]-glucosamine into the HA of SC using an organ culture of mouse skin. Based on the results, we confirm that HA is supplied from keratinocytes beneath the SC layer and exists in the normal SC.

## MATERIALS AND METHODS

**Materials** Male hairless mice (Hos/HR-1) were obtained from Nippon SLC (Shizuoka, Japan). Eagle's minimum essential medium, nonessential amino acids, and sodium pyruvate were obtained from Dainippon Pharmaceutical (Osaka, Japan). Trypsin was purchased from Sigma (St Louis, MO). Pronase E from *Streptomyces griseus* was obtained from Merck (Darmstadt, Germany). DE52 cellulose was from Whatman International (Maidstone, U.K.). Sephadex G25, Sephadex G50, and Sepharose CL-2B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). HA from pig skin (molecular weight,  $4\text{--}6 \times 10^4$ ) and human umbilical cord (molecular weight,  $8\text{--}12 \times 10^5$ ), *Streptomyces* hyaluronidase, and unsaturated oligosaccharide from HA were from Seikagaku kogyo (Tokyo, Japan). D-[1,6- $^3\text{H}$ (N)]Glucosamine hydrochloride ( $50\text{--}60$  Ci per mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO).

**Separation and solubilization of the tissues** Full-thickness skin-punch samples (diameter of disk, 18 mm) were taken from the dorsal skin of 12-wk-old hairless mice. The intact epidermal sheet was peeled from the dermis by heat separation ( $60^\circ\text{C}$ , 30 s). The epidermal sheets were then

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Abbreviations: HA, hyaluronan; SC, stratum corneum.

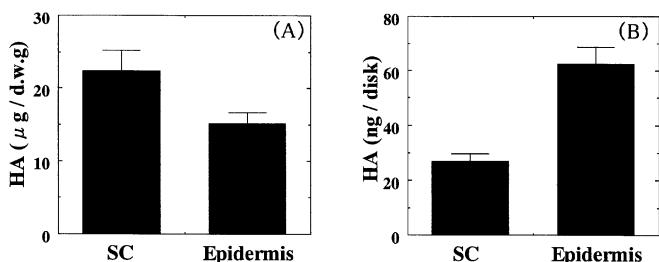
incubated for 2 h at 37°C, basal side downward, in 0.5% trypsin in phosphate-buffered saline, as described previously (Elias *et al.*, 1979). After the incubation, these SC sheets were washed with phosphate-buffered saline three times using a vortex. The SC sheets, epidermal sheets (including SC), and dermis were then washed with acetone, dried with N<sub>2</sub> gas, and weighed. Dried tissues were suspended in 50 mM tris(hydroxymethyl)-aminomethane (Tris) HCl, pH 7.8, and boiled for 20 min, and then submitted to proteolytic digestion with pronase E (1% wt/vol) for 1 wk at 40°C. The resulting solution was deproteinized by addition of trichloroacetic acid to a final concentration of 10%. The samples were centrifuged and supernatants were neutralized with NaOH.

**Quantification of HA** The neutralized extract of each sheet was chromatographed on a PD-10 column (Pharmacia). The void volume (2.5 ml) was discarded and the fraction eluted with 3.5 ml of distilled water was collected. This fraction was concentrated to 0.2 ml with a centrifugal evaporator centrifuge (EC-57C; Sakuma Seisakusyo, Tokyo, Japan). Each 0.15 ml of the concentrated samples was treated with 2.5 turbidity reducing units of *Streptomyces hyaluronidase* (Seikagaku kogyo) (Ohya and Kaneko, 1970) in 200  $\mu$ l of 25 mM sodium acetate buffer (pH 6.0) at 55°C for 8 h, and then the mixture was ultrafiltered using an Ultrafree C3GC system (molecular size cut-off, 10,000; Japan Millipore, Tokyo, Japan). High performance liquid chromatography (HPLC) analysis of the unsaturated tetrasaccharide of HA (*f*<sub>4</sub> Tetra-HA) and the unsaturated hexasaccharide of HA (*f*<sub>6</sub> Hexa-HA) was performed according to the method of Takazono and Tanaka (1984) and Shinmei *et al.* (1992). The HPLC system used in this study was constructed using two pumps (Model 880-PU; Japanspectroscopic, Tokyo, Japan), an autosampling injector (Model 851-AS; Japanspectroscopic), a stainless steel column packed with polyamine-bound silica (YMC gel PA-120; YMC, Kyoto, Japan), a dry reaction bath (DB-3; Shimamura Instrument, Tokyo, Japan), a fluoromonitor (Model FP-920; Japanspectroscopic), and an integrator (Model 807-IT; Japanspectroscopic).

The *f*<sub>4</sub> Tetra-HA and *f*<sub>6</sub> Hexa-HA in each sample were eluted with a gradient of 0–100 mM sodium sulfate for 45 min at a flow rate of 0.5 ml per min. To the eluant from the column was added 100 mM sodium tetraborate buffer (pH 9.0) containing 1% 2-cyanoacetamide, at a flow rate of 0.5 ml per min. The mixture passed through PEEK Tubing (0.5 mm internal diameter  $\times$  ~10 m) set in a dry reaction bath thermostatted at 137°C, and the effluent was monitored by the fluoromonitor (Ex, 331 nm; Em, 383 nm). The area of each peak corresponding to *f*<sub>4</sub> Tetra-HA and *f*<sub>6</sub> Hexa-HA was calculated by the integrator and converted to an amount of HA against the area of standard *f*<sub>4</sub> Tetra-HA and *f*<sub>6</sub> Hexa-HA (Seikagaku).

**Organ culture** Full-thickness skin-punch samples (diameter of disk, 18 mm) were taken from the dorsal skin of 15-wk-old hairless mice. After removal of subcutaneous tissues, the skin was floated, with the epidermis exposed to air, on Eagle's minimum essential medium containing 50  $\mu$ Ci [<sup>3</sup>H]-glucosamine per ml, nonessential amino acids, 1 mM sodium pyruvate, 500 U penicillin per ml and 500  $\mu$ g streptomycin per ml. No serum was added to the culture medium. The skin was then incubated for 3 d at 37°C in 5% CO<sub>2</sub>.

**Assay of HA synthesis in organ culture** After culturing, skin-punch samples were separated into SC sheets, epidermal sheets (including SC), and dermis, as described above. The neutralized extracts were diluted with Tris/HCl, pH 7.5, to a final concentration of 140 mM NaCl. The diluted solution was subjected to ion exchange chromatography on a column of DE52 cellulose (1  $\times$  ~1 cm). The column was developed stepwise with 0.2 M and 0.3 M NaCl. The fractions eluted with 0.3 M NaCl were



**Figure 1. Content of HA in each compartment.** The SC and epidermis were separated from normal mouse skin. The content of HA in each compartment was assayed using HPLC as described in the *Materials and Methods* section. Values were expressed as  $\mu$ g per g dry weight (A) or ng per disk (B). Values represent means with SEM from five individual samples.

collected and their radioactivity levels were counted. The identity of the purified HA was confirmed by a change in the elution position in Sephadex G50 gel chromatography after treatment with *Streptomyces hyaluronidase*. *Streptomyces hyaluronidase* was added to a final concentration of 4 U per ml, followed by incubation at 37°C overnight.

**Molecular mass determination of labeled HA** The fraction eluted with 0.3 M NaCl containing labeled HA was used for molecular mass determination. The fractions incubated with or without hyaluronidase were subjected to a 1  $\times$  ~62 cm column of Sepharose CL-2B. The column was equilibrated and eluted with 0.5 M NaCl solution at a flow rate of 39 ml per h at 25°C, and fractions (2.5 ml) were collected and assayed for radioactivity.

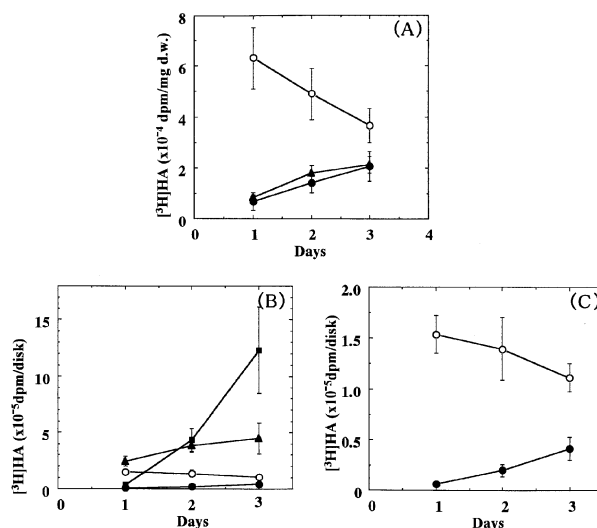
**Measurement of radioactivity** The sample solution (1 ml) was mixed in a vial with 10 ml of Scintisol EX-H (Dojindo Laboratories, Kumamoto, Japan), and radioactivity was measured using a scintillation counter (Aloka LSC-1000; Yokohama, Japan).

## RESULTS

**Quantification of HA content in SC** HPLC was used to quantify HA content in individual layers of normal mouse skin. This system can quantify HA of molecular mass greater than  $2 \times 10^5$ . The absence of granular cells in isolated SC samples was confirmed microscopically (data not shown). Seven days were required for extraction of SC samples by protease.

The dry weight content of HA in the SC was  $22.3 \pm 2.9 \mu$ g per g, almost the same as that in the epidermis ( $15.1 \pm 1.5 \mu$ g per g) (Fig 1A). The dry weight content of the dermis was  $738.6 \pm 31.6 \mu$ g per g (data not shown). When expressed as ng per disk, the content of HA in the SC ( $26.6 \pm 3.1$  ng per disk) was almost half that in the epidermis ( $62.4 \pm 6.4$  ng per disk) (Fig 1B), demonstrating that the SC contained almost half of the total HA content of the epidermis.

**Time course of radioactivity of labeled HA in the SC during organ culture** Normal mouse skin (punched-disk samples) was labeled with [<sup>3</sup>H]-glucosamine in an organ culture, and the SC, epidermis, and dermis were separately assayed for accumulation of [<sup>3</sup>H]-labeled HA. We confirmed using high molecular mass labeled HA that depolymerization of HA did not take place during the pronase treatment (data not shown). In the SC, [<sup>3</sup>H]-labeled HA was accumulated linearly over the 3 d culture period (Fig 2A–C).



**Figure 2. Time course of radioactivity of labeled HA in each compartment during organ culture.** Normal mouse skin was labeled with [<sup>3</sup>H]-glucosamine in organ culture. The SC (●), epidermis (○), dermis (▲), and conditioned medium (■) were separated at the indicated times. The accumulation of [<sup>3</sup>H]-labeled HA in each compartment was assayed as described in the *Materials and Methods* section. Values were expressed as dpm per mg dry weight (A) or dpm per disk (B, C). Values represent means with SEM from three individual organs.

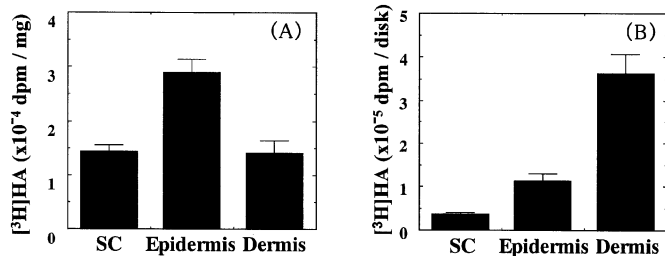
When expressed as dpm per mg dry weight, the radioactivity of labeled HA in the SC was half that in the epidermis and almost the same as that in the dermis after the 3-d culture (Fig 3A). The radioactivity of [<sup>3</sup>H]-labeled HA in the epidermis was six times greater than that in the dermis over the first day of culturing, but decreased linearly thereafter (Fig 2A).

When expressed as dpm per disk, the radioactivity of HA in the SC was about 10% of that in the epidermis after 1 d, and increased thereafter (Fig 2B, C). After 3 d of culturing, the radioactivity of HA in the SC reached about 40% of that in the epidermis (Fig 3B). The radioactivity of HA in the epidermis decreased gradually during the culture period (Fig 2B, C). The radioactivity of HA in conditioned medium increased linearly during the culture period, indicating the diffusion of HA from the organ cultured skin (Fig 2B).

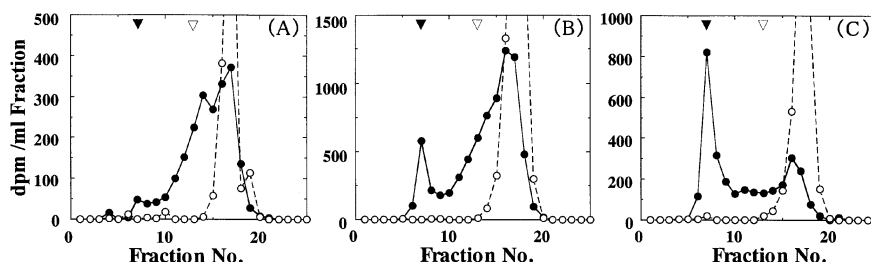
**Molecular mass distribution of labeled HA in the SC** A HA-rich fraction from each compartment (SC, epidermis, and dermis) was collected by stepwise chromatography using a column of DE52 cellulose. To estimate the molecular mass distribution of [<sup>3</sup>H]-labeled HA, HA-rich fractions were incubated with or without *Streptomyces* hyaluronidase. Then, each sample was subjected to Sepharose CL-2B column chromatography. Radioactivities of fractions 5–13 were entirely digested by *Streptomyces* hyaluronidase, showing that these radioactivities were due to HA (Fig 4A–C). Compared with the epidermis (Fig 4A, B), the SC had a smaller portion of high molecular mass [<sup>3</sup>H]-labeled HA (>1.0 × 10<sup>6</sup>), but a larger portion of low molecular mass [<sup>3</sup>H]-labeled HA (<6.0 × 10<sup>4</sup>). The dermis contained mainly [<sup>3</sup>H]-labeled HA of high molecular mass (>1.0 × 10<sup>6</sup>) (Fig 4C).

## DISCUSSION

In this study, we confirmed that the large amounts of HA produced in the epidermis are transferred to the SC. This finding is very interesting with regard to both the metabolism of HA in the epidermis and the function of HA in the SC.



**Figure 3. Radioactivity of labeled HA in each compartment after organ culture.** The accumulation of [<sup>3</sup>H]-labeled HA in each compartment after culturing for 3 d was assayed as described in the *Materials and Methods* section. Values were expressed as dpm per mg dry weight (A) or dpm per disk (B). Values represent means with SEM from five individual organs.



**Figure 4. Elution profiles from a Sepharose CL-2B column of the [<sup>3</sup>H]-labeled HA rich fraction from each compartment after organ culture.** The [<sup>3</sup>H]-labeled HA rich fraction from each compartment after culturing for 3 d was subjected to Sepharose CL-2B column (10 × 550 mm) chromatography as described in the *Materials and Methods* section: ●, [<sup>3</sup>H]-labeled HA rich fraction incubated without *Streptomyces* hyaluronidase; ○, [<sup>3</sup>H]-labeled HA rich fraction incubated with *Streptomyces* hyaluronidase; A, SC; B, epidermis; C, dermis. Values represent means from five individual organs. The elution peak of marker HA is indicated. ▼, human umbilical HA (120–80 × 10<sup>4</sup>); ▽, HA derived from pig skin (4–6 × 10<sup>4</sup>).

Previously, HA has been considered not to exist in the normal SC, based on the lack of histochemical signals detected in the normal SC using HA binding probes (Tammi *et al*, 1988; Alho and Underhill, 1989; Wang *et al*, 1992; Meyer and Stern, 1994; Agren *et al*, 1997a). No attempts have been made to quantitatively determine the presence or absence of HA in the normal SC, however. We previously reported the expression of *Has1* and *Has2* mRNA in mouse skin using *in situ* mRNA hybridization, and confirmed that both mRNA were abundantly expressed in the epidermis (Sugiyama *et al*, 1998). In that report, we also demonstrated strong expressions of both mRNA in the stratum granulosum. These findings led us to examine HA content in the SC. Surprisingly, we found that the SC contained a large amount of HA, in contradiction to numerous histochemical analyses using the HA binding protein, HABP. We can offer two possible explanations for the disagreement. The first concerns the binding activity of HABP to HA, which is known to be dependent on the molecular mass of HA [we also confirmed that the binding activity of HABP to low molecular mass HA (4–6 × 10<sup>4</sup>) was about 50% of that to high molecular mass HA (>10<sup>6</sup>) (data not shown)]. Our results showed, however, that HA exists in the SC in a low molecular mass form such that previous histochemical detections of HA might have been affected by low binding of HABP to HA. The other possibility is that binding sites of HA to HABP may have been masked by components of the SC.

The presence of HA in the SC is of particular interest from the viewpoint of epidermal metabolism. The epidermis is a closed compartment with no blood vessels, and macromolecules are thought to be too large to penetrate the basal lamina. But the half-life of HA in the epidermis is very short. Using an organ culture, Tammi *et al* (1991) showed that the half-life of HA in the epidermis was only 24 h. The mechanisms of HA degradation in the epidermis remain unclear, although they may involve HAase (Frost and Stern, 1997) of keratinocytes and/or reactive oxygen species (Agren *et al*, 1997b). Interestingly, Frost and Stern (1997) reported that the secretion of HAase from keratinocytes increased during the differentiation process. Tuhkanen *et al* (1998) reported that immunohistologic signals of CD44, a major HA receptor of the plasma membrane (Underhill, 1992), disappeared in the stratum granulosum. A portion of the HA produced in the epidermis may therefore be degraded in the differentiation process and transported with or without corneocytes to the SC. Alternatively, granular cells might have a system for supplying HA to the SC, as we previously reported that some strong signals of the mRNA of *Has1* and *Has2* were seen in the stratum granulosum (Sugiyama *et al*, 1998).

The function of HA in the SC is unclear. The SC is a heterogeneous structure composed of protein-enriched corneocytes embedded in a lipid matrix (Rawlings *et al*, 1994), and plays a very important role against dry stress. Accordingly, moisturization of the SC is highly important for maintaining its flexibility and desquamation. Such moisturizing is considered to be regulated by natural moisturizing factors (Horie *et al*, 1989; Yamamura and Tezuka, 1989; Rawlings *et al*, 1994) and lipids (Imokawa *et al*,

1989, 1991). HA is also well known to be a water-sorbed macromolecule (Laurent and Fraser, 1992). Moreover, we showed here that the SC has an HA content equal to nearly half that of the epidermis. HA might thus be a factor involved in the moisturization of SC. Alternatively, HA has been reported to have not only a hydrophilic but also a hydrophobic property (Scott *et al*, 1991; Ghosh *et al*, 1994). Indeed, Ghosh *et al* (1994) reported that HA interacts with phospholipids. Interaction of HA with lipids of lamella structure in the SC might serve to regulate mechanical properties of the SC. Interestingly, abnormal staining of intercellular HA in the SC has been demonstrated in both retinoic-acid-treated skin (Tammi *et al*, 1989) and psoriatic skin (Wells *et al*, 1991). Miyamoto *et al* found very small molecular mass HA ( $3.4 \times 10^4$ ) in the horny layer of n-hexadecan-treated hyperkeratinized tissue of guinea pigs (Miyamoto and Nagase, 1984). HA may be strictly modified (e.g., degraded, folded, and packed) during the differentiation from granular cells to corneocytes, as the transition to corneocytes requires dramatic changes in the extracellular matrix. Further studies on the changes of HA metabolism during the differentiation of keratinocytes would thus be of use. Finally, we note that the HA content in human SC is as high as that in mouse SC (data not shown). We are currently evaluating the content and molecular mass of HA in human SC under various skin conditions to clarify the function of HA in the SC.

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