Age-Dependent Changes of Hyaluronan in Human Skin

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Hyaluronan is a major component of the extracellular matrix of skin. The large volume of water of hydration associated with hyaluronan may be a mechanism for maintaining the normal hydration of skin. As such, decreasing levels of hyaluronan deposition might underlie the changes associated with the aging process. To test this hypothesis, hyaluronan levels were determined in extracts of skin obtained at autopsy from individuals of different ages. However, no significant differences in hyaluronan concentrations were found. The distribution of hyaluronan polymer sizes in various extracts did not change as a function of age as measured by size exclusion chromatography. However, major differences in hyaluronan extractability did occur as a function of age. Sequential extraction was performed utilizing 1) 0.1% Triton X-100, 2) 4 M guanidine-HCl, and 3) papain digestion, to release species of hyaluronan progressively more tightly associated with tissue. With advancing age, hyaluronan polymers became progressively more tissue associated. The proportion of hyaluronan released after papain digestion increased from 7% of the total in fetal to 23% of the total in senescent skin. Finally, histolocalization of hyaluronan was examined in full-thickness sections of human skin of different ages. Major differences in compartmentalization were found. We conclude that neither the concentration nor polymer size of hyaluronan changes as a function of age. However, enhanced association with tissue occurs, presumably through hyaluronan-binding proteins and alterations in the histolocalization of hyaluronan. Such observations may underlie some of the changes in human skin that occur with aging. *Key words: extracellular matrix/glycosaminoglycans/aging. J Invest Dermatol 102:385–389, 1994*

With advancing age, there is a decline in the quality of human connective tissue and its repair processes. Nowhere is this deterioration more evident than in skin. Particularly prominent is the decrease in hydration of skin as a function of age.

Hyaluronan (HA) is the predominant glycosaminoglycan (GAG) of human skin, constituting the major reservoir of HA in the body, more than 50% of the total [1]. We initially postulated a decline in the HA content of skin, and the large volume of water of hydration with which it is associated, as partially responsible for this age-related deterioration. To test this hypothesis, we examined biochemically the total HA content of human skin as a function of age, including relative extractability and polymer size, as well as the histologic compartmentalization of HA. For the histolocalization of HA in skin as a function of age, paraffin-embedded tissue sections were examined. The present study is part of a comprehensive evaluation of wound healing in skin as a function of age. The study aims to evaluate the role of HA and its associated synthetic and catabolic reactions, as well as the profiles of HA-binding proteins that decorate the molecule.

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Abbreviations: dH_2O, deionized H_2O; GAG, glycosaminoglycan; HA, hyaluronan; HABP, hyaluronan-binding protein; PBS-CMF, phosphate-buffered saline-calcium and magnesium free; PG, proteoglycan.

MATERIALS AND METHODS

Preparation of Samples For biochemical studies, full-thickness male skin specimens were obtained from two fetuses of 20 and 22 weeks gestation, two adults, 31 and 32 years old, and two elderly subjects, 81 and 89 years of age. Samples (2 × 4 cm) were obtained at autopsy and were taken from a non-sun-exposed area, the anterior aspect of the right thigh.

Differential Digestion After removal of subcutaneous fat, the minced tissue was homogenized in 0.1% Triton X-100 in phosphate-buffered saline-calcium and magnesium free (PBS-CMF) at pH 7.0 using a Brinkmann Polytron PT 3000 (Brinkmann Instruments, Burlingame, CA) at 9000 rpm with constant cooling. The homogenized material was spun at 6500 × *g* (Sorvall Superspeed RC 2, DuPont Instruments, Stanford, CA) for 2 h, the supernatant was removed, and stored at − 20°C. The pellets were suspended in a solution of 4 M guanidine-HCl, 5 ml/g wet weight. After overnight incubation, the centrifugation was repeated, the second supernatant removed, and 0.2 ml papain solution added containing equal parts of twice-crystallized papain, 25 mg/ml (Sigma Chemical Co., St. Louis, MO) and a buffer solution, consisting of 2 mM ethylene diamine tetraacetic acid (EDTA), 20 mM NaCl, 4 mM L-cysteine, and 0.25 M Na phosphate buffer at pH 5.0, per gram wet pellet. This was followed by incubation at 70°C for 16 h. The third extract was spun, separated, and stored as described above.

Enzyme-Linked Immunosorbent Assay (ELISA)-Like Assay for HA After the initial digestion, the HA levels were determined. The HA assay is an ELISA-like technique based on a biotinylated HA-binding protein (HABP) isolated from a tryptic digest of bovine nasal cartilage as described by Tengblad *et al* [2]. Briefly, homogenized cartilage was placed in 4 M guanidine-HCl and 0.5 M NaAc, pH 5.8, overnight. The supernatant was separated and dialyzed against distilled H_2O and 1 mM Tris-HCl, followed by lyophilization. Trypsin digestion was then performed, followed by dialysis against 4 M guanidine-HCl, 0.5 M NaAC, pH 5.8. The digest was incubated with EAH Sepharose 4B gel (Pharmacia, Piscataway, NJ) to which HA had been covalently bound. The guanidine concentration was reduced to

0.4 M. After washing the gel with 3M NaCl, the protein was eluted with 4 M guanidine HCl and biotinylated.

The assay procedure was that described by Fosang *et al* [3]. We utilized Covalink NH multiwell plates (Nunc, Naperville, IL) that had been precoated with a solution containing 184 μg sulfo-N-hydroxysulfosuccinimide, 200 μg HA, and 1.23 mg ethyldimethylaminopropyl-carbodiimide (EDC) per ml dH_2O. After having washed with a solution containing 116.9 mg NaCl and 10 mg $MgSO_4$ in 1 ml PBS-CMF (Buffer A), 300 μl blocking reagent was added to each of the wells, and incubated in humidified environment at 37°C for 30 min. The blocking reagent was dry milk powder 0.5% w/v PBS-CMF. Following this incubation, the plates were washed with Buffer A with the addition of 0.05% Tween 20 (Buffer B). Samples and standards were incubated with an equal volume of HABP solution for 1 h at 37°C, then added to the multiwell plate and incubated for an additional hour at 37°C. A Vectastain peroxidase avidin-biotin peroxidase complex (ABC) Kit (Vector Laboratories, Burlingame, CA) was used for signal amplification, and O-phenylenediamine (OPD; Calbiochem, La Jolla, CA) was used as a substrate. After developing for 20–30 min in the dark, the color was read at 492 nm. This procedure permitted determination of HA concentrations at the nanogram level. Each plate contained positive as well as negative controls. A standard curve using HA standards ranging from 40 ng to 3 μg HA/ml (Healon, Pharmacia, Piscataway, NJ) was run with each plate. Each experimental point was determined in triplicate.

Protein Determination For protein determinations, the Biorad Microassay Kit (Biorad Laboratories, Richmond, CA) was employed utilizing microtiter plates (Costar, Data Packaging Co., Cambridge, MA).

Size Distribution To determine the size profile of the HA polymers, Sepharose CL-4B column chromatography (Pharmacia AB, Uppsala, Sweden) was used [4]. A column (1 cm inner diameter) with 35 ml bed volume of Sepharose CL-4B beads was utilized in buffer containing 50 mM Tris-HCl and 150 mM NaCl , pH 8.0. Under continuous pressure (Gilson Minipuls 2 pump, Gilson Medical Electronics Inc., Middleton, WI), 40 1-ml fractions were collected on a Gilson Microfractionator. The HA levels in each fraction were then determined utilizing the ELISA-like assay.

Histochemical Studies of HA Staining was performed from paraffin-embedded full-thickness preparations of skin from 17 autopsy patients. The specimens were obtained from fetal skin (28, 30, and 34 weeks), neonates (7 and 24 d), infants within their first year of life (3 and 8 months), adolescents (11 and 14 years), young adults (21 and 22 years), middle-aged individuals (49 and 50 years), and finally, elderly individuals (70, 81, and 88 years).

After initially placing the tissue pieces in saline, we transfered them immediately to a solution of 2% formalin and 0.5% glutaraldehyde in 0.1 M PBS at pH 7.35 and then fixed them as described by Hellstroem *et al* [5]. Specimens were processed in the usual manner for paraffin embedding. Serial sections of 4 μm thickness were mounted on polylysine-coated glass slides for hematoxylin and eosin, as well as for HA staining.

To document specificity of HA staining, we combined 1 ml of hyaluronidase buffer (0.1 M sodium formate, 0.15 M NaCl, 0.1 mg/ml BSA, 0.1% Triton X-100, pH 3.7) with 100 turbidity-reducing units of Streptomyces hyaluronidase (CalBiochem, Behring Diagnostics, La Jolla, CA). Approximately 0.3 ml of this solution was placed onto deparaffinized control slides, followed by an overnight incubation in humidified environment at 37°C. The biotinylated HABP was diluted 1:100 with HABP-buffer solution (0.25 M Na phosphate, 1.5 M NaCl, 0.3 M guanidine-HCl, 0.08% bovine serum albumin [BSA], 0.02% NaN3, pH 7.0), and combined 1:1 with a solution of 0.1 mg/ml HA (Sigma).

On day 2, the hyaluronidase-control slides, together with the experimental slides, were incubated in 3% BSA at 37°C for 30 min. After rinsing with PBS-CMF, 0.3 ml of the diluted HABP solution was added to each slide. Coincidently, 0.3 ml of the HA and HABP-control solution were added to each HA and HABP-control slide. All slides were incubated overnight at 4°C.

On the final day, a solution of 98 ml methanol with 2 ml of 30% H_2O_2 was prepared for each well, containing 10 slides. All slides were rinsed with PBS-CMF, incubated, and transferred to the staining wells containing this solution for 3 min at room temperature. Immediately following, slides were rinsed with PBS-CMF, and then incubated with Vectastain ABC Peroxidase Kit PK 4000 (Vector) for 30 min at room temperature. After rinsing again with PBS-CMF, slides were incubated for 5 min at room temperature with 3,3′-diaminobenzidine (Peroxidase Substrate Kit, DAB SK-4100 (Vector), followed by a rinse with dH_2O. Slides were counterstained for 15 min in 0.25% methylgreen, rinsed in dH_2O, dipped in 70%, 95%, and 100% methanol, and finally in xylene.

Photographs were taken on an Olympus Vanox AHBT3 Microscope (Olympus Co., Woodbury, NY) fitted with an integrated Olympus C-35AD-4 camera on Kodak Gold Plus 100 film (Eastman Kodak Co., Rochester, NY).

Table I. Patient Information

Number	Age	Diagnoses
1	Fetus, 28 weeks	Liver laceration Intraabdominal hemorrhage Respiratory distress syndrome
2	Fetus, 31 weeks	Cystic abdominal mass Skeletal malformations Lung hypoplasia
3	21 years	Acute myelogenous leukemia Bone marrow transplant Fungal pneumonia
4	22 years	Acute lymphocytic leukemia Bone marrow transplant Aspergillosis
5	81 years	Gastric carcinoma Pulmonary embolism
6	88 years	Multiple brainstem strokes

RESULTS

Skin Samples The samples of skin were obtained from autopsy material from the anterior aspect of the right thigh. The clinical diagnoses of each subject are given in Table I.

Total HA Levels Skin samples were subjected to three sequential extraction steps. Buffered saline contained 0.1% Triton-X 100, 4 M guanidine-HCl, and papain-digestion (extracts 1, 2, and 3, respectively). No major differences in the total amounts of HA between the three age groups were apparent, with levels ranging from 23.2 μg HA/ml sample in fetal skin, 17.5 in the adult, and 22 in the senescent material. Two cases were evaluated in each age group. Small differences were obtained, from 2.6% to 7.6% variations occurring between individual samples in each group.

However, major differences in the distribution of HA in the different extracts were observed. With the first extraction in Triton X-100, levels of HA were comparable in the three age groups, 21.3 μg/ml in fetal, 15.7 μg/ml in the adult, and 19.7 μg/ml in senescent specimens. The fetal samples showed a steady decrease of HA concentration with continued extraction. Both adult and senescent samples, however, had increasing values after papain treatment ranging from 0.20 μg HA/ml in the fetal to 0.85 μg in the adult, and 1.30 μg in the senescent skin. The concentration of HA in the third extraction step was 4.3 times higher in the adult, and 6.7 times higher in the senescent material, compared to fetal skin, suggesting that with increasing age, HA became progressively more tightly bound.

Protein Concentration The distribution of protein in the different samples was also determined. Extract 1 had the highest level, with only minor differences occurring between the three age groups. Levels ranged from 3.25 mg protein/ml in the fetus, to 1.98 in the adult, and 2.83 in senescent skin. In fetal skin, 0.52 mg/ml following guanidine digestion and 0.2 mg/ml after papain treatment was noted. The adult preparation also had lower levels, from 0.35 mg/ml after guanidine to 0.31 mg/ml after papain. The protein concentration in the senescent sample was 0.24 mg/ml following guanidine, and 0.38 mg/ml following papain treatment.

Relative HA Concentrations The concentration of HA in μg/mg protein was calculated in the three compartments. In each of the skin samples, the predominant levels of HA was contained in the first extraction. In the fetal skin (extract 1, 8.4 μg/mg; extract 2, 3.2; extract 3, 1.0), in the adult (extract 1, 8.0 μg/mg; extract 2, 2.8; extract 3, 2.7), and in the senescent specimen (extract 1, 7.0 μg/mg; extract 2, 4.6; extract 3, 3.4). The ratios of HA changed from 67:26:7 in fetal skin to 59:21:20 in the adult, to 46:31:23 in the senescent material. These data are presented in Fig 1. A steady rise in

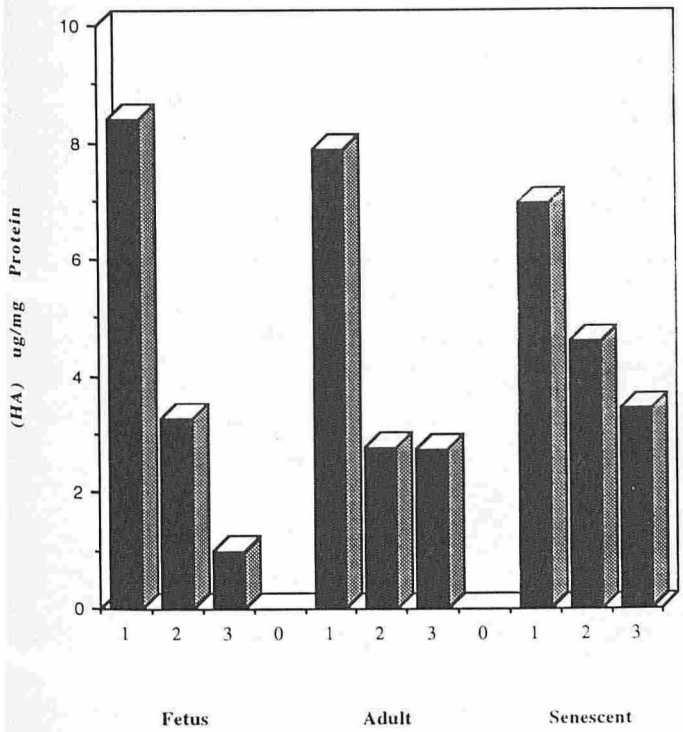

Figure 1. Differential extractability of HA in skin following sequential digestion. Note the shift in extractability with advancing age. Extraction procedures were 1) saline with 0.1% Triton X-100, 2) 4 M guanidine-HCl, and 3) papain digestion. Each sample was assayed in triplicate, with less than 5% variation occurring between such data points. Reassay of the same sample was also reproducible, with less than 5% occurring. Differences between individuals in each group varied from 2.6 to 7.6%.

the proportion of HA levels occurred with increasing age with progressively stronger extraction procedures.

Size Distribution The size distribution of the HA polymers in the three extraction steps was examined. Samples were chromatographed on CL-4B Sepharose columns. In all samples, comparable patterns were obtained for extract 1. The highest levels of HA were observed in the high molecular weight fractions, in the excluded volume from the column. In all of the other samples, broad, polydisperse profiles of HA polymer sizes were noted.

HA Histochemistry In fetal skin, significant differences in compartmentalization were detected before and after the middle of the third trimester. At 28 weeks of gestation, the epidermal monolayer did not contain HA, whereas a diffuse staining was found in the dermis (Fig 2*c,d*). At 34 weeks gestation, staining for HA was detected in the basal layer of the epidermis.

After birth, and at 7 and 24 d of age, increased levels of HA were observed in all epidermal layers, particularly the stratum spinosum. Maximum intensity of staining occurred in the basal layer and a lesser amount, in the upper stratum spinosum.

An apparent plateau in staining intensity was reached at 3–8 months of age. Compartmentalization in different layers of the epidermis was observed; the uppermost layers of the epidermis, the stratum corneum, lucidum, and granulosum showed no staining. Just under the stratum granulosum, the upper part of the stratum spinosum stained with medium intensity, with fading towards the inferior portion of this compartment. Again, an intense staining reaction was detected overlying the stratum basalis and, to a lesser degree, the uppermost portion of the papillary dermis. As with the other specimens, faint staining was observed surrounding the collagen fibrils of the dermis (Fig 2*e,f*). In samples from 11- and 14-year-old individuals and in the 21- and 22-year-olds, a similar staining pattern was observed. The intensity of staining in the upper stratum spinosum decreased slightly, with an increase in staining in the papillary dermis in the latter subjects (Fig 2*g–i*). This trend continued in specimens from individuals 49 and 50 years of age. Staining for HA was diffuse within the stratum spinosum, and the highest concentration was again observed in the papillary dermis, just underlying the basal lamina. The overall intensity of staining for HA was slightly less, compared to the skin specimens from younger subjects. This gradual retreat from the superior portions of the epidermis continued in the 70-year-old skin specimen. Additionally, overall staining intensity continued to decrease with age. The staining pattern in the senescent skin from 81- and 88-year-old subjects was quite different. The epidermis was nearly devoid of staining except for the stratum basalis. The papillary dermis, however, stained with great intensity, particularly surrounding collagen fibrils (Fig 2*j,k*). In addition to these changes, other histologic parameters associated with the aging process were noted. The stratum corneum increased remarkably in size with age whereas the stratum spinosum underwent a continuous decline until its final width to almost a monolayer was reached.

DISCUSSION

Hyaluronan is a key macromolecule of the extracellular matrix (ECM), prominent whenever rapid tissue proliferation, regeneration, and repair occur. It is involved in the structure and organization of the ECM. Bursts in HA deposition correlate with mitotic activity [6–9]. Elevated levels enhance cell detachment and migration in proliferating tissues [10], and decreases coincide with the onset of differentiation [11,12]. Hyaluronan is also important in ion solute transport and to the spacing of collagen fibrils. More than 50% of the HA of the body is present in skin [1]. Understanding the quantity, storage forms, turnover rates, and biologic properties in skin as a function of age is important to many aspects of skin pathobiology. Initially, we postulated that total HA in skin decreases with age, consistent with changes observed in the aging process. Instead we found that total HA in skin did not change with age. Differences in extractability were noted, however, with increasing tissue association occurring with age.

Hyaluronan exists in both free [13] and tissue-bound [14,15] forms. The sequential extraction buffers described in this communication, it was assumed, would resolve these categories of HA polymers. Extract 1 would contain most of the free form of HA. Incubation in 4 M guanidine should release HA strongly bound by ionic interactions, including associations with proteoglycans (extract 2). Finally, digestion with papain releases HA covalently linked to protein [15,16], and from tenacious noncovalent interactions (extract 3). Collagen, the major structural protein of skin, becomes progressively more cross-linked and more difficult to extract as a function of age. Extraction requires progressively stronger procedures: saline, acid, and finally pepsin digestion. This paradigm of collagen extractability was the model for HA extractions in the current studies.

Dissociation between HA and its binding proteins requires conditions stronger than those necessary to eliminate nonspecifically bound proteins [17]. The higher levels of bound HA found with increasing age suggest that the array of HA-binding proteins undergoes major age-dependent alterations, with increasing concentrations of such proteins occurring concomitantly. The HA-binding proteins or hyaladherens, are a class of molecules that are only now beginning to be recognized [18]. Among hyaladherens are fibrinogen [19], collagen [20–22], CD44 [23], RHAMM [24], albumin [25–29], and hyaluronidase itself, as well as proteins whose functions are unknown [15]. It is likely that other hyaladherens will be identified. They are far more ubiquitous than previously assumed. Age-dependent changes in skin hyaladheren profiles must occur and may play an important role in the aging process.

The compartmentalization of HA in different layers of the skin was determined using a biotinylated HA-binding peptide staining technique on paraffin-embedded tissue sections. We included fetal skin at two different timepoints, before and after the middle of the

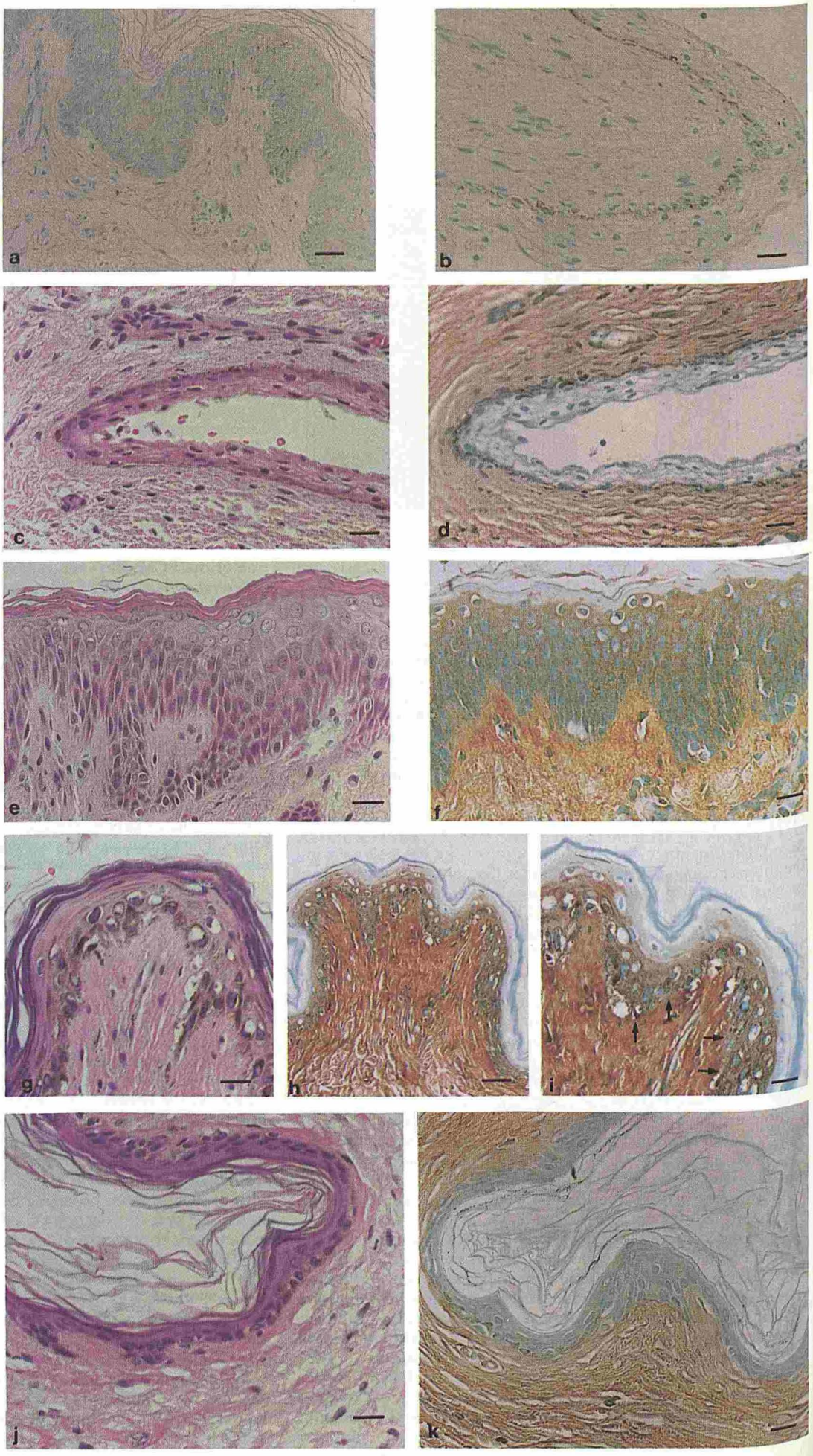

Figure 2. Staining for hyaluronan in normal human skin as a function of age. *a)* *Streptomyces* hyaluronidase control. *b)* Hyaluronan and HABP control. *c)* Fetal skin, 28 weeks gestation, hematoxylin/eosin (H + E) staining. *d)* Fetal skin, 28 weeks gestation, HA staining. The epidermis does not stain at this age, in marked contrast to the underlying dermis. *e)* Skin of infant (3 months); H + E staining. *f)* Skin of infant (3 months); HA staining. Intense staining of the papillary dermis and the upper portion of the stratum spinosum, fading out towards the stratum basalis. *g)* Adult skin, 21 years; H + E staining. *h)* HA staining, intense staining of the papillary dermis and the stratum spinosum, reaching up into the stratum granulosum. *i)* Adult skin, 21 years. At higher magnification the separation of the stratum basalis from the papillary dermis by the basement membrane is apparent (see *arrows*). Also remarkable is the high tissue level of HA throughout the stratum spinosum. *j)* Senescent skin, 81 years; H + E staining. *k)* HA staining. Except for a faint signal in the upper portion of the stratum spinosum, no HA is detectable in the epidermis. In contrast, the staining reaction in the papillary dermis is intense. Bars, 200 μm in *a, c, d, h;* 100 μm in all others.

third trimester, because a transition from scar-free fetal wound healing to adult-like healing with scar formation occurs between these two time points [30]. The fetal mode of wound repair correlates with elevated levels of HA [31]. We observed significant differences in HA distribution before and after the aforementioned timepoint: at 28 weeks of gestation the epidermal layer had no staining for HA, with uniform diffuse HA observed in the underlying dermis. At 34 weeks gestation intraepidermal staining had begun to occur.

From the third month of postnatal life, a constant staining pattern was observed. The uppermost portion of the stratum spinosum had intermediate concentrations of HA, with higher levels occurring in the basal layer of the epidermis and the upper portions of the papillary dermis, embracing the basement membrane on either side. It is tempting to posit the existence of soluble factors that stimulate HA deposition emanating from the basal lamina. The intense mitotic activity taking place in the basal layer of the epidermis may be associated with this HA [6–9]. Our findings confirm initial observations by Tammi *et al* [32,33]. However, more recently they describe HA distribution with peak values in the middle [34] and uppermost [35] spinous cell layers, respectively. Such variations may result from differences in age among their specimens or from differences in sampling location.

With increasing age, a steady decline of HA occurred in the upper epidermal layer, with concomitant increases in the basal layer of the epidermis and the upper portions of the papillary dermis. At senescence, HA was still present in the upper dermis, but was entirely absent in the epidermis. A significant reduction in the cellularity of the epidermis, and in particular the stratum spinosum, occurs with age, with a parallel increase in the thickness of the stratum corneum. The shift of HA towards lower dermal layers also occurred with aging. Decreased accessibility of biotinylated HABP to HA binding sites might be occurring as a function of age, and may be the basis of diminished staining. Additional experiments with graded trypsin predigestion steps are required to address this point more fully. In summary, we have documented herein major changes in HA compartmentalization that occur within the dermis and epidermis as part of the aging process. Alternatively, with increasing age, changes occur in the types and concentrations of hyaladherens that decorate the HA polymers, such that the availability of binding sites for the HA-binding peptides used in these histolocalization studies become modified. Major changes in absolute levels of total extractable HA do not occur and increasing resistence to extraction supports this hypothesis.

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