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Review article

Emerging evidence for the essential role of hyaluronan in cutaneous biology

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ABSTRACT

Hyaluronan (HA), a linear non-sulfated glycosaminoglycan, participates in a variety of biological processes in the skin, such as cell-matrix interactions and activation of chemokines/cytokines, enzymes, and growth factors. In these activation events, HA acts as a damage-associated molecular pattern (DAMP). This review discusses the progress in functional research on HA, and its associated factors, in several aspects of cutaneous biology; *e.g.*, immunity and wound repair.

1. Introduction

Hyaluronan (HA) was isolated in the early 1930s [1]. HA and other glycosaminoglycans (GAGs) were originally thought to be structural, and to fill the extracellular space necessary for the orientation and organization of the matrix. However, advances in glycobiology have shown that HA actively participates in a variety of cell communication events. Also, HA reportedly initiates and controls events associated with inflammation; for example, cytokine/chemokine production and secretion [2], leukocyte recruitment [3], and inflammatory cell maturation and migration [4–6]. In such settings, HA released after injury functions as a damage-associated molecular pattern (DAMP); that is, as a molecular signal of host injury. The function of HA is dependent on its size. High-molecular-weight (HMW) HA mixtures have been reported to be anti-inflammatory and anti-angiogenic. Clinically, HMW HA has been used for the treatment of arthritis. However, reports that HA exerts both pro- and anti-inflammatory effects have long been a source of confusion. Studies investigating these apparently conflicting effects have yielded considerable progress in understanding the mechanism by which HA participates in inflammation. Such progress includes how the enzymes that mediate HA synthesis and degradation encode information within the linear carbohydrate chain of HA, and the functional implications of such encoding. It is now apparent that the presence of HA of different sizes should be considered in any investigation of the complex and coordinated steps of inflammation and tissue repair. This review discusses these aspects of HA biology with an emphasis on its implications for inflammatory processes in human disease.

2. Overview of HA

2.1. Structure

Meyer's laboratory determined the chemical structure of HA, a linear unsulfated GAG composed of repeating disaccharide units ([-D-glucuronic β1, 3-*N*-acetyl-D-glucosamine [β1, 4-]) [7]. HA, which is the largest polysaccharide found in vertebrates, with an end-to-end length of about 1 μm (typically in the range of 10^4 disaccharides), forms hydrated matrices (Fig. 1). HA is the only polysaccharide produced at the cell surface and released into the extracellular matrix as an HMW GAG.

2.2. HA in the skin

HA is present in many tissues but particularly abundant in the skin. Indeed, the skin is the largest single source of HA in humans and other mammals. As early as 1969, it was shown that HA was present in both the epidermis and the dermis, and subsequently that HA is the major GAG synthesized in human epidermis. HA in

Abbreviations: HA, hyaluronan; HABP, hyaluronan-binding protein; DC, dendritic cell(s); LPS, lipopolysaccharide; DAMP, damage-associated molecular pattern.

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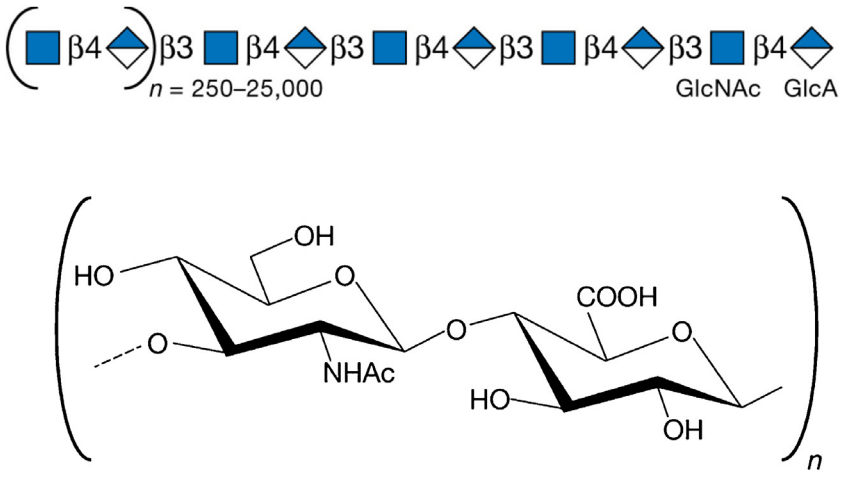

Fig. 1. Hyaluronan (HA) consists of repeating disaccharides composed of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA). Adapted from Vincent Hascall and Jeffrey Esko, Hyaluronan, in: Ajit Varki (Eds.), Essentials of Glycobiology, third edition, Cold Spring Harbor Laboratory Press, New York, 2017, Chapter 16.

the human epidermis is mostly extracellular, localized between the basal and spinous cells [8]. HA is also reported to be present in the normal stratum corneum and plays a role in maintaining its moisture level. Interestingly, the epidermal localization of CD44 completely overlaps with that of HA. HA solutions exhibit high viscosity and viscoelastic properties, which are particularly relevant to its cushioning and lubricating effects as a component of the aqueous humor of the eye and synovial fluid. Topical application of *Streptomyces* hyaluronidase reduced epidermal thickness and accelerated terminal differentiation. Thus, HMW intercellular HA likely participates in the epidermal response to barrier injury by decelerating keratinocyte differentiation. Further, HA-CD44 interactions promote keratinocyte differentiation, lamellar body formation/secretion, and permeability barrier homeostasis [9].

2.3. HA synthases

HA is synthesized by HA synthases (HAS), which are summarized in supplementary Table S1. In mammals, three HAS genes, *HAS1*, *HAS2*, and *HAS3* encode homologous proteins predicted to contain five to six membrane–spanning segments and a central cytoplasmic domain. Itano et al. by culturing three *HAS* transfectants, showed that HAS1 and HAS3 generated HA with a broad size distribution (2×10^5 to approximately 2×10^6 Da) whereas HAS2 generated very HMW HA (mean, $> 2 \times 10^6$Da) [10]. The *Has*2-deficient mouse exhibits an embryonic lethal phenotype at the time of heart formation, whereas *Has1*- and *Has3*-deficient mice and *Has1/3* double-deficient mice show no obvious phenotypic abnormality. Mack et al. explored the roles of HA in the response to cutaneous injury and found that *Has1/3* double-deficient mice express functional Has2 and exhibit significantly faster wound closure than Has1- or 3-deficient mice [11]. Epidermal keratinocytes express all three HASs. The synthesized HA is of HMW ($> 2 \times 10^6$ Da) in both keratinocyte cultures and the normal epidermis. The epidermal HA level is significantly increased after epidermal trauma caused by tape stripping, and this HA response is associated with strong induction of *HAS2* and *HAS3* expression [12]. All-trans retinoic acid, epidermal growth factor (EGF), and keratinocyte growth factor (KGF) increase the epidermal HA level and stimulate cell proliferation. Hydrocortisone, transforming growth factor-beta (TGF-β), and 4-methylumbelliferone inhibit HA synthesis and reduce keratinocyte proliferation. HA synthesis thus seems to parallel active tissue growth; indeed, in the epidermis acanthosis is associated with an increased HA concentration. In the human skin disorder psoriasis, the epidermis shows acanthosis associated with a remarkable increase in the thickness of the HA positive epidermal layer [13]. Increased accumulation of HA and increased HAS expression has been noted in autoimmune renal injury. Skin biopsies from patients with acute eczematous dermatitis show an increased level of HA and an elevated *HAS3* mRNA level in areas of spongiosis.

2.4. HA degradation

Animal cells also express a set of catabolic enzymes that degrade HA, i.e., the hyaluronidases (HYALs), which hydrolyze the hexosaminidil β (1→4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid in HA and summarized in Table 1. Humans have genes encoding six hyaluronidases—*HYAL1*, *HYAL2*, *HYAL3*, *HYAL4*, *PH-20*, and *HYALP1*—the products of which are present in the major parenchymal organs and in human serum and urine [14]. These enzymes are active at acidic pH [14]. Mutations in the *HYAL1* gene are associated with mucopolysaccharidosis type IX [15]. *Hyal1*deficient mice were viable but exhibited osteoarthritis [16]. HYAL2 is a glycosylphosphatidyl inositol (GPI)-anchored cell-surface hyaluronidase with very weak hyaluronidase activity compared with HYAL1 [17]. *Hyal2*-deficient mice were viable but exhibited congenital defects in frontonasal and vertebral bone formation and suffered from mild thrombocytopenia. Chowdhury et al. reported recently that *Hyal2*-deficient mice exhibit severe pre-weaning lethality and accumulation of HA in the heart, lungs, and serum, which lead to cardio-pulmonary dysfunction [18].

Atmuri et al. reported that HYAL3 does not play a major role in HA catabolism, but may compensate for HA degradation in nonskeletal tissues in the absence of HYAL1 [19]. HYAL4 appears to have chondroitinase, but not hyaluronidase, activity [20]. PH-20 is restricted to the testes and has an essential role in fertilization. HYALP1 is a Pseudogene. Recently, Yoshida et al. reported that KIAA1199 (CEMIP; Cell Migration Inducing and Hyaluronan-binding Protein) is a unique HA-binding protein with a key role in HA catabolism in the dermis and the arthritic synovium [21]. Furthermore, Yamamoto et al. reported that transmembrane protein 2 (TMEM2) is the novel cell-surface hyaluronidase that cleaves extracellular HMW-HA into 5 kDa fragments before internalization and degradation in the lysosome [22]. Reactive oxygen species are also involved in HA degradation after tissue injury, although HMW HA absorbs reactive oxygen species to protect tissues. The turnover of HA is rapid. It has been assumed that an adult human has 15 g of HA, about one-third of which turns

Table 1
Summary of catabolic enzymes that degrade HA.

Gene	Degraded HA sizes	Knockout phenotype*
HYAL1	Tetrasaccharides	Viable but exhibited osteoarthritis
HYAL2	20 kDa (50 disaccharides)	Pre-weaning lethality and accumulation of HA in the heart, lungs, and serum
HYAL3	Compensate in the absence of HYAL1	No evidence of hyaluronan accumulation
HYAL4	Have chondroitnase activity but not hyaluronidase activity	
HYAL5 (PH-20)	Tetrasaccharides	Fertile and normal in body size, behavior and health condition
KIAA1199 (CEMIP) (via the cell membrane-associated clathrin-coated pit endocytic pathway)	10-100 kDa	
TMEM2	5 kDa	

* Phenotype of mice homozygous for a null allele.

over daily. The half-life of HA in epidermal tissues is approximately 1 day, suggesting active local catabolism.

2.5. HA-binding proteins

Several important functions of HA are dependent on HA-binding proteins, which are widely distributed in the body. A class of proteins that binds specifically to HA was first discovered in cartilage. This class is referred to as the link-module family of hyaladherins. These proteins contain a link module that interacts specifically with HA. Aggrecan and versican are anchored to HA in tissues, with the link module at the amino terminus and the C-type lectin domain of the carboxyl terminus cross-linked by fibulins. Absence of the link module in aggrecan results in failure to anchor the proteoglycan. Versican, a major component in blood vessels and the dermis, is produced by smooth muscle cells and fibroblasts. The level of versican is increased after vascular injury. Mice deficient in hyaluronan and proteoglycan link protein 1 (HAPLN1) show a defect in cartilage development and delayed bone formation (short limbs and craniofacial abnormalities). Most mutant mice die shortly after birth as a result of respiratory failure [23]. Some cell-surface receptors have extracellular domains with link module motifs; *e.g.*, CD44, LYVE1 (lymphatic vessel endothelial hyaluronan receptor), HARE/STABILIN-2 (hepatic hyaluronan clearance receptor), and STABILIN-1.

3. HA in skin inflammation

3.1. Wound healing

The concentration of HA increases very rapidly in experimental skin wounds and peaks after 3 days. HA provides a temporary matrix for the migration of inflammatory cells and proliferation of fibroblasts. Abundant HA surrounds migrating cells in the wounded human oral epithelium and the cells flanking the wound site, and colocalizes with CD44 on migrating keratinocytes. Kaya et al. showed that suppression of CD44 resulted in animals with defective HA accumulation in the superficial dermis and defective keratinocyte proliferation in response to growth factors [24], as well as an impaired local inflammatory response and tissue repair. Application of HA enhances skin-wound healing *in vivo*. Fetal wound healing is characterized by a lack of fibrous scarring. The HA content in fetal wounds remains high for longer periods than in adult wounds, suggesting that HA reduces collagen deposition, leading to reduced scarring.

HA forms a temporary complex with fibrin and other extracellular matrix proteins, which supports the influx of fibroblasts and endothelial cells into the wound site for the early response to tissue injury and subsequent formation of granulation tissue. HA may suppress inflammation via free-radical scavenging, antioxidant effects, and/or exclusion of tissue-degrading enzymes from the immediate cellular environment, and from other structural components of the extracellular matrix.

The effects of HA are dependent on its molecular weight. HMW HA has beneficial effects on wound healing. Arnold et al. evaluated seven daily topical treatments of full-thickness skin wounds in pigs with ultrapure HA of defined molecular size. HMW HA (> 1000 kDa) enhanced, whereas LMW HA decreased, the rate of early wound contraction as compared with intermediate HA (100 kDa) and the saline control [25]. David-Raoudi et al. studied the effects of native HA, HA fragments of 12 and 880 saccharides on human foreskin-derived fibroblast proliferation and the expression of matrix-related genes. All three types of HA promoted cell adhesion and proliferation, and increased matrix metalloproteinase-1 and -3 expression, while HA-12 enhanced type I collagen and TGF-β 1 expression [26]. The HA fragments (1.2–3.6 kDa) induced type I and type III collagen synthesis in an X-irradiation model of lung fibrosis [27]. HA and its fragments activated Akt and extracellular-regulated kinases 1/2 and p38 partly, through CD44 [26]. These data are consistent with previous reports on human dermal and NIH 3T3 fibroblasts [28,29]. The mechanism of this stimulatory effect is not clear. It has been proposed that HA induces the detachment of cells from their matrix and facilitates mitosis. However, investigation on other fibroblasts showed contradictory data. HMW HA decreased the proliferation of fetal rabbit and embryogenic chick skin fibroblasts [30,31]. Furthermore, the formation of a pericellular HA matrix regulates autocrine TGF-β signaling and the persistence of the myofibroblast phenotype [32].

Fragmented HA of 4–25 disaccharide units (about 1.6–10 kDa) induced angiogenesis in a chick chorioallantoic membrane assay, whereas intact HA did not [33]. HA of 3–10 disaccharides (1.2–4 kDa) stimulated endothelial cell proliferation by inducing protein tyrosine kinase activity, but native HA inhibited proliferation. These effects are of benefit for the healing of acute and chronic wounds.

3.2. Role of HA in injury recognition and regulation of inflammation

Although HA is a natural product that lacks immunogenicity, HA generated during injury is involved in the resulting inflammation. Taylor et al. reported that HA increases the production of CXCL2/MIP2 and IL-1β during sterile injury [34,35]. Moreover, HA fragments smaller than 500 kDa stimulate the expression of genes encoding proinflammatory factors, leading to the production of cytokines. Kobayashi and Terao showed that CD44 mediates a dose-dependent increase in the production by human fibroblasts of the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-8 [36]. Both Toll-like receptor (TLR) 2 and TLR4 are required for the expression by macrophages of genes encoding proinflammatory factors in response to HA fragmentation, and TLR2/TLR4 deficiencies protect the host from acute inflammation but may impede epithelial cell repair [37]. The

initiation of a sterile intrinsic inflammatory event induces HA accumulation at the injured site. HA is important in this context, as it is an abundant GAG in the extracellular matrix of many tissues and is available for release in soluble form upon tissue injury. The sterile tissue is normally exposed to external microbial challenge after injury. During this process, local innate immune defenses are triggered to suppress and eliminate microbial invasion. Lipopolysaccharide (LPS), a bacterial endotoxin, activates TLR4 and promotes factor nuclear factor kappa B (NF-κB)–mediated production of proinflammatory cytokines by many cell types. Progress has been made in understanding the mechanisms involved in the negative regulation of TLR signaling, which is essential to prevent detrimental effects of an excessive inflammatory response. Several intracellular proteins, including TNFα-induced protein 3 (TNFAIP3/A20) [38,39] have been identified as negative regulators of TLR signaling. TNFAIP3/A20-deficient mice develop severe inflammation and cachexia, are hypersensitive to both LPS and TNF, and die due to the failure to regulate TNF-α–induced NF-κB activation [38,39]. HA binds to CD44, and has been shown to engage a receptor complex comprising CD44, MD-2, and TLR4, which results in the activation of TLR4 [34]. Interestingly, HA and CD44 trigger A20 activation and subsequent suppression of TLR4 activation [40]. Thus, the presence of free HA at sites of injury may modulate the host reaction to bacterial endotoxin. This observation is consistent with previous reports of an anti-inflammatory effect of HA on a variety of cell types [37,41,42]. Moreover, CD44 suppresses innate immune inflammatory responses by promoting the expression and function of negative regulators by macrophages responding to pathogens [43]. Thus, HA status reflects changes in the tissue environment and regulates the host inflammatory response.

The size of HA is a determinant of its function. Kawana et al. reported that LMW HA (3 and 22 kDa) inhibited TLR signaling independently of CD44 expression [44]. Similarly, small extracellular HA oligosaccharides did not induce the production of cytokines, such as MIP2/Cxcl2 and IL-1β, but small intracellular HA oligosaccharides induced the production and release of IL-1β [34,35]. These observations suggest that the mechanisms underlying the host responses are dependent on its size and localization.

Furthermore, HA oligosaccharides act as a danger signal through recognition by TLR4. Kim et al. reported that HA tetrasaccharides modify the inflammatory response induced by poly (I:C)activated TLR3 signaling through TLR4 [45]. This cross-talk appears to be mediated in part by the induction of TRAF1, a negative regulator of TLR3 [46]. Bernard et al. showed that ultraviolet-B (UVB) damage is detected by TLR3, and that self-RNA is a DAMP that serves as an endogenous signal of solar injury [47]. Therefore, HA oligosaccharides may prevent excess production of inflammatory cytokines, which can harm the host after UV irradiation. Phagocytosis is critical for the induction of inflammation by other TLR ligands. Forrester and Balazs reported that LMW HA (< 100 kDa) promotes phagocytosis by macrophages, but HMW HA (1000–2000 kDa) exerts an inhibitory effect [48].

3.3. HA as a link between innate and adaptive immunity

The early inflammatory response to tissue injury has been proposed to involve recognition of components of damaged cells by pattern recognition receptors (PRRs), such as TLRs. These components are known as DAMPs. Several lines of *in vitro* and *in vivo* evidence support an important role for PRRs. However, interpretation of the functions of DAMPs is hampered by the multiple microbial products present in a wound, and potential contamination by small amounts of microbial products of the reagents used to study these responses. HA is of interest as a DAMP because it is particularly abundant in skin [8]. Small tetra- and hexa-saccharide fragments of HA influence dendritic cells (DCs) in culture via TLR4 [4,5].

We investigated the function of HA breakdown *in vivo* in mice overexpressing *HYAL1*, and thus producing HA fragments without inflicting injury or altering the microbial milieu. This *in vivo* approach, combined with direct comparison with the response to purified HA tetrasaccharides, demonstrates that HA breakdown initiates DC migration from the skin and affects sensitization to

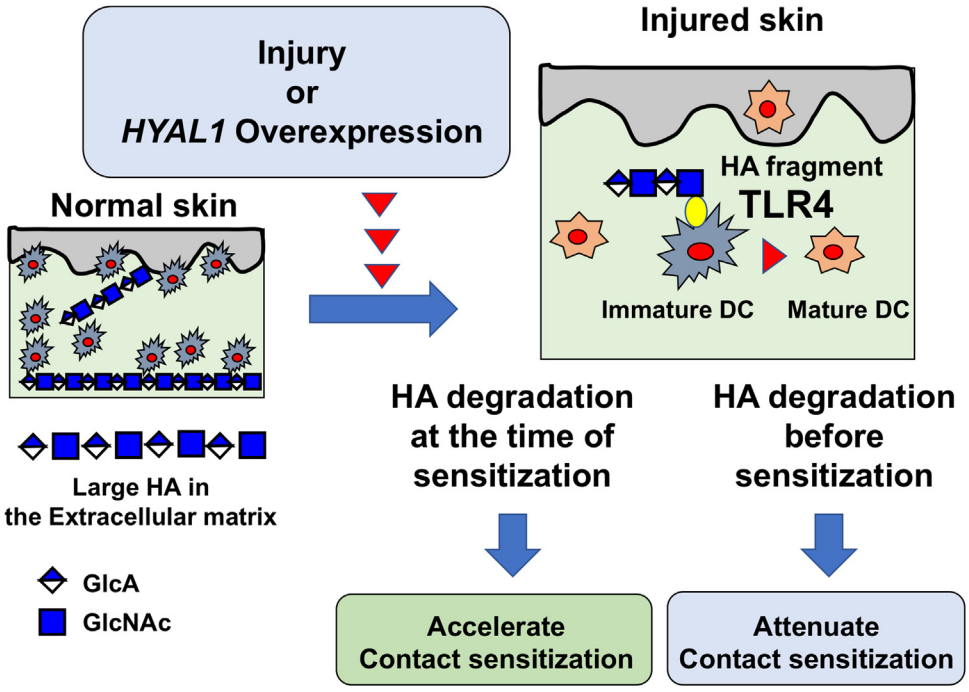

Fig. 2. Schematic diagram showing the activation by hyaluronidase, or by an increase in the amount of small fragments of HA, of cutaneous dendritic cells (DCs), leading to modulation of Toll-like receptor (TLR) 4-mediated induction of contact allergy. Accordingly, HA may link innate and adaptive immunity.

topical antigens [49]. Thus, the action of hyaluronidase, or an increase in the quantity of small HA fragments, activates cutaneous DCs and modulates the induction of contact allergy. A recent study has further advanced these observations and identified a major change of the antimicrobial defense and inflammation of both the skin and the large intestine after expression or addition of hyaluronidase [50]. These observations suggest an important physiologic role of HA breakdown. Our model system has confirmed the role of HA in the cutaneous immune response in the absence of the many other variables present following injury or infection, and the dependence of this response on TLR4. Somewhat surprisingly, we did not observe inflammatory responses typically attributed to TLR4 in our model system. These findings provide new insight into the influence on immunity of DAMPs, and in particular HA (Fig. 2).

4. Conclusion

HA modulates various mechanisms of innate immunity, such as barrier formation and the functions of DCs in the skin. New findings compel a major revision of previous models that predicted that HA breakdown triggers inflammation after injury—in contrast, HA fragments modify adaptive immune responsiveness to the environment. Such a response is most relevant after wounding and may prevent the development of autoimmunity and an excessive reaction to tissue damage, as observed in TLR2-/-TLR4-/- mice after lung injury [37]. These findings suggest a new approach to modulating allergic responses, to accelerate when desirable or inhibit when unwanted. Fragmentation of HA may also accelerate antigen presentation and the development of immune responses, enhancing the effectiveness of vaccination. Cutaneous DCs play a major role in the pathophysiology of several common human skin disorders, including psoriasis and atopic dermatitis. We believe that further studies of HA catabolism will enhance our understanding of human immunology and promote the development of novel therapeutic interventions for skin diseases.

Conflict of interest

None declared.

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