Minireview

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Hyaluronan and Homeostasis: A Balancing Act*

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Markku I. Tammi‡, Anthony J. Day§, and Eva A. Turley¶‖

From the ‡Department of Anatomy, University of Kuopio, FIN-70211, Kuopio, Finland, §Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom, and ¶London Regional Cancer Center, London, Ontario N6A 4L6, Canada

Hyaluronan was first described as the mucus of the vitreous body of the eye (1). Its deduced structure revealed an acidic glycosaminoglycan made entirely of a repeating disaccharide (D-glucuronic acid β-1,3-*N*-acetylglucosamine-β1,4) (2), which exists *in vivo* as a polyanion. The ability to synthesize hyaluronan is probably a comparatively recent innovation in the evolution of metazoan organisms (3), but it is also present in the capsule of some pathogenic bacteria. Molecules of hyaluronan are generally of very high molecular mass, ranging from about 10^5 to 10^7 Da, depending upon the tissue, but can also exist as smaller fragments and oligosaccharides under certain physiological or pathological conditions. Hyaluronan exhibits unusual physicochemical properties in concentrated solutions because of a combination of its random-coil structure, its large size, which results in molecular entanglement, and its capacity to interact with water molecules. A molecule of hyaluronan, therefore, has a large hydrodynamic volume and forms solutions with high viscosity and elasticity that provide space filling, lubricating, and filtering functions (4).

It was originally thought that hyaluronan acts as an inert molecular "stuffing" in connective tissues. However, the identification and study of specific hyaluronan-binding proteins, referred to as hyaladherins, have revealed that hyaluronan mediate many other important functional activities. The first well studied hyaladherins were link protein and aggrecan, which together with hyaluronan form the well known, massive multimolecular proteoglycan aggregates (5, 6). These expansive complexes have an important role in the formation and stability of extracellular matrices, in particular providing tissues such as cartilage with load-bearing capabilities. Direct evidence for this has now been provided by the observation that removal of the link protein gene (7) or loss of functional aggrecan (8) results in skeletal defects, particularly in cartilaginous tissues, that give rise to short limbs, cleft palate, and other craniofacial abnormalities.

A major development occurred in the field about 10 years ago with the cloning of two cell surface hyaluronan receptors, CD44 (9, 10) and RHAMM[1] (11). Their discovery first revealed a role for hyaluronan in directly regulating cell motility, invasion, and proliferation. These receptors bind to high molecular weight hyaluronan but can also interact with smaller forms of hyaluronan. Indeed, several studies suggest that fragmentation of hyaluronan enhances its ability to activate cell-signaling pathways (12–14). Therefore, a novel mechanism may exist that permits sorting of signals based upon ligand size. Deletion of either CD44 (15, 16) or RHAMM[2] is not lethal. Deletion of CD44 in mice results in a complex phenotype with animals exhibiting defective trafficking of leukocytes (15, 16), altered responses to tissue injury (15–18), and transformation by oncogenic viruses such as SV40 (16). CD44−/− animals are compromised in their ability to respond to injury. Surprisingly, −/− mice exhibit an exaggerated granuloma response to *Cryptosporidium parvum* infection (16) and enhanced hepatitis after concanavalin injection (18). These results contrast with the ability of anti-CD44 antibodies to reduce many inflammatory responses (19) and of targeted disruption of CD44 expression to inhibit both wounding and delayed type hypersensitivity responses in skin (20, 21) and to abrogate experimental colitis (22). Furthermore, although CD44 has been implicated in promoting tumor progression (23, 24), SV40-transformed CD44−/− cells are highly tumorigenic, and re-introduction of CD44 reduces their tumorigenicity (16). These results suggest a complicated role for CD44 in inflammation and neoplasia (23, 24). However, because CD44 binds to multiple ligands and participates in growth factor signaling, the role for hyaluronan in these CD44-regulated processes remains, for the most part, to be determined.

Another recent major breakthrough has been the identification of three vertebrate hyaluronan synthases (for reviews, see Refs. 25 and 26), and this, along with data from experimental modification of their expression (see below), solidified evidence for a key role of hyaluronan in morphogenesis and in many forms of cancer. It is expected that ongoing research to identify and characterize enzymes, which degrade hyaluronan (hyaluronidases), as well as assessment of the function of a group of intracellular hyaluronan-binding proteins will add further to our knowledge of the biological roles of hyaluronan.

There is an emerging picture of an exquisitely regulated balance between the production, sizing, secretion, and removal of hyaluronan that is central to its functions in development, homeostasis, and disease. This concept is well illustrated in morphogenesis of the heart and in ovulation and fertilization discussed below. Other minireviews in this series describe the molecular basis of hyaluronan-protein interactions (27), the nature of the signaling pathways that are activated as a consequence of these interactions (28), and the alteration of hyaluronan expression in pathology (29). Further information on hyaluronan and hyaladherins can be found in a group of excellent web reviews.[3]

Synthesis, Catabolism, and Compartmentalization of Hyaluronan

The paradigm of a balanced regulation of hyaluronan synthesis and catabolism contributing to tissue function was originally noted during embryonic development. An excellent example of this is provided by the study of heart valve morphogenesis where cushion cells first migrate from the endocardium to the myocardium in a hyaluronan-rich matrix (30, 31). Subsequent differentiation of heart valves is accompanied by a reduction in local hyaluronan that is achieved by both a decrease in its synthesis and an enhancement of its receptor-mediated uptake and degradation. Direct evidence supporting an instructive role for hyaluronan in heart development has now been provided by the demonstration that genetic deletion of one of the three hyaluronan synthases (HAS2) leads to developmental abnormalities of the heart, including the valves (32) (Fig. 1). Malformation results, in part, from a lack of tissue swelling that normally leads to the division of the primordial heart tube into atria, ventricles, and arterial outlet trunks (Fig. 1), similar to the null mutation of the proteoglycan versican (33). Furthermore, there is a defect in the ability of HAS2−/− endocardium cells to undergo

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‖ To whom correspondence should be addressed: London Regional Cancer Center, 790 Commissioners Rd. E, London, Ontario N6A 4L6, Canada. Tel.: 519-685-8651; Fax: 519-685-8646; E-mail: eva.turley@lrcc.on.ca.

[1] The abbreviations used are: RHAMM, receptor for hyaluronan-mediated motility; HAS, hyaluronan synthase(s); COC, cumulus cell-oocyte complex; IαI, inter-α-trypsin inhibitor; TSG-6, tumor necrosis factor-stimulated gene-6.

[2] C. Tölg and E. A. Turley, unpublished data.

[3] See "Science of Hyaluronan Today" at www.glycoforum.gr.jp/.

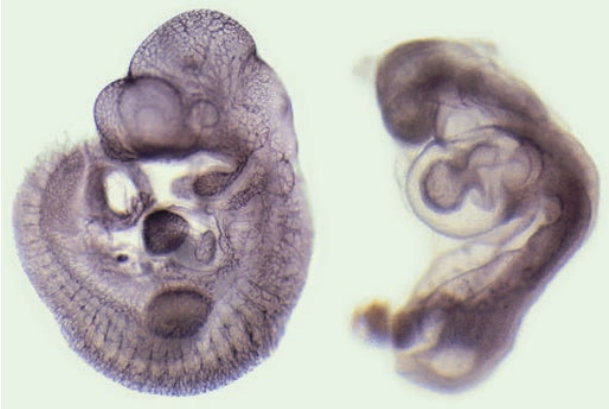

FIG. 1. **Developmental defects in the 9.5-day Has2−/− mouse.** The whole mount embryo was stained with an antibody against CD31, an endothelial marker protein. As compared with the wild type embryo (*left*), the HAS2−/− embryo (*right*) shows the cardiac tube invested in an enlarged pericardium and incompletely divided into atria and ventricles. The wild type heart contains more blood erythrocytes. The vasculature is poorly developed, the extracellular space collapsed, and the size of the HAS2−/− embryo reduced; however, somites are present. For further details, see Ref. 32.

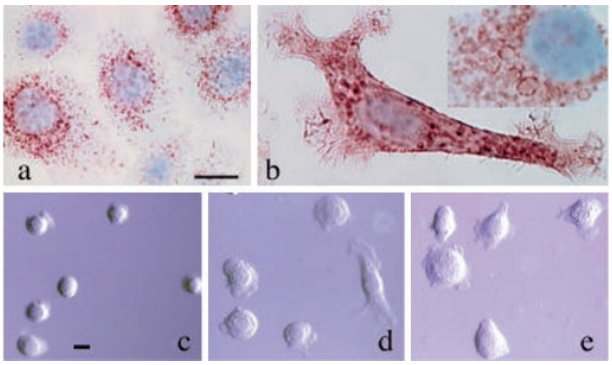

FIG. 2. **Changes in cellular morphology associated with HAS2-directed hyaluronan synthesis.** *Upper panels*, epidermal keratinocytes with a round and flattened epithelial phenotype (*a*) were treated with epidermal growth factor (*b*) that specifically up-regulates HAS2 and increases hyaluronan synthesis 3–5-fold (94). The pericellular hyaluronan bound to plasma membrane receptors such as CD44, and that still associated with the hyaluronan synthase was visualized by a probe containing the hyaluronan binding region of aggrecan and link protein (*brown color*). Note the elongated, partially lifted keratinocyte morphology in *b*, resembling that of mesenchymal cells and indicating elevated migratory activity. This phenotype is also associated with a markedly increased level of hyaluronan in intracellular, perinuclear vesicles, specifically demonstrated by first removing extracellular hyaluronan by *Streptomyces* hyaluronidase (*inset*). The *lower panels* show the influence on keratinocytes of reduced (*c*) and increased (*e*) expression of HAS2 6 h after trypsinization and replating. Stable transfections of HAS2 antisense cDNA retarded the extension of cellular lamellipodia (*c*) as compared with control cells with an empty vector (*d*). The spreading rate of transfectants receiving HAS2 sense cDNA was similar to that of controls or slightly faster (*e*). *Scale bars*, 10 μm.

a transition to a migratory, mesenchymal morphology, as detected in organ culture *in vitro* (32). This inability to migrate is rescued *in vitro* by the addition of low concentrations of hyaluronan or by transfection with a plasmid encoding HAS2 (32). A transition from an epithelial to mesenchymal morphology and stimulation of motility may be common with HAS2 up-regulation because the same phenomenon occurs in epidermal keratinocytes (Fig. 2).[4]

Synthesis of Hyaluronan: HAS1, -2, and -3—Hyaluronan synthases (HAS) were first characterized in the bacterium *Streptococcus pyogenes* (SpHAS) (34). Subsequently, three related isoenzymes (HAS1, HAS2, and HAS3), all homologous to the streptococcal enzyme and to a lesser degree to invertebrate chitin synthases (35), have been found in human, mice, *Xenopus*, and chicken (25, 36). To date, most of our knowledge of how this family of HAS genes produces hyaluronan derives from studies of the bacterial enzyme. The SpHAS is a 48-kDa transmembrane protein that passes through the plasma membrane 4 times (37) and requires the association of about 16 molecules of cardiolipin for full activity (38). Its cytoplasmic UDP-*N*-acetylglucosamine and UDP-glucuronic acid transferase sites add the alternating monosaccharides, most probably to the reducing end of the growing hyaluronan chain with continuous extrusion through the plasma membrane using a pore provided by the enzyme itself. Mouse and *Xenopus laevis* HAS1 also synthesizes hyaluronan without other proteins (39, 40). The functional unit size of *Xenopus* HAS1 is 85–92 kDa, clearly larger than its 69-kDa polypeptide, indicating that it works catalytically as a monomer but is associated with other material, possibly lipid (40). Recently, a completely different kind of HAS was cloned and characterized in the pathogenic bacterium *Pasteurella multocida*, which encodes two separate domains that are homologous to glycosyltransferases. In these organisms, synthesis of hyaluronan occurs on the non-reducing end of the growing chain (41).

The existence of three HAS isoenzyme genes in all vertebrates studied so far and their location to different chromosomes (25) predict distinct expression patterns and not completely overlapping functions. Indeed, Northern blots indicate that the production of each HAS is differentially scheduled during the embryonic development and that there are tissue and cell-specific variations in their expression. Genetic deletion of the three HAS genes in mice indicates that only HAS2 is vital to development, resulting in death at day 10 because of a failure in the development of the heart, as noted above (25, 32). The specific roles that HAS1 and HAS3 play are not yet as well documented. However, overexpression of HAS1, -2, or -3 in several cell types indicates that there are distinct differences in their requirement for cellular UDP-*N*-acetylglucosamine and UDP-glucuronic acid, in elongation rates, and in the final polymer size (35, 36). For instance, HAS3 produces lower molecular weight hyaluronan than HAS2 (42, 43). Given increasing evidence that lower molecular mass hyaluronan (<200,000 Da) more efficiently activates intracellular signaling pathways via cell-associated hyaladherins than high molecular weight hyaluronan (12–14), the differential regulation of the HAS may have important consequences for the modulation of cell behavior. HAS2, for instance, may be important in contributing high molecular weight hyaluronan that is required for formation of extracellular proteoglycan complexes, particularly abundant in cartilage (44). Because the synthesis rate and product size of hyaluronan appear to depend on the cellular background (43), hyaluronan synthases are also likely to be subject to regulation by other proteins.

Modulating the expression of HAS genes in cells *in vitro* provides a tool for a more direct assessment of the role of hyaluronan in cell behavior. For instance, overexpression of HAS genes in Chinese hamster ovary cells resulted in greater than 1000-fold enhancement of hyaluronan production, inhibited cell migration, and reduced the expression of cell surface CD44 (42), consistent with a study that showed down-regulation of hyaluronan receptors with the addition of high levels of exogenous hyaluronan (45). However, overexpression of either HAS1 or -2 in melanoma cells strongly enhanced cell motility (46). Accordingly, transfection of the HAS2 gene in sense and antisense orientations stimulates and inhibits, respectively, the migration of epidermal keratinocytes in an *in vitro* wound healing assay.[4] The level of HAS2 expression influences lamellipodial outgrowth, a key function in the migration process (Fig. 2*c*). These results indicate that an optimal size and amount of hyaluronan is required to promote cell motility (47), and this response may be cell background-dependent (48). An altered balance in the ratio of synthesis to catabolism is strongly associated with neoplastic progression, and studies where HAS1 or HAS2 expression is increased by transfection have now provided direct evidence for a role of hyaluronan in tumor metastasis (46, 49), as discussed in another minireview of this series (29).

Uptake and Catabolism of Hyaluronan: Hyaladherins and Hyaluronidases—The removal of hyaluronan appears to be as important as its synthesis in both morphogenesis and tissue homeostasis. For instance, it has been estimated that about a third of hyaluronan in the human body is removed and replaced each day. In many instances, removal is achieved by endocytic uptake, either within the tissue where it is made or in lymph nodes and the liver (50). The

[4] K. Rilla, M. Lammi, R. Sironen, K. Törrönen, M. Luukkonen, V. C. Hascall, R. J. Midura, M. Hyttinen, M. I. Tammi, and R. Tammi, manuscript in preparation.

catabolic rate of hyaluronan greatly varies between tissues. Labeled hyaluronan in the epidermal compartment of human skin organ cultures disappears with a half-life of about 1 day (51), in contrast to ~20 and ~70 days in the cartilage (52) and vitreous body (50), respectively. Hyaluronan undergoes fragmentation in the presence of reactive oxygen species, which can enhance hyaluronan turnover (53). CD44, which plays a major part in the formation of cell-associated matrices can, under certain conditions, also mediate hyaluronan endocytosis (for instance during morphogenesis of tissues such as the lung and skin (31), during long bone growth (31), and in adult tissues such as cartilage (54)). However, the hyaluronan receptor that is most efficient in the internalization and degradation of hyaluronan is a novel liver endothelial cell receptor (55) that forms a large molecular mass complex of several subunits. A lymph vessel-specific receptor for hyaluronan, LYVE-1, which is a homologue of CD44, has been identified and shown recently to participate in the endocytic uptake of hyaluronan in the lymphatics (56).

The mechanisms of hyaluronan uptake by cells appear unique because clathrin-coated pits and caveolae, the most common vehicles for wrapping and detaching receptor bound cargo into endosomal vesicles, seem not to be operative (57). Mechanisms by which internalized hyaluronan is broken down have not been studied in detail but likely involve endosomal and lysosomal hyaluronidases (58). In some circumstances hyaluronan is degraded outside the cell by extracellular hyaluronidases. For example, a hyaluronidase, PH20, is attached to the sperm surface via a glycophosphatidylinositol anchor and is required for sperm penetration through the hyaluronan-rich matrix surrounding the oocyte. Thus, it is required for successful fertilization (59). This extracellular hyaluronidase is similar to hyaluronidase found in the bee venom gland (60). Five hyaluronidase genes and one pseudogene have been recently cloned (61). One of them (*Hyal1*) is widely expressed in contrast to PH20 whose expression is restricted to the testes (62). The functional importance of the high level of *Hyal1* in serum remains obscure because its sharp pH optimum of 3.7 predicts little activity outside lysosomes. Naturally occurring mutations in *Hyal1* result in mild mucopolysaccharidoses, a finding consistent with its function as a lysosomal enzyme (63). Curiously, *Hyal2* presumably resides in lysosomes (64) but is also displayed as a glycophosphatidylinositol-anchored plasma membrane protein. It was recently suggested that *Hyal2* mediates the oncogenic transformation and lung cancer caused by Jaagsiekte sheep retrovirus (65). However, no hyaluronidase activity could be demonstrated in NIH3T3 fibroblast and HeLa cell lysates transfected with human *Hyal2* despite their enhanced susceptibility to oncogenic transformation, suggesting that Hyal proteins may perform several functions (65).

Cellular Distribution of Hyaluronan: Hyaladherins

Hyaluronan is present in the extracellular matrix, on the cell surface, and inside the cell (Fig. 2, *a* and *b*). It is useful therefore to broadly divide the functions of hyaluronan into those associated with the organization of the extracellular matrix, those associated with a formation of a hyaluronan coat on the cell surface, those associated with receptor-mediated signaling, and those associated with the intracellular presence of hyaluronan. Extracellular hyaluronan is found in tissues that are comprised primarily of extracellular matrix, for example cartilage, where its role as an important structural component of the matrix has been reviewed elsewhere (66). This section will focus on pericellular and intracellular hyaluronan.

For a hyaluronan-rich pericellular matrix to form, it is necessary for it to be anchored to the cell surface. In many instances this is mediated by CD44, but nascent chains of hyaluronan that remain attached to the HAS machinery can also contribute to coat formation (67, 68). Other hyaluronan receptors of unknown function with homology to the link protein family (69) or completely unrelated to CD44 or RHAMM have been described (70, 71). Retention of hyaluronan as a coat at the cell surface allows capture and incorporation of extracellular hyaluronan-binding proteins, such as aggrecan (44), into the immediate environment of the cell. Evanko *et al.* (72) have recently shown that a hyaluronan coat incorporating versican is required for the proliferation and migration of smooth muscle cells *in vitro*. Coat formation is rapid, and its presence is related to events involving cell detachment, such as during mitotic cell rounding (73). A remarkable example of rapid pericellular matrix formation *in vivo* occurs prior to ovulation during the expansion phase of the cumulus cell-oocyte complex (COC), which is made up of an oocyte surrounded by ~1500 closely adherent cumulus cells. A massive up-regulation of hyaluronan synthesis drives the expansion of the COC volume by up to 20-fold (74). This matrix is stabilized by inter-α-trypsin inhibitor (IαI) (75), a serum-derived hyaladherin, likely in conjunction with tumor necrosis factor-stimulated gene-6 (TSG-6), another hyaluronan-binding protein, which is expressed at the initiation of COC expansion (76–78). IαI and TSG-6 form a stable complex (78), which facilitates the cross-linking of molecules of hyaluronan to stabilize matrix formation. Abolition of functional IαI in mice leads to severe female infertility because of impaired expansion of the COCs and poor fertilization of ovulated oocytes, which were devoid of matrix (79).

Hyaluronan-CD44 and hyaluronan-RHAMM (CD168) interactions have been reported to result in the activation of signaling cascades that contribute to cell motility and proliferation. Why some hyaluronan-CD44 interactions signal, some promote endocytic uptake, and others permit retention of hyaluronan on the cell surface has not yet been resolved. This may be determined, in part, by the presence of other cellular hyaladherins. For instance, in some cell backgrounds, RHAMM is required for motility even though CD44 is clearly expressed at the surface of these cells (14, 80). In addition, studies describing effects of hyaluronan on signaling and cell behavior often use different amounts and molecular weight preparations that may affect the outcome of experiments. For instance, the amounts of hyaluronan used to stimulate cell signaling vary from nanograms (32, 47, 81) to micro- or even milligrams (14, 81–84). Because the purity of preparations used may vary (85), studies using the higher concentrations of hyaluronan need to be interpreted with caution.

Unlike genetic deletion of HAS2 noted above, the deletion of CD44 or RHAMM genes in mice does not result in embryonic lethality. It is therefore likely that a family of cell surface hyaladherins exists that can complement the function of CD44 and RHAMM in morphogenesis (70). However, it will be interesting to assess whether or not adult knockout animals are as resistant to injury and disease as their wild type counterparts. Interpreting the signaling capabilities of CD44 and RHAMM strictly in the context of binding to hyaluronan must be viewed with some caution given that both bind to other extracellular matrix proteins such as fibronectin (86), which itself has been implicated in signaling during response-to-injury and cancer.

There is growing evidence for the presence of intracellular hyaluronan (Fig. 2, *inset*) and intracellular hyaladherins. Intracellular hyaluronan has been detected in the cytoplasm of vascular smooth muscle cells during late prophase/early prometaphase of mitosis and in key subcellular compartments such as the nucleus and lamellae during cell locomotion and following serum stimulation (87, 88). Intracellular hyaluronan can be derived from either the extracellular environment (87) or from an as yet unidentified intracellular source (88) and may be involved in nuclear function, chromosomal rearrangement, and other events associated with cell proliferation and motility. A vertebrate homologue of the cell cycle control protein Cdc37 is a hyaluronan-binding protein that is found in the cytoplasm around the nuclear membrane. Cdc37 associates with a variety of kinases, including Raf and pp60v-Src protein (89). Another cytoplasmic hyaluronan-binding protein, P-32, has been associated with RNA splicing (90). An intracellular form of RHAMM is associated with podosomes, lamellae, the actin cytoskeleton, microtubules, and the cell nucleus and associates with the mitogen-activated protein kinase Erk-1 and calmodulin (91–93). The role that these hyaladherins might play in transducing a signal from either extracellular or intracellular hyaluronan promises to be an exciting and active area of research for the future and is discussed in more detail in another minireview in this series (28).

Summary

The investigation of the functions of hyaluronan, which began with its discovery in the 1930s (1), has evolved slowly. Re-examination of the fascinating and complex biology of hyaluronan is now occurring both as a result of increasing awareness of the key and complex role played by components of the extracellular matrix in the regulation of developmental, physiological, and disease pro-

cesses and of the relatively recent cloning of many new hyaladherins, synthases, and hyaluronidases. During the coming decades, unraveling the hyaluronan enigma will undoubtedly uncover paradigms that shift our understanding for its roles in the regulation of cell homeostasis and matrix biology.

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