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TRAFFICKING OF HYALURONAN IN THE INTERSTITIUM AND ITS POSSIBLE IMPLICATIONS

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ABSTRACT

The mechanisms underlying the pathological changes in lymphedematous tissue are far from clear, and it is becoming apparent that plasma proteins may not be the only key factors responsible for holding water in the interstitium. This review focuses on an "old" macromolecule - hyaluronan (HA) which is one of the major components of the interstitium and has a close relationship with the lymphatic system. Growing recognition of the multiple functions of this macromolecule for important physiological and pathological events may be helpful in identifying the crucial changes in tissues subjected to lymphatic circulation insufficiency and ultimately in the search for rational therapeutic approaches to prevent or reverse these tissues changes.

Hyaluronan is a major constituent of the extracellular matrices in which most tissues differentiate and also an essential component of many extracellular matrices in mature tissues.

The components of interstitium are principally the same in all tissue. They are collagen, elastin, glycosaminoglycans including hyaluronan (HA), chondroitin sulfate, and plasma protein. It is believed that most of the macromolecules are immobilized in the interstitium. The present study, however, shows that HA shares the same pathway as plasma proteins in trafficking through the interstitium and undergoing catabolism mainly outside the extracellular matrix (1).

HA is synthesized at and extruded through plasma membranes of fibroblasts and other mesenchymal cells. Its deduced structure reveals an acidic glycosaminoglycan made entirely of a repeating disaccharide (D-glucuronic acid ß-1,3-N-acetylglucosamine-ß-1,4) (1). The disaccharide units have anionic groups that are fully charged at physiologic pH (2). Molecules of HA 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 and pathological conditions (2). HA exhibits physicochemical properties in concentrated solutions due to a combination of its random-coil structure and large size (2). A molecule of HA has a large hydrodynamic volume and forms solutions with high viscosity and elasticity that provide space-filling, lubricating, and filtering functions (2). Thus, HA is a biopolymer with extraordinary biophysical properties that contributes to the extracellular matrix structure and interstitial homeostasis (3,4).

In the last 20 years, paradigms have shifted from regarding HA only as a molecular "cotton wool" that fills certain extracellular spaces to the view that as is a

center around which many matrix macromolecules are organized. A great range of biological functions has been attributed to the molecule, and these have numerous physiological, clinical and diagnostic implications.

A close relationship has been found between HA metabolism and lymphatic drainage both clinically and experimentally (5,6) as the HA content is significantly increased in lymphedematous tissue due to the impairment of lymph circulation.

The objective of the present paper is to discuss the physiological and metabolic character of HA and the possible impact that it may have on maintaining body homeostasis under conditions of lymph drainage impairment when the close relationship between HA and the lymphatic system is revealed.

HA Trafficking as a Function of the Lymphatic System

Most of the components of extracellular matrix (ECM) are immobilized in the interstitium. HA is the only macromolecule that undergoes rapid turnover with most degradation occurring not locally, but outside the ECM. It was discovered in 1981 that HA is present in normal blood and is transported from peripheral tissues to the blood circulation through lymph (7). When tritium labeled polysaccharide was injected into the circulation of rabbits and humans, most degradation of HA was not found to occur within the skin itself; rather, HA was transported through the lymphatic system to distant lymph nodes where >90% of the HA in afferent lymph is degraded by the lymph node endothelium. The remaining 10-15% of HA exits via the efferent lymphatics and from there, to the blood vasculature, where it is rapidly endocytosed by the liver sinusoidal endothelium (8,9).

The development of analytical methods has allowed the determination of HA in the nanogram range in lymph from different tissues such as skin, intestine and thoracic duct. Interstitial lymph has a higher concentration of HA than lymph from other organs. The largest amount of HA (7-8g per average adult human, ~50% of the total in the body) resides in the skin where it is present in both the dermis (~0.5 mg/g wet tissue) and epidermis (~0.1mg/g wet tissue). Taking into account the difference in density between cells and interstitial matrix, the actual concentration of HA in the matrix around cells in the epidermis (estimated to be 2-4 mg/ml) is an order of magnitude higher than in the dermis (estimated to be ~0.5mg/ml) (9). The metabolism of HA is very dynamic in skin. Keratinocytes in epidermis actively synthesize and catabolize HA; the half-life of a HA molecule is surprisingly short, less than a day. Cells in dermis actively synthesize more HA than they catabolize. A large proportion of the HA molecules escape from this tissue only to be rapidly captured by receptors on reticuloendothelial cells in lymph nodes and in the liver (10). Although the magnitude of HA turnover in some tissue such as epidermis is high, there is little evidence to support the presence of an extracellular neutral pH active hyaluronidase activity (2). The polysaccharides are degraded in lysosomes.

It has been estimated that almost one-third of the total HA in the human body is metabolically removed and replaced during an average day (11). An estimate of the total amount of HA metabolized in humans, based on thoracic duct lymph studies is 150mg/day, but due to metabolism in lymph nodes, the total turnover could be considerably larger (1).

During washout of tissue through lymphatic vessels, the crucial factor that may influence the concentration of HA in the interstitium is lymph flow *(Fig. 1)*. Any factors that alter the lymph flow such as net capillary filtration may indirectly influence the content of interstitial HA. It was shown that the lymphatic removal of HA from the lung increased when lymph flow was increased by elevating left atrial pressure or by intravenous injection of *Escherichia coli*

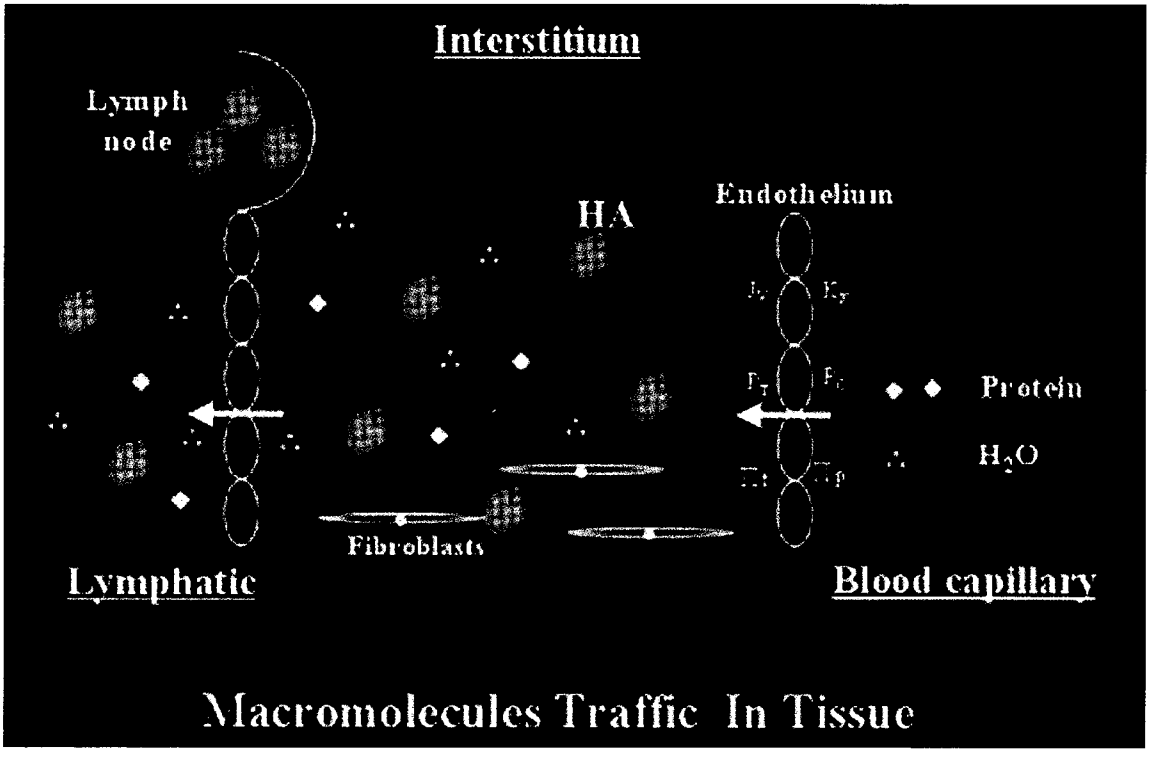

Fig. 1. Schematic diagram of macromolecular trafficking through the interstitium. HA is synthesized by mesenchymal cells in the interstitium and enters the afferent lymphatic vessels for transport to lymph nodes. The content of HA in the tissue is determined largely by the lymphatic circulation.

(1). The turnover of HA can be further increased after tissue injury or sepsis (1).

Because about one-half of the total body HA is localized in skin, the rapid turnover (15-20 hr) in this tissue should be a major determinant of the turnover of HA in the whole body. Due to the fact that a large amount of HA is carried away through lymph circulation every day, the fate of HA in the tissue of lymphangiodysplasia or lymphatic damage in which the lymph may be stagnant in the tissue is particularly interesting. In clinical studies, HA content in the interstitial fluid was found to be significantly higher in the lymphedematous limbs of patients (30 µg/ml) compared with the non-edematous contralateral limb (8 µg/ml) (5). A similar result was found in another experimental study in which the HA content in the rat tail skin after blockage of lymphatics was also significantly higher (50 µg/ml) than before blockage (2 µg/ml) (6). In an initial model of chronic lymphedema, tritiated HA was injected to follow its metabolism. The clearance of injected HA was much delayed in rat tail skin after lymphatic blockage, and the half-life was ~105-110 hr, compared with ~70-75 hr in the controls (6). Both clinical and experimental studies indicate that blockage of lymph outflow directly influences the capacity of affected tissue to dispose of HA and therefore alters the catabolism of this macromolecule (5,6).

Both clearance and turnover of HA involve HA receptor-mediated endocytosis rather than fluid phase pinocytosis (12). HA has diverse biological roles; it acts as a vital structural component of connective tissues; forms loose hydrated matrices that allow cells to divide and migrate (for example, during

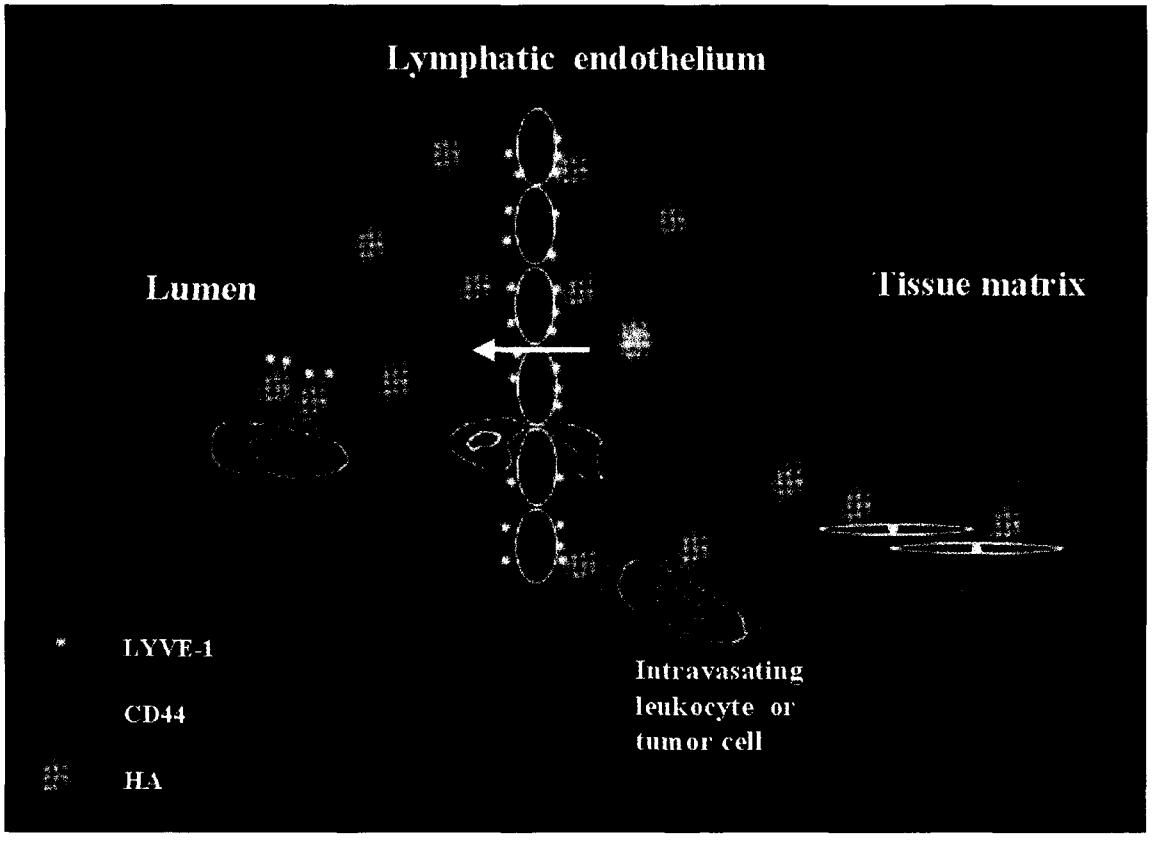

Fig. 2. Schematic diagram of the role of LYVE-1 in the lymphatic system. LYVE-1 receptors are expressed on both luminal and abluminal surface of lymphatic endothelial cells. HA molecules enter the afferent vessels and are transported to lymph nodes. The cell surface HA receptor CD44 mediates leukocyte passage across lymphatic vessels through the binding of HA molecules that are also bound with LYVE-1.

development) (13); mediates immune cell adhesion; and activates intracellular signaling (10). This wide range of functional activities may seem surprising given hyaluronan's simple structure, which is dependent on the large number of HA-binding proteins that exhibit significant differences in their tissue expression, cellular localization, specificity, affinity and regulation (14). Characterization of the molecular basis of HA recognition by proteins, and how this is modulated *in vivo*, will be a key determinant in understanding the biology of HA. HA binding proteins have been grouped together as a family termed hyaladherins, which are further subdivided into matrix and cell-surface hyaladherins (receptors) (15).

The hyaladherin implicated in recognition, capture and transport of HA from tissue into lymphatics is the recently discovered specific receptor LYVE-1, lymphatic vessel endothelial HA receptor (16). LYVE-1 appears to be equally exposed to both the luminal and abluminal surfaces of lymphatic vessels. It is believed that the role of LYVE-1 in the lymphatic system is to remove tissue HA from interstitium to lymph (12). LYVE-1 might shuttle across the lymphatic endothelium and transport HA from tissue to lymph by transcytosis (12) *(Fig. 2)*. Efficient macromolecular transport is one of the key roles of the lymphatic endothelium (17), and the process has been studied in detail by means of tracer labeling experiments with

isolated perfused renal lymphatics (18). The capacity to mediate HA – internalization *in vitro* – suggests a physiological role for LYVE-1 in lymphatic HA turnover. Unlike HA binding receptors, LYVE-1 displays exquisite specificity for binding HA and no affinity for the glycosaminoglycans chondroitin-4 sulfate, chondroitin-6 sulfate, or heparan sulfate; therefore, neither glycosaminoglycan blocks binding uptake of HA by LYVE-1. The majority of HA turnover in tissue such as skin and digestive tract is known to occur in draining lymph nodes. This process is highly efficient, and studies have shown that some 80% or more of HA entering afferent lymphatics is degraded during passage through the nodes. But a recent study points to the oligomeric HA receptor "HARE" (HA receptor for endocytosis) (19) rather than LYVE-1 as the major receptor for HA uptake in lymph nodes.

Extracellular matrix HA is a large polymer that plays an important role in maintaining tissue integrity. It is reasonable to believe that this important function is dependent on rapid HA turnover through the efficient and constant transportation of lymphatic endothelium via LYVE-1 receptor. The distribution, function, as well as the contribution of LYVE-1 receptor in lymphedematous tissue and during lymphedema formation remain unknown.

Among cell surface receptors for HA, CD44 is widely expressed on epithelial, mesenchymal, and lymphoid cells (10). CD44-HA interactions are known to participate in a wide variety of cellular functions, including retention of pericellular matrix, internalization/degradation of HA, cell-cell aggregation, matrix-cell and cell-matrix signaling and cell migration (10). The selective antisense transgene suppression of CD44 in keratinocytes, achieved using a tissue-specific promoter strategy, resulted in an obvious skin phenotype not observed in the knockout mouse (20). The skin of the keratinocyte-deficient mice was highly hydrated and easily torn. Closer examination revealed an aberrant accumulation of hyaluronan in the epidermis, likely due to inhibited CD44 receptor-mediated endocytosis of HA (20). CD44 is mostly absent from lymphatic vessels and is thus unlikely to play a major role in lymphatic HA homeostasis (16,21-23).

The Possible Implications of HA Trafficking

The critical importance of HA in the body is underlined by the observation that deletion of HA synthesis in knockout mice results in early death of the embryo (24). Numerous studies, both old and recent, have supported the concept that HA plays a central role in the physical and chemical properties of the pericellular milieu as well as extracellular matrices. The network-forming, viscoelastic, and charge characteristics of HA are fundamental to the biomechanical properties and homeostasis of many tissues (13). The physiological function of tissue HA trafficking is unknown. The lack of optimal pH (3.7) for skin hyaluronidase to digest HA in the physiological extracellular environment gives little chance for the polymer to be digested *in situ* (1), and the breakdown products have additional properties including the capacity to stimulate chemokine release (25), induce nuclear factor (NF)-κB activation (26), and promote angiogenesis (27).

There is increased evidence that HA trafficking through interstitium to the lymph circulation is associated with important physiological events, and the removal of HA appears to be as important as its synthesis (12). When the balance between the synthesis, sizing, secretion, and removal of HA is broken, it may interfere with the functions of HA in development, morphogenesis, tissue homeostasis, and disease. Thus the impaired HA transportation due to lymphatic circulation failure may have a connection with the pathological phenomenon of chronic lymphedema. Lymphedema has long been considered as a high protein edema (28). The formation of edema has been attributed

largely to the accumulation of plasma protein in the tissue, therefore raising the osmotic (oncotic) pressure and absorption of water. However, this view seems not always to be supported by clinical observations (5,29). The protein concentration in the edematous tissue may even be lower than in the non-edematous tissue. It is suspected that there are other factors, too, accounting for holding water in the lymphedematous tissue. HA might be one of these factors.

Tissues enriched in HA have the tendency to trap water and swell (30). Increased HA content in the interstitium may accelerate edema formation and persistence. The osmotic pressure of a HA solution is strongly concentration dependent, and the polymer can therefore act as an osmotic buffer regulating the extracellular water content. Furthermore, it can be shown that a HA network exerts a high resistance towards water flow and that it therefore can form flow barriers in the tissue (31). HA affects microcirculatory exchange through its influence on interstitial volume exclusion, hydraulic conductivity, and diffusivity of macromolecules (32). Based on calculated lymph flow and lymph concentration of HA, there is a significant fraction of the total HA in skin (greater than 1%) removed via the lymphatics in a 24 h period (10). As such, increasing levels of HA deposition might underlie edema associated with lymphatic disorders.

The importance of HA to physiologic transport has been investigated *in vivo*. Increasing the HA in the perfusion solution into adjacent joint cavity of rabbit knee from 0.0 g/L to 0.5 g/L reduced the hydraulic conductivity governing trans-synovial flow by 39-64% (33). Raising the HA to the levels typically present in synovial fluid *in vivo* (3-6 g/L) resulted in a marked change in pressure-driven flow with a plateau of constant flow at pathologic pressures above 12 cm H_2O. It is hypothesized that the surface HA would raise the local osmotic pressure at the synovial surface, which would resist the flux of water. After treatment of the synovium with hyaluronidase, the hydraulic conductance increased fivefold. The addition of HA to peritoneal dialysis solution (34) could decrease the peritoneal fluid absorption rate, possibly through decreasing peritoneal tissue hydraulic conductivity, and a higher concentration of HA in dialysate resulted in a more marked decrease in peritoneal fluid absorption (absorption to peritoneal tissue as well as direct lymphatic absorption), possibly through both decreasing tissue hydraulic conductivity and increasing fluid viscosity. Based on the experimental demonstration of delayed clearance of HA after blockage of lymphatic circulation as well as the increased HA content in tissue fluid of lymphedematous patients, it is reasonable to believe that impaired HA traffic might to a large extent account for the persistent swelling in the tissue. Further investigations are needed to obtain direct evidence of the correlation of HA with the presence of edema.

The impairment of HA trafficking may also contribute to initiating inflammation in the edematous tissue. A prominent function of the lymphatic system is the provision of fluid drainage of immune cells and foreign antigens from the tissue to the peripheral lymph nodes, where the latter are sampled by professional antigen presenting cells and phagocytes (16). Lymph fluid contains high levels (40-50μg/ml) of HA (35), and its flux through the lymphatics is known to increase in inflammatory disease such as rheumatoid arthritis and psoriasis and in response to bacterial infection (36,37). The lymph vessels thus act as a conduit both for migrating inflammatory cells and for HA. This process may be fulfilled by the adhesion between CD44 receptor of inflammatory cells and LYVE-1 receptor on lymphatic endothelial cells mediated by HA (12). In conditions of lymph circulatory failure, not only the HA is stagnated in the interstitium, but the transport of bacteria, antigens and inflammatory cells is also impaired. The increased incidence of infection in lymphedematous tissue is a

well-known clinical phenomenon (38). Recently there is an increasing body of information on the important role of HA in inflammation. High levels of HA were found both locally and in blood during various other inflam-matory diseases (39). With the discovery of HA receptors, we have a much firmer basis for the concept that HA plays a role in regulating cellular activity, e.g., motility. HA thus is an active partner of inflammation. First, under the regulation of inflammatory cytokines, the inflammatory cells enhance their capacity to bind HA and express its receptors. Through the CD44/HA interaction with vascular endothelial cells, parenchymal cells and tissue substrates, HA mediates the extravasation of lymphocytes and macrophages from blood circulation and their migration into sites of inflammation (40-43). Secondly, through LYVE-1 mediated HA trafficking, it may regulate the entry of leukocytes into the lumen of afferent lymphatic capillaries. For example, in the skin, resident $CD44^+$ epidermal Langerhans cells are known to migrate to draining lymph nodes in response to proinflammatory cytokines and HA breakdown products produced during tissue inflammation (44). The initial entry of these cells into the lumen of lymphatic capillaries could conceivably be facilitated by interaction with LYVE-1. HA complexes on the abluminal surface of the endothelium or in overlapping cell junctions. Not only is HA important in the initial stages of leukocyte extravasation but its accumulation in the lesions may promote inflammatory cell retention as a substrate for those cells (30).

The importance of CD44-HA interaction in inflammation was demonstrated by the experiment (45) in which the use of anti-CD44 antibody induced a rapid loss of CD44 from both leukocytes and synovial cells and displayed an inhibitory effect on cell-extracellular matrix interactions in synovium in murine arthritis. As a result, the administration of such an antibody abrogated tissue swelling and leukocyte infiltration, two major components of inflammation (45). Current therapies for chronic inflammatory disease typically act through the nonspecific down-regulation of immune cell activation (46). The correlation of HA and CD44 in inflammation was shown by the study (47) in which topical hyaluronidase decreased both HA and CD44 expression in a dose-dependent manner in both the epidermal culture system and in human skin. The expression of genes for pro-inflammatory cytokines in the edematous tissue was found to be higher in the untreated lymphedema patients than after complex decongestive physiotherapy (48), including interferon-gamma receptor (IFN gamma R), tumor necrosis factor receptor-alpha (TNF-alpha), integrin alpha 4 beta 1 (VLA-4), tumor necrosis factor receptor p55 (TNFR1) and CD44. Together, the stagnation of HA as well as bacterial and inflammatory cells may facilitate the persistence of inflammatory events in the lymphedematous tissue. The mechanisms of CD44-HA interactions in inflammation might provide potential targets for therapy (46,49).

The third possible implication of impairment of HA traffic in tissue may be its influence on the metabolism of collagen and the promotion of tissue fibrosis. Previously, thickening of lymphedema skin was thought to bear a close relationship with repeated cellulitis. Whereas the development of fibrosclerosis parallels the incidence of mild infection, infection is not the only factor that may influence tissue fibrosclerosis. As a large hydrophilic molecule, HA is known to modulate the extracellular packaging of collagen and fibrin, leading to increased fiber size and porosity of extracellular substrates (50). The correlation of HA content and skin fibrosis has been in a disorder of HA metabolism associated with generalized folding and thickening of the skin (51). The activity of HA synthase in cultured dermal fibroblasts was increased, and biopsy specimens of the skin displayed extreme cutaneous thickening and folding as well as gross accumulation of HA.

Significant advances have been made in

the last decade in our understanding of the biology of HA. However, this knowledge is far from complete. Nevertheless, this amazing polysaccharide should not be neglected as we explore the complex mechanisms underlying lymphedema and develop new therapeutic approaches to the disease.

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