Hyaluronan in intestinal homeostasis and inflammation: implications for fibrosis

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de la Motte CA. Hyaluronan in intestinal homeostasis and inflammation: implications for fibrosis. *Am J Physiol Gastrointest Liver Physiol* 301: G945–G949, 2011. First published August 18, 2011; doi:10.1152/ajpgi.00063.2011.—The causes of fibrosis, or the inappropriate wound healing, that follows chronic intestinal inflammation are not well defined and likely involve the contributions of multiple cellular mechanisms. As other articles in this series confirm, inflammatory cytokines clearly play a role in driving cell differentiation to the myofibroblast phenotype, promoting proliferation and extracellular matrix deposition that are characteristic of fibrotic tissue. However, controlling the balance of cytokines produced and process of myofibroblast differentiation appears to be more complex. This review considers ways in which hyaluronan, an extracellular matrix component that is remodeled during the progression of colitis, may provide indirect as well as direct cues that influence the balancing act of intestinal wound healing.

mesenchymal cells; colitis; extracellular matrix

CHRONIC INFLAMMATION IS WIDELY considered a major predisposing factor to fibrosis in all organs including the intestine (22) and fosters the accumulation of myofibroblasts and extracellular matrix (ECM) in tissue culminating in impaired organ function. This review outlines a novel pathway to chronic inflammation of the intestine that is mediated by alterations of the pivotal ECM component hyaluronan (HA) and discusses the indirect and direct effects HA may have on fibrosis.

HA and Components of the System

HA is a natural and abundant component of the ECM of most tissues including the intestine, where it serves a function in maintaining structure, flexibility, and the hydrated environment of tissue. Under conditions of stress, HA is produced and organized into structures that act as leukocyte adhesion molecules (5, 10, 12, 14, 24) and contribute to inflammation in a variety of organs (depicted in Fig. 1*B*). During the past decade we have come to appreciate that HA production in the intestine may also be an innate response mechanism aimed at maintaining tissue homeostasis during times of injury or microbial challenge. Data from our laboratory provide evidence that HA plays a part in the initiation of intestinal inflammation (4, 5, 10), and we have proposed a mechanism whereby HA contributes to chronic inflammation in inflammatory bowel diseases (IBD) (6).

A variety of cell types including structural cells such as smooth muscle and fibroblasts, as well as barrier-forming epithelial and endothelial cells, produce HA (5, 10, 24). HA is composed solely of repeating disaccharides of *N*-acetylglucosamine and glucuronic acid and forms a nonbranched polymer.

HA does not contain protein, nor is it naturally sulfated, nitrated, or chemically substituted in any other way (3). Polymers of HA normally present in the body in sites such as the joints and skin reach a molecular weight of 10^7 Da. Since protein synthesis is not required, HA production proceeds rapidly on cell surfaces by the action of one or more of three synthetic enzymes, the HA synthases (HAS1, HAS2, HAS3), embedded in cell membranes (40) as shown schematically in Fig. 1*A*. The importance of HA in biology is underscored by the facts that *1*) the HAS2 mutation is embryonically lethal and *2*) there are multiple receptors that recognize HA and signal different functions.

Known Roles of HA and the Associated Receptors and Enzymes in the Intestine During Homeostasis and Inflammation

Four receptors that signal upon HA interaction have been well described (9, 21, 26, 30), and their cellular distribution and roles are illustrated in Fig. 1*D*. CD44 is the most well-known and characterized HA receptor and is present on most cells including leukocytes. CD44 mediates leukocyte binding to HA and contributes to inflammatory leukocyte infiltration into tissue (17, 27). CD44 is also responsible for cellular HA turnover and maintaining ECM integrity. Cells internalize HA bound to surface CD44 into endocytic vesicles to degrade and recycle the sugar components. The macrophage, using this CD44 process, has been shown to be pivotal in clearing the inflammatory HA matrix and resolving inflammation (35). RHAMM (receptor for HA-mediated motility) is an important receptor that mediates cell migration and proliferation, but is not specifically associated with inflammatory events. The Toll-like receptors (TLR) 4 and 2 are increasingly recognized as receptors for cleaved HA matrix fragments. TLR4 and TLR2 signal inflammatory cytokine and chemokine production, ster-

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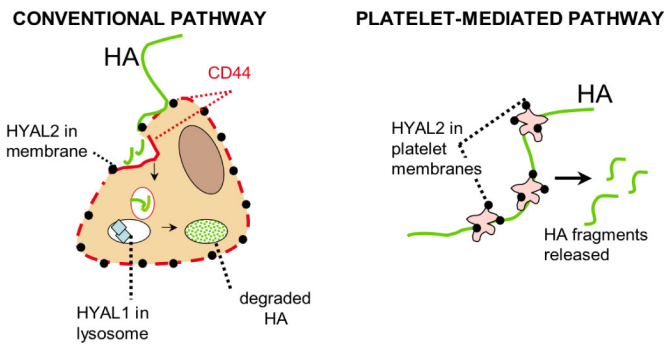
A Synthesis

B Matrix assembly

HOMEOSTATIC ECM PRODUCTION

LEUKOCYTE ADHESIVE CABLE FORMATION
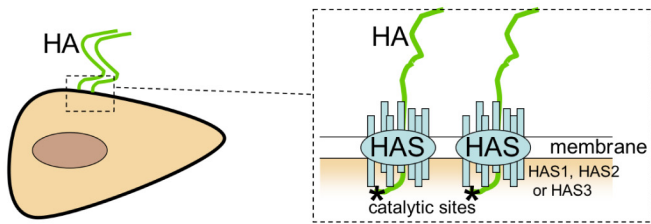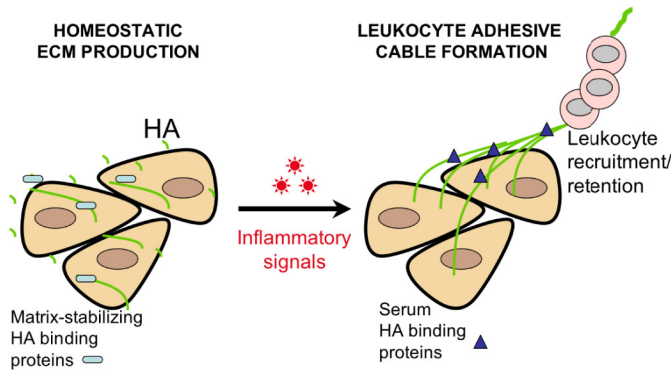
C Cell mediated catabolism

CONVENTIONAL PATHWAY

PLATELET-MEDIATED PATHWAY
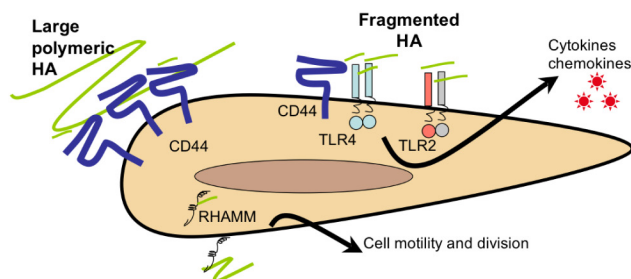
D Cellular recognition

Fig. 1. Metabolism and recognition of hyaluronan (HA) by cells. Diagrammatic representation of the mechanisms through which HA is made (*A*) and incorporated into normal and pathological matrices (*B*) as well as degraded (*C*) and recognized (*D*) during the processes associated with wound healing and inflammation. HAS, HA synthase; HYAL, hyaluronidase; RHAMM, receptor for HA-mediated motility.

cytokine release and inflammation in the absence of infection and overt tissue damage.

Multiple inflammatory cytokines promote different aspects of fibrosis including myofibroblast differentiation and proliferation, as well as excessive deposition of components such as collagen I and fibronectin. How ECM components themselves impact the fibrotic process is much less appreciated. HA, in its fragmented form, has the ability to induce proinflammatory and profibrotic cytokines (32) such as IL-1β (36, 42), TNF-α (2, 18, 36), IL-12 (8, 36), MIP-1α (15, 23), IL-6 (6, 25), and IL-8 (6, 33). HA, in its large polymeric form, also contributes to leukocyte recruitment from the circulation (10, 17, 27). Therefore, in multiple ways, HA may serve an indirect role in fibrosis.

For many years investigators have appreciated that increased matrix HA was associated with tissue inflammation, notably in the liver (20). Clinical tests were developed that measure circulating HA to detect and predict severity in hepatic diseases (37). Increased matrix HA has also been observed in samples of inflamed tissue from patients and animal models including lung, skin, kidney, and, as our group has demonstrated, the colon of IBD patients and mice with colitis (5, 10). However, the mechanistic role of HA in these diverse conditions is only starting to be defined. This review highlights three cellular mechanisms involving mesenchymal cells (4, 5), endothelial cells (10) and platelets (6), through which HA can impact the intestinal inflammatory process, and hence downstream pathologies such as fibrosis and tissue remodeling that leads to loss of function.

Histological comparisons of human noninflamed and inflamed colon tissue (Fig. 2) highlight typical, yet striking, changes that include *1*) increased influx of leukocytes and *2*) expansion and disorganization of the muscularis mucosae smooth muscle cells, along with *3*) epithelial loss and crypt destruction. Cytokines produced by inflammatory leukocytes, such as IL-1β, TNF-α, IL-12, and IL-6, are known to influence the behavior of intestinal mesenchymal cells, including smooth
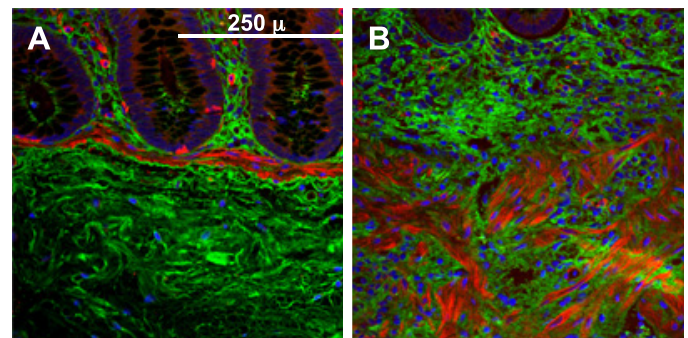

Fig. 2. Muscularis mucosae mesenchymal cells (red) and HA (green) are fluorescently detected in confocal micrographs of representative colon sections. *A*: in noninflamed control colon samples, the mesenchymal cells form a thin layer beneath the colonic crypts, where HA is present in a delicate reticulated network. In contrast, in inflamed colons of some Crohn's disease patients (*B*), the muscularis layer is greatly expanded and disorganized. Dense deposits of HA lie beneath the remaining epithelium, and between the mesenchymal cells. The increased number of blue [4,6-diamidino-2-phenylindole (DAPI)]-stained nuclei embedded in the HA in *B* is indicative of the inflammatory infiltrate. Leukocytes and HA in close proximity to the muscularis cells likely provide signals that mediate hyperplasia. (Mesenchymal cells are immunostained with an antibody that recognizes anti-TNF-induced protein 6.)

ile inflammation, and angiogenesis (21, 33, 34). The term "endogenous danger signal" is now commonly used to describe the group of host-origin triggers, many of which are ECM fragments including the prototypical HA (23), which mediate

muscle cells (SMCs). In light of the histological observation, we questioned whether the cell-cell interaction was bidirectional and, therefore, SMCs were actually contributing to inflammation. Our initial work focused on determining whether the smooth muscle cells played a role in leukocyte recruitment during the development of inflammation. Virus infection, double-stranded RNA (viral mimetic, poly I:C), and other agents that induce endoplasmic reticulum stress caused SMCs to be adhesive for mononuclear leukocytes (monocytes, T cells, B cells) (4, 5, 14). HA was determined to be the SMC-produced adhesion molecule, and binding leukocytes interacted with HA via their CD44 receptors (4). Using specific histochemical detection and confocal fluorescence microscopy, we were able to see large HA structures and show that CD44-positive leukocytes engaged with these structures, providing evidence that SMCs were capable of influencing recruitment or retention of inflammatory cells in tissue. Staining of inflamed intestinal tissue from IBD patients confirms that a HA-rich ECM forms around the SMC and throughout the inflamed mucosa (Fig. 2) and that leukocytes are in intimate contact with HA (5).

To help determine whether the HA produced in inflamed tissue was a contributor to or an effect of inflammation, we tracked progression of ECM modifications in a mouse model of colitis. Oral administration of dextran sodium sulfate (DSS) (2.5% in drinking water) induces colitis in conventionally housed C57/BL6 mice over a 2-wk time course, after which time animals are euthanized because of severe weight loss. We have observed the normally delicate HA matrix at the base crypts in the distal colon of untreated mice reorganizes into a dense deposit of HA in the colonic submucosa of mice treated with DSS for 7 days (10). Importantly, the HA deposition precedes leukocyte infiltration indicating that HA is not the consequence of inflammation, but rather promoter of the process. Additionally, through these studies we observed that HA was present within blood vessels, a point that was originally overlooked in human tissue because of the very abundant HA staining of the inflamed colon mucosa and submucosa in IBD patients.

Interestingly, cultured human intestinal microvessel endothelial cells (HIMEC) upregulate leukocyte adhesive HA in response to TNF-α, which is in contrast to SMC HA production (4). This differential expression is likely a result of expression of different HA synthetic enzyme expression; HIMEC upregulate HAS3 enzyme gene expression in response to TNF-α, whereas SMCs increase HAS2 expression in response to stress response activating agents, but not TNF-α (C. de la Motte and S. A. Strong, unpublished data). Interestingly, Crohn's disease and ulcerative colitis patient HIMEC produce more leukocyte adhesive HA after TNF-α treatment, compared with non-IBD controls (10). Endothelial cells are the gatekeepers of leukocyte extravasation from the blood into tissue and express many leukocyte adhesion molecules such as E-selectin, VCAM-1, and ICAM-1. In vitro studies show that TNF-α-induced HIMEC may bind up to a third of adhered mononuclear leukocytes via a HA-mediated mechanism (10), which is impressive considering the array of more traditional leukocyte adhesion molecules known to be expressed on TNF-activated endothelium, including HIMEC.

Accumulation of HA in the vasculature and the surrounding intestinal mucosa appears to promote inflammation. Although modulation of HA synthase expression frequently corresponds with the production of HA, the total deposition of HA in tissue reflects the balance between synthesis and breakdown. There are several mechanisms to degrade HA in tissue, including non-HA-specific mechanisms (e.g., reactive oxygen species release, glycosidase activity) and HA-specific mechanisms based on the action of hyaluronidase (HYAL) enzymes. There are two active somatically expressed enzymes that participate in HA catabolism, HYAL1 and HYAL2. The current HA catabolism model (31) suggests that HYAL2 on cell surfaces clips large pieces (>20 kDa) of HA from the matrix that are bound up by surface CD44 and internalized into endosomes. The endosomes join with HYAL1-containing lysosomes, and in this acidic environment HA is cleaved to two to six sugar (~400–1,200 Da) oligosaccharides. The cellular degradation pathway has definite implications for inflammation (35). Upon damage to tissue, a rapidly made HA "provisional matrix" is formed to protect tissue from dehydration and infection and to provide structure. The macrophage is responsible for removing the temporary HA matrix, and this then allows the permanent matrix to be deposited. If HA is not cleared by macrophages, inflammation does not resolve and tissue restoration does not occur, as has been demonstrated in mice lacking the CD44 receptor on their macrophages (35).

The distribution of hyaluronidases has been investigated in the DSS model of colitis (6). In inflamed colon, most cells are immunohistochemically positive for HYAL1 with the strongest staining in infiltrating leukocytes, whereas in control tissue, the HYAL1 staining appears most evident in epithelium (C. de la Motte, unpublished observations). The strongest HYAL2 staining in inflamed tissue was observed in platelets, yet some infiltrating leukocytes also expressed the enzyme. Normal circulating human platelets also contain HYAL2, without any apparent HYAL1, as detected by histology, immunoblot analysis, and RNA transcript analysis. Although the optimal pH of HYAL2 activity is under debate [that is, some investigators think the enzyme only works under acidic conditions, whereas others have demonstrated HYAL2 activity at neutral pH when the enzyme works with CD44 (7)], we have clearly observed that platelets actively cleave HA from the surface of activated endothelium under the pH of cell culture conditions. In contrast to the HA catabolism model (31) (Fig. 1C) used for HA clearance by macrophages, platelets do not internalize the cleaved HA fragments but leave them in the external environment. This is potentially important because HA fragments serve as endogenous danger signals and promote innate immunity.

Distinct Roles of Large HA Polymers vs. Small Fragments

HA in the native ECM exists in large polymeric form that does not promote inflammation or angiogenesis. However, in fragmented/depolymerized form, HA signals many inflammatory and proangiogenic responses by leukocytes, endothelium, and mesenchymal cells, most often through TLR4 and TLR2 signaling (19, 32). HA in the matrix therefore acts as a sentinel of tissue damage and drives necessary immune and reparative responses theoretically in proportion to the magnitude of damage. Such a mechanism may be operative in the microvasculature of tissues as well. Platelet-cleaved HA fragments derived from activated endothelium have been shown to activate mono-

cytes to produce proinflammatory cytokines, such as IL-6 and IL-8 (6). If the leukocytes have migrated into tissues, the subsequent cytokine release may influence mesenchymal cell activation and fibrosis; if the cytokines are released intravascularly they could lead to further endothelial HA production, driving a cycle of inflammation that continues down the vessel (6). In IBD patients, two physiological differences may contribute to an accelerated inflammatory response: *1*) IBD patient HIMEC appear to produce more surface HA (substrate), which could generate more HA fragments to trigger inflammation; and *2*) IBD patients frequently have higher circulating levels of platelets that conceivably would create HA fragments at a higher rate than controls, thus contributing to disease.

Evidence for a Role in Fibrosis

HA may also directly affect the process of fibrosis. Fragmented HA promotes wound healing, an observation that has been appreciated for over 25 years (1, 41). These data suggest that HA potentially affects the dysregulated form of wound healing, fibrosis. Numerous studies have shown that large-molecular-weight HA inhibits fibroblast migration and proliferation whereas depolymerized small fragments of HA promote wound closure through effects on structural cells as well as by promoting angiogenesis. HA fragments may also drive cytokine release from fibroblasts themselves (11). A recent body of work from the laboratories of Steadman and Phillips (16, 28, 29, 38, 39) defines a multistep pathway of fibrosis/wound healing and demonstrates that HA is essential to TGF-β-induced myofibroblast differentiation, an early step in the fibrotic process. Very recently, Li et al. (13) demonstrated using an in vivo lung injury model that targeted HAS2 overexpression in myofibroblasts promotes fibrosis through a CD44-mediated mechanism, compelling evidence supporting a direct role of HA in contributing to the pathological processes.

Future Directions and Challenges

The causal relationship of HA to intestinal fibrosis is still circumstantial. This article presents opportunities whereby HA could affect the process by promoting inflammation and cytokine release, as well as by affecting myofibroblast differentiation and proliferation. Numerous parameters still need to be defined with respect to the size and concentration of HA fragments necessary to mediate the cellular effects. Understanding the mechanisms whereby the fragments are generated, as well as how they are recognized, remains elusive. Fortunately, highly purified, endotoxin-free, specific-size HA fragments are now commercially available to help us define the size requirements for HA-mediated effects on the fibrotic processes. Biochemical assays are being fine tuned to aid in determining HA-fragment size from tissue samples. Additionally, animals with gene deletions for *1*) the HA synthases (has1-, has3-, conditional has2-null mice), *2*) the major somatic hyaluronidases (hyal1, hyal2), and *3*) the HA receptors (CD44, TLR4, TLR2) are now available. These models will be important tools for gaining an understanding of the in vivo role of each of the elements of the HA-mediated inflammatory and fibrotic pathways at work in colitis.

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