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The Role of Hyaluronan and the Extracellular Matrix in Islet Inflammation and Immune Regulation

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Abstract

Type 1 diabetes (T1D) is a disease that in most individuals results from autoimmune attack of a single tissue type, the pancreatic islet. A fundamental, unanswered question in T1D pathogenesis is how the islet tissue environment influences immune regulation. This crosstalk is likely to be communicated through the extracellular matrix (ECM). Here, we review what is known about the ECM in insulinitis and examine how the tissue environment is synchronized with immune regulation. In particular, we focus on the role of hyaluronan (HA) and its interactions with Foxp3+ regulatory T-cells (Treg). We propose that HA is a “keystone molecule” in the inflammatory milieu and that HA, together with its associated binding proteins and receptors, is an appropriate point of entry for investigations into how ECM influences immune regulation in the islet.

Keywords

Hyaluronan; TSG-6; CD44; TLR2; TLR4; Toll-like receptors; Foxp3; regulatory T-cells; TR1; Treg; IL-10; TGF-beta; extracellular matrix; diabetes; islets; Islet inflammation; Immune regulation

Introduction

Type 1 diabetes (T1D) is a disease which results from autoimmune attack of the pancreatic islet. Most current models of T1D pathogenesis begin with an immune response to infections, toxins or other islet-tropic insults that is initially normal but subsequently cascades into autoimmune-mediated destruction of β -cells. These models emphasize critical roles first for islet injury and then subsequently for immune dysregulation in diabetes pathogenesis (1).

While the populations of regulatory T-cells that mediate immune tolerance are the focus of intense investigation, the influence of the inflammatory milieu within which they function remains poorly understood. A central, unanswered question in T1D pathogenesis is therefore how the islet tissue environment participates in immune regulation.

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The crosstalk between the inflammatory milieu and the immune response during insulinitis is likely to be communicated in large part through the extracellular matrix (ECM). Here, we examine how changes in the ECM that follow injury are synchronized with immune regulation and review what is known about the ECM in inflamed islets. In particular, we focus on the role of hyaluronan (HA), a long polysaccharide that is a prominent in inflamed tissues, and its binding partners. We propose that HA is a “keystone molecule” in the inflammatory milieu and an appropriate point of entry for investigations of how the ECM influences immune regulation in T1D.

Hyaluronan is a keystone molecule in the inflammatory ECM

A basic challenge in approaching the ECM (or other similarly complex systems) is identifying what is important. The ECM is a dynamic, highly interdependent network of hundreds of molecules that challenges reductive analyses. Conceptually, a schematic of this network might resemble a web similar to those used by ecologists to map the complex interactions between species in a forest (2).

While characterizing complex ecosystems, ecologists realized that certain nodes within such interdependent networks tend to have disproportionate effects on the composition and health of the ecosystem. They coined the term “keystone species” to describe these organisms. To borrow this concept, we propose that HA, together with its receptors and binding partners, is a “keystone molecule” in the inflammatory milieu. By this we mean that HA is at the center of a complex network of ECM molecules that together exert decisive effects on the nature of inflammation.

Hyaluronan has several unique features that qualify it as a keystone molecule in the inflammatory response:

1. First, HA is known to play important roles in most aspects of the tissue response to injury, including wound healing, angiogenesis, cell trafficking, and proliferation (3).
2. Second, HA physically interacts with a large and diverse network of ECM molecules present in inflamed tissues. These include HA binding molecules called hyaladherins such as tumor necrosis factor-stimulated gene (TSG-6), versican, Inter- α -inhibitor (I α I), CD38 and others (4). HA binding interactions with its receptor CD44 are also impacted by other ECM molecules, including osteopontin and fibronectin.
3. Third, HA is highly abundant in inflamed tissue and its production can increase as much as 200 fold following injury (5).
4. Fourth, fluid shifts caused by HA are responsible for many of the physiologic changes associated with inflammation. Because the repeating disaccharides of *N* acetyl glucosamine and glucuronic acid that make up HA carry strong negative charges, HA synthesis generates oncotic forces that result in edema, vascular permeability changes, and leukocyte egress at sites of injury (6). Studies utilizing magnetic resonance imaging (MRI) to detect edema associated with islet inflammation (7) may well be tracking the effects of HA.
5. Fifth, the length of HA strands in the ECM is a sensitive barometer of the inflammatory milieu. Uninjured tissues are characterized by modest amounts of high molecular weight (>1000 kDa) HA (HMW-HA). Following tissue injury, there is an accumulation of low molecular weight (<250 kDa) HA (LMW-HA) and short (<30 kDa) HA oligomers (sHA) generated through enzymatic degradation of HMW-HA by endogenous hyaluronidases as well as catabolism by a diverse group

of microbial hyaluronidases, mechanical forces, and oxidative stress (8). Normally, LMW-HA is cleared within 14 days of injury and enhanced HA production ceases as collagen production increases in conjunction with wound healing. However, chronic wounds are characterized by large amounts of LMW-HA. Both the size and the amount of HA in the tissue environment are therefore tightly linked to the stage of an injury response and its resolution (9;10).

We find that HA is present in the peri-islet ECM of healthy mouse islets, and is best visualized following Carnoy's (acid, ethanol and chloroform) fixation ((Unpublished data – manuscript in review) and Fig. 1A). In addition to peri-capsular HA, normal islets contain a number of hyaladherins. We find that versican, TSG-6, I- α -I, and bikunin, are normally produced in the mouse pancreatic islet. Immunocytochemistry and mRNA analysis demonstrated that hyaluronan synthase 3 (Has3), versican, and bikunin are produced in α -cells, while Has1, Has3, TSG-6, ITIH1/ITIH2, and bikunin are produced in β -cells (Unpublished data – manuscript in review)

LMW-HA is an Endogenous Danger Signal

HMW-HA and LMW-HA have highly divergent effects on the behavior of lymphocytes and stromal cells. These effects are mediated through pattern recognition pathways.

LMW-HA functions as a Damage-Associated Molecular Pattern molecule (DAMP) and as an endogenous “danger signal” (11). DAMPs are host-derived molecular indicators of tissue stress and catabolism that promote inflammation in the setting of sterile injury. DAMPs and the pattern recognition receptors (PRR) with which they interact integrate the tissue environment with an appropriate immune response (12). LMW-HA promotes the activation and maturation of dendritic cells, the release of pro-inflammatory cytokines such as IL-1 β , TNF- α , IL-6 and IL-12, drives cell migration, promotes proliferation by numerous cell types, and promotes the activity of matrix metalloproteases (10;11). In addition to driving inflammation, LMW-HA plays a vital role in promoting angiogenesis and tissue regeneration in normal wound healing. In chronic wounds, however, LMW-HA is linked to prolonged inflammation and fibrosis (3).

Many of these pro-inflammatory effects of LMW-HA are mediated through interactions with the pattern-recognition receptors TLR2 or TLR4 (13–16). LMW-HA also upregulates negative regulators of TLR signaling, including IL-1R-associated kinase-M (17). While HA molecules of all sizes share the same repeating disaccharide structure, only LMW-HA can signal through TLR and function as a DAMP at sites of active inflammation (13;18). TLR signaling may also be relevant to islet homeostasis (19). Other HA receptors, including RHAMM and CD44, may contribute to LMW-HA effects that are atypical for TLR agonists, such as angiogenesis (20).

HMW-HA Provides Tissue Integrity Cues

High molecular weight HA (HMW-HA) predominates in uninflamed tissues (21;22) and is anti-inflammatory in a variety of *in vitro* and *in vivo* systems. HMW-HA is reported to impair phagocytosis by macrophages, diminish production of inflammatory cytokines by monocytes and other cell types, limit oxidative damage, and apoptosis (23–26). HMW-HA administration is similarly anti-inflammatory in a variety of *in vivo* systems including bleomycin-induced lung toxicity (14) and collagen-induced arthritis (27). In contrast, total inhibition of HA production using 4-methylumbelliferone was associated with heightened atherosclerosis in a mouse model of vascular disease (28).

Most of the anti-inflammatory functions attributed to HMW-HA are associated with CD44, the primary cell surface HA receptor. This has been demonstrated using blocking antibodies, siRNA, and in studies with CD44^{-/-} mice (29;30). Despite known roles for CD44 in cell activation, survival, and migration, indications are that CD44 is important in the resolution, rather than the propagation of inflammation. Indeed, CD44^{-/-} mice have an impaired ability to resolve inflammation and defects in wound healing in multiple model systems (18;31–37).

The anti-inflammatory properties of HMW-HA are dependent on its length and ability to crosslink multiple CD44 molecules on the cell surface. HA binding to CD44 is mediated by low-affinity hydrogen bonds such that interactions with multiple receptors are required for efficient binding and downstream signaling (38). The ability of HMW-HA to crosslink spatially isolated CD44 molecules and the inability of LMW-HA to do so may explain how a chemically homogenous molecule such as HA can produce such different effects based on its size. Consistent with this model, anti-inflammatory effects attributed to HMW-HA to multiple systems can be recapitulated using antibody-mediated CD44 crosslinking (29;39–43).

We have proposed that CD44 crosslinking functions as a form of pattern recognition that discriminates between active inflammation, characterized by LMW-HA, and healing tissue, characterized by HMW-HA (39). This model does not exclude other roles for CD44 but would attribute the homeostatic and anti-inflammatory properties of HMW-HA to CD44 crosslinking.

It is unclear how CD44 crosslinking communicates anti-inflammatory signals. One possibility is that the ability of CD44 crosslinking potentiates low-level TCR signaling that contributes to the induction and function of regulatory T-cell populations (44–48). Another possibility is that CD44 crosslinking induces anti-inflammatory cytokines, including IL-10 (29;40) and TGF- β (29;49;50). CD44 may also negate pro-inflammatory signals communicated through TLR signaling and NF κ B translocation (30;51).

The Importance of Hyaladherins and HA superstructures

HA can form a variety of higher order structures that impact its interactions with leukocytes. These structures influence the longevity, viscosity, and other physical properties of HA and also confer specific leukocyte-binding properties not attributed to free HA (4).

The nature of these higher order HA structures is determined by interactions with a diverse group of HA-binding proteins, known as hyaladherins. Hyaladherins may be just as important as HA size in coordinating the tissue environment with the immune response (4;52).

An example of a hyaladherin with established roles in preventing HA degradation and in crosslinking CD44 is TSG-6, a secreted protein which covalently links HA strands to inter- α -inhibitor (I α I) present in serum, forming complex, crosslinked HA networks. Such structures form the basis of provisional wound matrix, the scaffold that is a crucial early component of healing tissue. In addition to generating HA networks, TSG-6 also inhibits enzymes involved in ECM catabolism, including hyaluronidases (53). While we find that TSG-6 is present in healthy islets from C57Bl/6 and Balb/c mice (unpublished data), TSG-6 was not seen in islets at baseline from NOD mice, suggesting there may be strain-specific differences in expression. In that study TSG-6 was expressed by infiltrating leukocytes in 8 week old pre-diabetic NOD mice and, less robustly, in non-diabetic 25 wk old mice; future studies are needed to clarify the role of this molecule in T1D (54). Supplementation with

TS6-6 to promote HA integrity is an experimental strategy for immunotherapy with known benefit in autoimmune arthritis and other inflammatory settings (55).

Other hyaladherins inhibit HA-CD44 interactions. Versican is a pro-inflammatory hyaladherin (56) that inhibits interactions between HA and CD44 (57). We find that versican treatment also abrogates HMW-HA mediated IL-10 production (unpublished data), suggesting that the pro-inflammatory properties of versican may result from inhibition of HA interactions.

The pro-inflammatory effects of HA in poorly controlled diabetes illustrates why HA organization matters. Hyperglycemia drives overproduction of HA and this contributes to accelerated atherosclerosis through effects on vascular smooth muscle cells (58). Similarly, HA in the eye is depolymerized due to the effect of free radicals and advanced glycation end products and this may contribute to vitreous body liquefaction and proliferative retinopathy in diabetes (59). HA that is unincorporated into structures such as basement membranes or provisional wound matrix is subject to degradation and generation of pro-inflammatory LMW-HA (60). It is likely that within islets the organization of HA, as well as its amount and size, are important factors that influence HA function.

HMW-HA and CD44 in Regulatory T-cell Function and Stability

In healthy individuals, the development of T1D is prevented by populations of FoxP3+ regulatory T-cells (Treg), a specialized subpopulation of CD4+T-cells that maintain immune homeostasis (61). However, despite much data implicating Treg in the development of T1D, global defects in immune regulation have proved elusive. Most studies report equivalent function *in vitro* and comparable numbers of circulating Treg between T1D subjects and controls, although there are indications that Treg numbers may be diminished in intestinal lymphoid tissue and in islets in T1D (62;63). Most significantly, therapies designed to boost systemic Treg numbers have yet to result in long term prevention of T1D.

One possible explanation for the conflicting data on Treg in T1D is that Treg are inherently defective *in vivo* in ways that are not readily apparent *in vitro*. In support of this, FoxP3+ T-cells isolated from inflamed islets and other sites of immune destruction function well *in vitro* but do not prevent autoimmunity *in vivo* (64;65). Defects in IL-2 production (66) or IL-2R signaling (67) are examples of heritable factors that may not be obvious under culture conditions but could impact Treg stability and function once in the setting of inflamed islets.

Another, non-mutually exclusive explanation is that the tissue environment is altered in T1D in ways that promote immune dysregulation. The tissue environment is known to affect Treg function in ways that may not be evident *in vitro*. Illustrating this, in healthy animals, Treg are anergic (non-proliferative) in culture while *in vivo* their proliferation rate is high (68). The inflammatory milieu is shaped by a myriad of host and environmental factors and might thereby promote immune dysregulation in genetically susceptible individuals. Examples of environmental factors that can suspend the suppressive function of Treg are microbial ligands of Toll-like receptor 2 (TLR2) (69).

We and others have reported that HMW-HA promotes the function and stability of Foxp3+ Treg. Firan et al. (70) were the first group to demonstrate an association between HMW-HA binding and Foxp3 expression. They demonstrated that CD4+CD25+ T-cells selected by HMW-HA binding had superior suppressive function, suggesting that HMW-HA binding plays a role in promoting Treg cell functions (70). We subsequently reported that HMW-HA enhanced the function and viability of Treg, particularly in the setting of low IL-2 (39;71). Moreover, HMWHA promotes Treg function via increased Foxp3 levels and production of IL-10 (29). Notably, expression by Treg of CD44 variant isoforms that bind HMW-HA is

associated with high levels of Foxp3 expression (36). Liu et al. further demonstrated that expression levels of CD44 are correlated with Treg suppressive function and production of IL-10 (72).

A second population of regulatory T-cells that respond to HMW-HA are TR1. These are CD4⁺FoxP3⁻ regulatory T-cells that mediate immune tolerance to self and foreign antigens via production of prodigious amounts of IL-10. We recently reported that intact HA, in the context of an antigenic signal through the T-cell receptor (TCR), induces conventional T-cells to become TR1 regulatory cells. Moreover, this treatment engendered a regulatory phenotype in these cells which persisted even after they were withdrawn from contact with HA such that they could be transferred into an *in vivo* mouse model of colitis to prevent disease (40). Consistent with a role for HMW-HA in IL-10 production, HMW-HA was found to promote elevated IL-10 levels in intestinal biopsies upon oral administration (73) and production of IL-10 by synoviocytes in culture (74).

A third population of regulatory T-cells reported to be responsive to HMW-HA are NKT cells. NKT cells have significant immunoregulatory activity due to their rapid secretion of large quantities of cytokines following CD1d-dependent stimulation. Stimulation of CD44 through Ab cross-linking or HMW-HA caused NKT cells to secrete cytokines, up-regulate activation markers and resist activation-induced cell death (75). Together with the data on Foxp3⁺ Treg and TR1, these data suggest that matrix integrity cues promote immune tolerance through effects on regulatory T- cells.

Hyaluronan and the Islet ECM in Autoimmune Diabetes

From *in vitro* studies and work on islet transplantation it is known that the islet ECM makes decisive contributions to insulin production, β -cell homeostasis and proliferation (19;76–81). As part of these studies, aspects of the islet ECM in uninflamed islets have been characterized; these data are the subject of several reviews (82–84). Substantial progress has also been made towards recapitulating such signals using artificial matrices in order to facilitate islet transplantation and differentiation from stem cells (78). As examples, fibronectin and other RGD-containing components are incorporated into artificial matrices to promote integrin-mediated effects on insulin production (85) while collagen scaffolds have been used to mimic the islet-basement membrane interactions that are critical to islet survival (76;86). In contrast to this progress in understanding the ECM in healthy islets, the nature of the ECM in insulinitis is mostly unknown.

One ECM structure which has received substantial attention is the peri-islet ECM. In mice, this has been proposed to function as a physical barrier to leucocyte migration into islets and to thereby maintain immune privilege (84). Degradation of this ECM marks the onset of destructive autoimmune insulinitis in NOD mice, although in humans this same pattern has not been observed, to our knowledge. The ECM surrounding mouse islets is a basement membrane comprised of laminin, collagen (84). Heparan sulfate (HS) is also present and has been proposed to play a role in prevention of islet damage (87). Autoimmune insulinitis in NOD mice is associated with remodeling or destruction of the ECM surrounding islets (84) suggesting the presence of heparanase (which degrades HS) (88) and metalloproteinases (which break down collagen) perhaps secreted by populations of macrophages. In particular the loss of HS containing structures during insulinitis has been proposed to be important in the progression to diabetes perhaps through loss of protection from oxidative damage (88) or via loss of ECM interactions that make critical contributions to β -cell survival and proliferation (19;76–81). However, in humans the peri-islet ECM is different from that in mice (89) and its role in T1D pathogenesis remains to be defined.

HA is known to colocalize with infiltrating lymphocytes in the insulitis seen in NOD mice (90). We have observed similar accumulations in both NOD mice (Fig. 1B) and in another autoimmune model, the DORMO mouse (unpublished data). However, a completely different pattern was visualized in diabetic mice 5 days after treatment with 200 mg/kg STZ where only modest amounts of HA were seen in association with minimal infiltrates (Fig. 1C). A similar pattern of minimal HA was seen in mice treated with a sub-diabetogenic low-dose STZ regimen (50 mg/kg $\times$ 3 day) and euthanized at weekly intervals over a month (data not shown). These data suggest that different patterns of HA accumulation are associated with different forms of islet damage and that STZ treatment is not equivalent to autoimmune insulitis in this regards. We are currently investigating whether this HA is HMW-HA or LMW-HA and whether HA accumulation drives lymphocyte infiltration or merely reflects its presence. Weiss et al. found that injections of hyaluronidase and anti-CD44 Ab 1 hour before cell transfer of diabetogenic splenocytes prevented the development of diabetes (90), but it was unclear whether this was through effects on T-cell trafficking, apoptotic killing of antibody –targeted cells, or via effects on the islet environment (90). Thus, much about HA, as with other aspects of the ECM in insulitis, remains to be explored.

A model for how chronic inflammation and HA fragmentation might contribute to immune dysregulation in T1D

Conceptual frameworks are needed to facilitate characterization of the ECM in normal insulitis as well as in T1D. To address this, we propose that autoimmune insulitis can be approached from the perspective of an injury response.

Injured tissues are known to progress through a series of sequential, highly choreographed changes that first contain and then resolve damage (91). The immune response to injury follows a parallel arc. In chronic wounds, however, the progression from inflammation to resolution is “stuck” and unable to progress to resolution. Increasingly, research into chronic inflammation and impaired wound healing is focused on the role of immune regulation (92).

Most current models of T1D pathogenesis begin with an injury response that is initially normal (1). The available data from NOD mice suggest that the initial inflammatory response to remodeling or injury is unremarkable (93;94). However, the subsequent progression through the stages of an injury response is delayed in autoimmunity in ways that are similar to a chronic wound. Autoimmune insulitis is typified by elevated levels of inflammatory cytokines and reactive oxygen species (95) an excess of proteolytic enzymes (96), and the prolonged presence of macrophages and monocytes for weeks after the onset of inflammation (93). In insulitis induced using low-dose STZ, by comparison, the presence of macrophages peaked between 5–10 days after injury and these cells were gone by day 18 (97).

Given the established role for HA size in synchronizing immune responses with the tissue environment in wounds, we hypothesize that HA size likewise links inflammation to Treg stability and function at sites of sterile injury (Fig. 2). We propose a model where in healing tissues a predominance of HMW-HA provides signals that promote the activity of regulatory T-cells. However, in chronic inflammation, a predominance of LMW-HA inhibits these effects and instead drives further inflammation. The local balance of LMW-HA vs. HMW-HA would thereby provide contextual cues that influence immune tolerance. In this model, factors that promote prolonged inflammation in the context of a predisposing genetic background could influence the progression to T1D. Carried forward, this model would suggest that it may be possible to promote immune tolerance by supporting the integrity of the ECM, for example via enhanced expression of TSG-6 or reduced HA catabolism. This would be a novel and exciting approach towards T1D prevention.

Moving Forward

The necessary tools and collaborations are in place to open up the study of ECM in autoimmune diabetes in the near future. We now have access to clinically relevant, human specimens. The JDRF nPOD (network for Pancreatic Organ Donors) program is a burgeoning, invaluable resource for obtaining appropriate samples. As this resource expands and includes more samples from individual donors with recent onset T1D, much more will be learned. The characterization of islet ECM during the progression of disease can be supplemented with work in the NOD mouse, the BB rat and other models (e.g. STZ treatment).

Comprehensive and integrated studies of islet ECM are slowly becoming possible. Gene expression arrays and proteomic tools have limited utility for studying the ECM given the prominence of carbohydrates and complex synthetic pathways. However, analogous tools for high throughput glycomics and glycoproteomics are now in development (98;99). Moreover, other tools, including siRNA and tissue specific knock-out mouse models, are increasingly available for use in ECM studies.

Finally, there is evidence of growing collaboration between immunologists, endocrinologists and matrix biologists (57;84;88;100). Traditionally, these groups of scientists have not interacted extensively but this is clearly changing and is critical for the advancement of work in this area.

Conclusions

The tissue environment is known to have decisive effects on Treg behavior and immune regulation. From transplant studies it is also known that the islet ECM plays critical roles in islet survival and function. However, it is unknown how the immune response and the islet ECM interact during autoimmune insulinitis and the extent to which these interactions contribute to the pathogenesis of T1D. The ECM influences the course of chronic inflammation in other tissues and we hypothesize that this is likely to be the case in insulinitis. We propose that the size of HA and signaling through pattern receptors may be one way by which the inflammatory milieu is synchronized with local immune responses. However, much remains to be learned about the ECM in autoimmune insulinitis, particularly in humans. The nature of ECM in insulinitis and its role in T1D pathogenesis are a frontier in autoimmune diabetes research.

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Figure 1. HA staining in islets in the setting of autoimmunity versus toxin mediated damage. Histology from Balb/c control mice (**A**) and NOD mice at 4 weeks (**B**) of age, the typical age of onset of insulitis. **C**. Histology from a Balb/c mouse one week following treatment with high dose (200 mg/kg $\times$ 1 day) STZ. (Figures courtesy of Matt Warren.)

Figure 2.

A model for how danger signals and tissue integrity cues communicated via HA size could influence immune regulation in autoimmune diabetes. Following islet injury due to a virus or toxin, inflammation ensues. In healthy individuals, this progresses through the stages of an injury response that culminates in the resolution of the injury. In such individuals, tolerance is maintained by HMW-HA together with hyaladherins such as TSG-6 that signal tissue integrity and promote Treg function. However, in autoimmune diabetes, inflammation persists and progresses to autoimmune insulinitis. In these individuals, persistent LMW-HA provides danger signals that suspend immune regulation. In this model, the size of HA provides contextual cues that exert decisive effects on the maintenance of immune tolerance and the development of T1D.