

Hyaluronan cross-linking: a protective mechanism in inflammation?

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Production of the glycosaminoglycan hyaluronan is increased at sites of inflammation, often correlating with the accumulation of leukocytes. Mounting evidence suggests that this polysaccharide can be organized into a wide variety of molecular architectures by its association with specific binding proteins, leading to the formation of fibrils and cable-like structures involving a large number of hyaluronan chains. We propose that hyaluronan cross-linking is part of a protective mechanism, promoting adhesion of leukocytes to the hyaluronan complexes rather than enabling contact with inflammation-promoting receptors on the underlying tissues. Leukocytes are thus maintained in a non-activated state by appropriate receptor clustering or receptor co-engagement. Additionally, hyaluronan networks might serve as scaffolds to prevent the loss of extracellular matrix components during inflammation and to sequester proinflammatory mediators.

Hyaluronan–protein complexes: new forms and functions

Hyaluronan (HA) is an ubiquitous polysaccharide with diverse biological roles, including acting as a crucial structural component of extracellular matrix and as an important mediator of leukocyte adhesion and migration [1,2]. Recently, the concept has emerged that the function of HA is dictated by the particular proteins associated with it (Box 1), where HA probably represents a versatile scaffold that can be used to build a variety of complex structures [3,4]. However, the full range of possible structures that can be formed by HA is only just beginning to be appreciated. In addition to the well-known aggregates of proteoglycans and proteins that form arrays along a single HA molecule, providing tissues, such as cartilage, with their load-bearing properties, HA chains can also be cross-linked to generate huge superstructures with different architectures and functional activities. For example, HA cross-linking is essential to stabilize the nascent ‘cumulus’ matrix that forms around the oocyte prior to ovulation and the same mechanisms are likely to occur at sites of inflammation, where HA synthesis is upregulated [5,6]. In arthritis, this might be protective, by altering the hydrodynamic properties of HA in synovial fluid and stabilizing cartilage, preventing the loss of

matrix components and perhaps facilitating repair. Additionally, massive cable-like structures or HA fibrils can be generated in response to various stimuli [e.g. inflammation, viral infection, endoplasmic reticulum (ER) stress and hyperglycaemia] and these have specific leukocyte-binding properties not attributed to free HA [7–10]. We propose that the presentation of HA in a cross-linked form leads to receptor clustering on leukocytes (or co-receptor engagement through the presence of accessory molecules on the cables) and that, although this is proadhesive, it is likely to be counter-inflammatory. Here, we describe what is known currently about the various mechanisms of HA cross-linking, as well as the structure and composition of the complexes that are formed. It is our opinion that HA cross-linking represents an important new pathway in the regulation of inflammatory processes.

Cross-linking of HA during inflammation-like and inflammatory processes

HA cross-linking in ovulation

Much of what we know regarding the mechanisms of HA cross-linking comes from recent studies on cumulus matrix expansion, which is necessary for successful ovulation and fertilization; ovulation has many things in common with inflammation and can be described as an inflammation-like process [11]. In the pre-ovulatory follicle a large, HA-rich, viscoelastic matrix forms rapidly around the oocyte, giving it protection during ovulation and facilitating sperm capture *in vivo*. Four proteins, namely inter- α -inhibitor (I α I), pre- α -inhibitor (P α I), pentraxin 3 (PTX3) and TSG-6 (35 kDa-secreted product of the tumour necrosis factor-stimulated gene-6), are involved in stabilizing this matrix through HA cross-linking (Figure 1). Indeed, deletion of the *bikunin* gene (NM_001633), which disrupts the biosynthesis of both I α I and P α I [5], leads to a defect in cumulus expansion resulting in female infertility [12]. It is well established that the heavy chains (HCs) of I α I and P α I become covalently attached to HA during cumulus expansion and that this modification is necessary for the structural integrity of the cumulus matrix (reviewed in Ref. [5]). The HA-binding protein TSG-6 (Figure 1b) has been found to have a crucial role in the formation of the HC–HA complexes (Figure 1c) because these were absent in the *Tsg-6*^{-/-} mouse, which is also infertile [13]. Recently, one mechanism by which HCs are transferred from the intact

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Available online 7 October 2005

Box 1. Basic concepts and theories: HA and its binding proteins

Hyaluronan is a glycosaminoglycan of high molecular mass (usually between 10^5 – 10^7 Da) comprised solely of a repeating disaccharide of glucuronic acid (GlcA) and *N*-acetyl-glucosamine (GlcNAc) that, unlike other glycosaminoglycans (e.g. CS and DS), is neither sulfated nor attached to a core protein (see Figure 1a for the chemical structure). The lack of any chemical variation in HA and its conformational flexibility make it a versatile molecule that can form a variety of periodic multi-molecular structures through its interaction with specific HA-binding proteins, known as hyaladherins [3,40]. It is thought that these capture, stabilize and propagate particular conformations of the polysaccharide leading to HA-protein complexes with different architectures and specific functional activities [3,4]. Most hyaladherins bind to HA through a common structural domain termed a Link module [4,40], including the inflammation-associated protein TSG-6 and CD44, the major cell-surface receptor for HA. The 3D structures for the TSG-6 and CD44 HA-binding domains are the only ones to have been determined to date [36,41], including the TSG-6 Link module in its HA-bound conformation, which has enabled the accurate modelling of a hyaladherin–HA complex [42]. Given that hyaladherins generally footprint between 10 and 12 sugars of HA [41,43], and that an HA molecule of 2 MDa (i.e. the size found in synovial fluid) is made up of more than 5000 repeating disaccharides, a single chain of HA is able to accommodate in the order of 1000 protein molecules. Saturated complexes of this type do exist in the form of link protein-stabilized assemblies of CS proteoglycans built upon an individual HA molecule (recently predicted to adopt a super-helical structure [42]), which have an important function in maintaining the integrity of extracellular matrix, for example in cartilage, skin, blood vessels and brain [43].

I α I molecule onto HA has been elucidated [6,14–16]. In this regard, TSG-6 acts as an essential cofactor forming a covalent intermediate with HC1 or HC2 followed by the transfer of the HC onto HA. TSG-6 released on transfer can form new complexes with I α I, thus functioning as a true catalyst in this reaction [16].

Deletion of the *Ptx3* gene (NM_002852) also leads to impaired fertility due to poor incorporation of HA into the cumulus matrix [17]. However, PTX3 (a multimeric protein with 10 or 20 identical protomer subunits; Figure 1d) does not interact directly with HA but binds TSG-6 at a site on the Link module, independent of the HA-binding surface. Therefore, PTX3 and TSG-6 are likely to form multi-molecular complexes that act as foci for the attachment of multiple HA chains (Figure 1d).

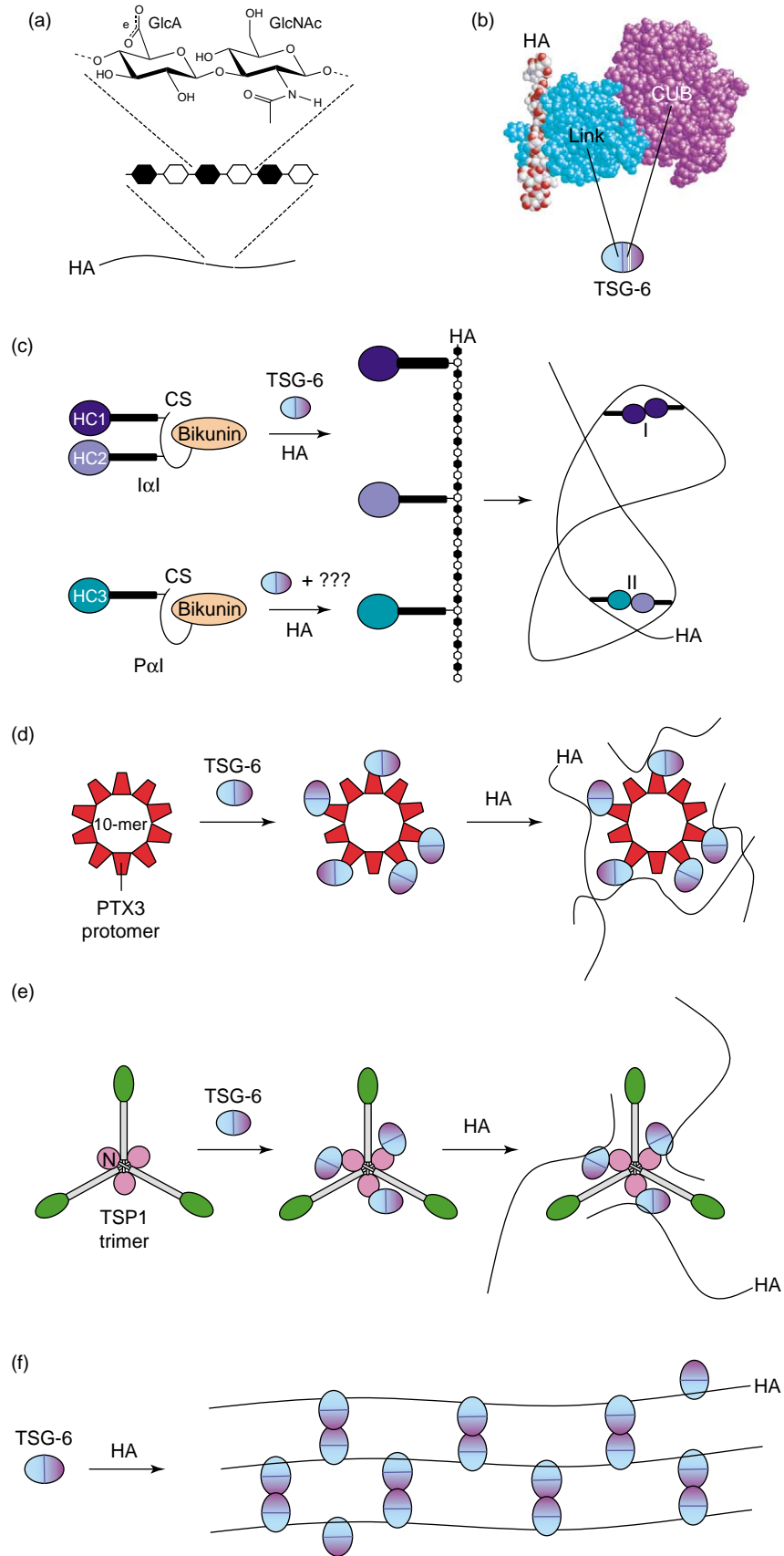
Recently, TSG-6 has been found to bind to thrombospondin 1 (TSP1), where this interaction enhances the TSG-6-mediated transfer of HCs onto HA [18]. As can be seen from Figure 1e, TSP1 is a trimeric protein and could also act in a manner analogous to PTX3 serving to cross-link three HA chains through its binding to TSG-6. Given that TSP1 is expressed in the ovarian follicle before ovulation, it seems probable that this protein contributes to this process [18].

Clearly, therefore, at least two distinct mechanisms of HA cross-linking occur in the context of cumulus matrix [6] and TSG-6 has a central role in its stabilization. However, the precise organization of the matrix and the complex network of interactions remain to be determined (Box 2).

HA cross-linking in inflammation

The cross-linking mechanisms described in the previous section are also relevant to inflammation. In this regard, PTX3, TSP1 and TSG-6 are all expressed in response to inflammatory mediators [18–21], and although I α I is usually only found in serum (being synthesized in the liver [5]) ingress into tissue spaces occurs as a consequence of increased vascular permeability at sites of inflammation, where the synthesis of HA and P α I are also upregulated [7,16]. Thus, wherever HA and these species meet there will follow the formation of HC–HA, PTX3–TSG-6–HA and TSP1–TSG-6–HA complexes [6,16,18]. For example, HC–HA has been identified in synovial fluids from arthritis patients [5,22] and in the bronchial secretions of asthmatics (R. Forteza and A.J. Day, unpublished). Furthermore, TSG-6 is expressed in both of these situations and complexes that we think are likely to correspond to the intermediates of the transfer reaction (i.e. TSG-6–HC1 and TSG-6–HC2 [16]) have been detected, although there is disagreement regarding the composition of these species [15,16,20]. Importantly, HA purified from the pooled synovial fluids of rheumatoid arthritis patients was demonstrated to have, on average, 3–5 HCs per 2MDa HA molecule, where the level of the modification appears to correlate with the degree of HA aggregation and disease severity [5,22]. Electron microscopy of the HC–HA complexes indicated that the HA chains were cross-linked by HC–HC interactions (as illustrated in Figure 1c). Aggregation of HA in synovial fluid should increase both its overall hydrodynamic size

a unusual proteoglycan comprised of three protein subunits, bikunin (a serine protease inhibitor) and two heavy chains (HC1 and HC2), which are covalently linked by a CS moiety [5]; the HCs are attached to the CS chain by ester bonds and this is anchored to bikunin through a standard glycosaminoglycan linkage. It has been demonstrated that the HCs can become covalently transferred onto HA by a reaction where TSG-6 acts as both a cofactor and catalyst [13,16]; TSG-6 forms a covalent intermediate with HC1 or HC2 through an initial trans-esterification step [15], followed by a second trans-esterification that transfers the HC onto HA [16]. P α I is related to I α I but only has a single heavy chain (HC3) linked to the CS. HC3 can also be transferred onto HA but in this case, although TSG-6 is necessary for the reaction, an unknown factor is also required [16]. This modification of HA leads to its aggregation, which is believed to occur by the non-covalent interaction of the HCs [22]. At present it is not known whether these interactions are homotypic (I) or heterotypic (II) in nature. (d) PTX3 is a member of the long pentraxin family and occurs as disulfide-linked multimers of 10 subunits of ~45 kDa each; two decamers can associate to form a 20-mer [21]. Although PTX3 is not a HA-binding protein, it can interact with the Link module of TSG-6 at a site distinct from its HA-binding surface, thus enabling TSG-6 to bind to both PTX3 and HA simultaneously [17]. The schematic model shows how complexes formed between TSG-6 and multiple protomer subunits could act to cross-link multiple HA chains (potentially up to 20), forming a node within the extracellular matrix. Complexes of this type are likely to be formed in cumulus matrix [17] and at inflammatory sites. (e) TSP1 is a disulfide-linked homotrimer of 150 kDa subunits that has been found to interact with the Link module of TSG-6 probably through its N-terminal 'N'-module [18]. The binding sites on TSG-6 for HA and TSP1 are non-overlapping, therefore, it is possible that TSG-6–TSP1 complexes could bring together three HA chains to form another kind of matrix node. This could be used to incorporate TSP1 into HA-containing matrices. The C-terminal domain of TSP1 (green) has been shown to mediate the interaction of TSP1 with CD47, which is present on T lymphocytes, and this interaction has been implicated in T-cell proliferation in rheumatoid arthritis [26]. (f) TSG-6 is also likely to cause direct cross-linking of HA chains to form elongated HA fibres through self-association of the TSG-6 protein [10]. This is represented here in a simplified 2D fashion involving just dimerization through the CUB module; however, this is also likely to involve Link module–Link module interactions leading to 3D bundles of aligned HA strands. Stable complexes of this type, formed through the incubation of TSG-6 with HA, have been shown to enhance or induce the binding of HA to CD44 on lymphocyte cell lines [10]. (d) reproduced with permission from Ref. [6].



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Figure 1. TSG-6-mediated HA cross-linking. (a) HA is a large glycosaminoglycan consisting entirely of the repeating disaccharide $\beta(1\rightarrow4)$ -GlcA- $\beta(1\rightarrow3)$ -GlcNAc (Box 1). (b) TSG-6 [19,20], is comprised mainly of contiguous Link and CUB modules, with the HA-binding site located within the Link module; in this model a short segment of HA (4 disaccharides in length) is docked into the binding groove as described in [42]. TSG-6 has been implicated in HA cross-linking through four distinct mechanisms (c-f). (c) $I\alpha 1$ is

Box 2. Outstanding questions

- What other types of HA cross-linking can occur? How are the various cross-linking mechanisms mixed and matched in tissues and what other architectures can be formed? In this regard, it is anticipated that many new HA structures and cross-linking mechanisms are yet to be identified, particularly in the context of inflammatory and inflammation-like processes, and that they will have a diverse range of cell-binding and other properties.
- How does the fine structure of cross-linked HA affect its functional activities (e.g. cell binding, sequestration of inflammatory mediators and mechanical properties)? For example, it is expected that the composition of proteins in a cable will not only dictate its structure but also affect the way the HA chains are presented to leukocyte receptors. Although there are no data, at present, regarding the affinity of HC–HC interactions (or indeed which HCs bind to which), conceivably there might be a large range of possible affinities, making the level and ratio of HC attachment of particular significance. In addition, the type and amount of accessory molecule associated with the cables could fine-tune cable structure and function.
- In keeping with the highly dynamic nature of HA [3], it seems probable that HA chains will be cross-linked by transient associations and, thus, there will be a constantly changing network of interactions. Research is required to determine the rheological and mechanical properties of cross-linked HA.
- Do cables enable long distance cell-to-cell communication? It has been observed that cables connect many nuclei and, thus, might be part of a novel information transfer system [32]. Further work is required to determine the structure of the intracellular portion of the cables and how they traverse from inside to outside the cell. Furthermore, the mechanism by which cables are generated in a directional fashion needs to be determined.

and viscosity (i.e. opposing the effects of HA dilution and fragmentation seen in arthritis), reducing its loss from the synovial cavity. HC–HA might also act as a ‘sink’ for oxygen free radicals [5,16], which would serve to attenuate inflammatory reactions.

HC modification of HA is also likely to occur within joint tissues because intense staining for I α I has been seen at the articular surface of osteoarthritic cartilage [23]. Furthermore, PTX3 is expressed in arthritis [24], where the staining pattern for PTX3 in the synovial intima and blood vessels is similar to that seen for TSG-6 [25]. Therefore, it is possible that PTX3–TSG-6–HA complexes are formed at these locations. TSP-1 is also present in the capillaries of rheumatoid synovium and accumulates on the surface of fibroblast-like synoviocytes, cells that express TSG-6 [26,27]. The formation of cross-linked HA networks at the surface of synovium and cartilage could serve to protect these tissues during inflammation by providing a barrier to prevent the loss of matrix components and perhaps acting as a temporary scaffold to direct and enhance formation of new matrix. This hypothesis, although speculative, is consistent with the anti-inflammatory properties of TSG-6, which has been shown to be highly protective of cartilage matrix and enhances its repair in mouse models of rheumatoid arthritis [19,28]. Although this protective activity also involves other functions of TSG-6 (i.e. its inhibition of neutrophil migration and its inhibitory effect on the plasmin network [28,29]), its role in HA cross-linking could contribute to its chondroprotective effect.

HA cables and fibrils have enhanced binding to leukocytes

Formation and structure of HA cables

HA cable-like structures were first identified emanating from cultured mucosal smooth muscle cells (M-SMCs) following stimulation with poly I:C, a viral mimic [7,30]. It is now known that they are also made by a wide variety of cells as a response to ER stress (e.g. colonic and aortic SMCs, lung mesenchymal cells and dermal fibroblasts [8]) and by proliferating renal mesangial cells exposed to high concentrations of glucose [31]. As shown in Figure 2a, an individual cable, which can be greater than 200 microns in length [32], supports the binding of a large number of monocytes. The cables appear to be formed by the coalescence of HA threads often arising from the surface of several M-SMCs [7,8]; there is increasing evidence to suggest that the HA is synthesized at the nuclear membrane and is organized into cables inside the cell before emerging into the extracellular space [9,32].

I α I and P α I have been found to be crucial structural components of the cables [7,8,30,33], which is probably a result of covalent transfer of their HCs onto HA, giving rise to stabilizing HC–HC cross-links (Figure 2c). For example, in the case of poly I:C-stimulated M-SMCs, it has been shown that the cables only form (in culture) in the presence of serum and that antibodies against I α I inhibit their production [7]. However, cables are also made by kidney proximal tubular epithelial cells in the absence of serum due to their direct expression of P α I [33,34]. The proteoglycan versican is also associated with cables [8,30,32] and could have a role in extending and hydrating these structures, as well as providing a means of sequestering proinflammatory chemokines through interactions with its chondroitin sulphate (CS) and dermatan sulphate (DS) chains (Figure 2c).

Interaction of cables with mononuclear leukocytes

Cables have been found to be proadhesive specifically for non-activated mononuclear leukocytes, including monocytes, T and B lymphocytes [35] (C.A. de la Motte, unpublished). This interaction is mediated predominantly through CD44 [7,35], which is in an inactive state on circulating leukocytes; HA binding to CD44 can be induced in several different ways, including by direct CD44 cross-linking (e.g. by certain antibodies [10,36]). Given that monocytes do not adhere to HA patches present on M-SMC or to free HA [7,32], this indicates that the HA in cables is presented in such a way as to facilitate receptor binding. One mechanism by which this could occur is by promotion of CD44 clustering with appropriately cross-linked HA (Figure 2d). Alternatively, accessory molecules on the cables could interact with a co-receptor on the leukocyte surface and circumvent the need for CD44 ‘activation’. In this regard, versican has been shown to have a crucial role in the adhesion of cables to mononuclear leukocytes because antibodies to this proteoglycan can inhibit leukocyte binding by ~80% [30]. Conceivably, other accessory molecules could also fine-tune the adhesive function of the cable (Figure 2 and Box 2).

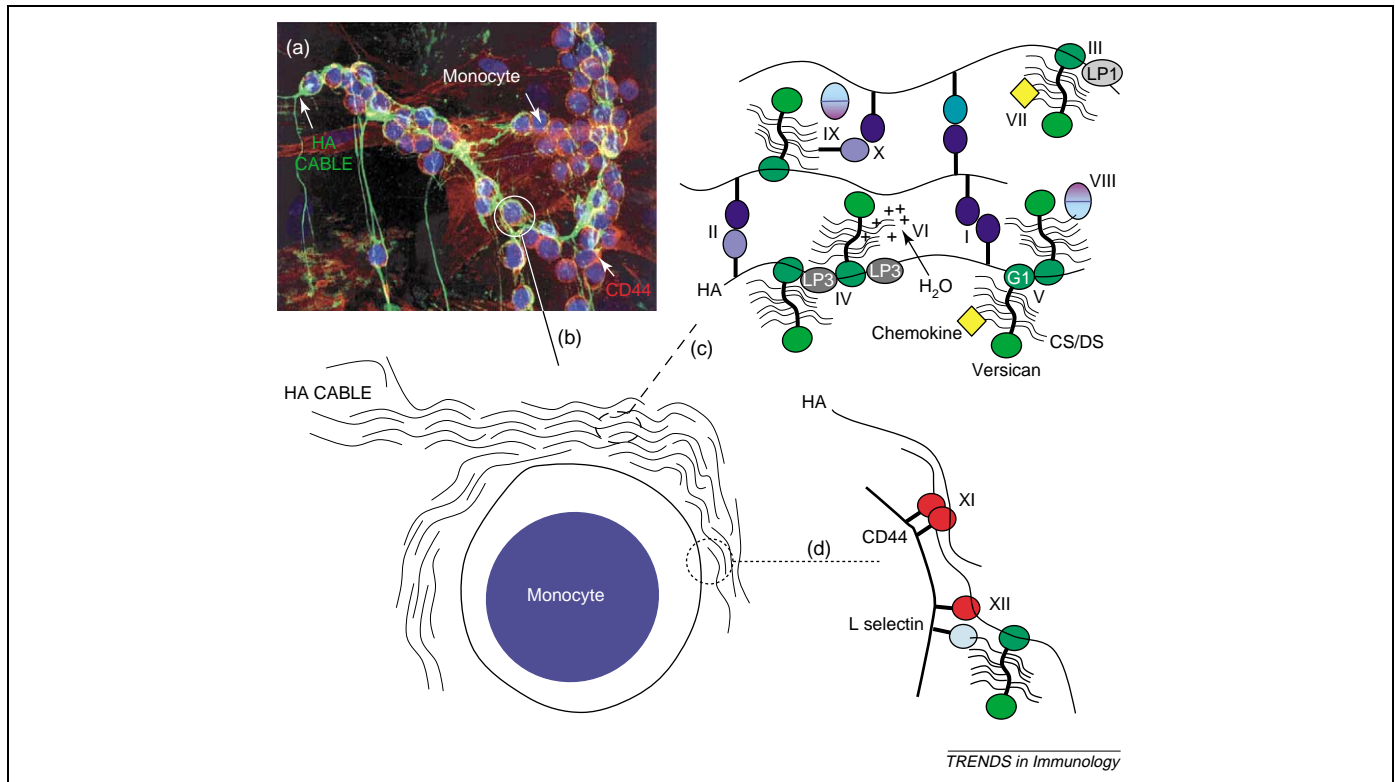


Figure 2. HA cables support leukocyte attachment. **(a)** A confocal micrograph of an HA cable (stained with HABP; green) synthesized by mucosal smooth muscle cells (M-SMC) following their stimulation with the viral mimic poly I:C [7,30,32]. Non-activated monocytes (nuclei stained blue with DAPI) bind to these HA cables through CD44 (red) on their cell surfaces. However, they do not adhere to HA 'patches' present on the surface of the smooth muscle cells (not shown) indicating that the organization of the cable into a cable-like structure provides it with specific proadhesive properties. **(b)** A cartoon expanding a region of the confocal micrograph illustrating the association of the cable with a single monocyte. It can be seen that a cable is comprised of a large number of individual HA molecules, where an extended HA chain of 2 MDa has a length of ~5 micron, and a monocyte has a diameter of 15–25 microns. **(c)** A region of the cable is magnified to show some of the possible intermolecular interactions that contribute to cable formation and function. The HCs of IxI and PzI have been shown to be required for cable formation [7,30,33]. Therefore, it is likely that HC–HC contacts serve to cross-link the individual HA chains by either homotypic (I) or heterotypic (II) interactions, as described in Figure 1c; presumably the HCs are covalently linked to HA through some form of transfer reaction, however, in this case this is likely to occur in a TSG-6-independent manner (C.A. de la Motte and A.J. Day, unpublished). The CS/DS proteoglycan versican also associates with the cable structures [7,30]. Versican is a HA-binding protein, which belongs to the Link module superfamily [4,40] and interacts with the polysaccharide through its N-terminal G1-domain [43]. Cartilage link protein (LP1) has been shown to form cooperative complexes with versican (III) and it is possible, therefore, that the related, and ubiquitously expressed LP3 [43], could stabilize the binding of versican to HA (IV). Versican might also interact with HA cooperatively in the absence of link proteins (V) [43,44]. Such associations could serve to form stiffened and extended regions of the HA chain (e.g. with the super-helical conformation described recently [42]); however, it is clear from the extensive staining with HABP that much of the HA is not saturated with proteins. The CS and/or DS chains of versican are also likely to have an important structural role, through the attraction of positive counter ions and 'inflation' of the cables due to an osmotic potential (VI). Additionally, versican has been reported to bind various chemokines (yellow) through its CS and/or DS chains [45,46], where these interactions could sequester the chemokines and negatively regulate their function (VII); TSG-6 also binds to these GAGs (VIII) and could become sequestered. Under-sulfated regions of the CS chains might become covalently modified with HCs (IX), as seen in human follicular fluid [47]. This reaction could be mediated by TSG-6 because chondroitin (i.e. non-sulfated CS) can act as a weak substrate for TSG-6-dependent HC transfer [14]. This would provide an alternative means of HA cross-linking (X) and could have an effect on the local structure of the cable. Thus, TSG-6 might be able to influence cable function even though it does not appear to be required for their formation. **(d)** Two possible ways in which the HA cable might interact with receptors on the monocyte surface are illustrated. The correct presentation of cross-linked HA chains could promote the clustering and functional activation of CD44 (XI). Alternatively, there could be attachment of the HA cable through the co-engagement of HA with CD44 and of versican (on the HA cable) with a counter receptor on the surface of the monocyte (XII). L-selectin is a reasonable candidate for this given that it has been shown to bind to the CS/DS chains of versican through its C-type lectin domain and is present on monocytes [46]. (a) reproduced with permission from Ref. [48].

Cables might prevent immune activation and promote healing

In inflammatory conditions (e.g. inflammatory bowel disease, asthma and atherosclerosis), there is a clear correlation between ER stress, HA synthesis and leukocyte accumulation [8,9]. Therefore, it has been suggested that HA cables act as distress signals to promote interactions with leukocytes and thus might perpetuate chronic inflammation [8]. However, it seems equally probable that cables function to control leukocyte activation. For example, infiltration of monocytes contributes to the pathogenesis of diabetic nephropathy, where transforming growth factor- β 1 (TGF- β 1), induced in response to direct proximal tubule cell–monocyte adhesion, has been implicated in progressive renal interstitial fibrosis [34]. Here the CD44-dependent

binding of monocytes to HA cables [33], produced constitutively by the proximal tubule cells, inhibits intercellular adhesion molecule-1 (ICAM-1) mediated contact and thus prevents their activation [34]. Similarly, the vascular cell adhesion molecule-1 (VCAM-1) receptors of virally-stimulated colon M-SMC are masked by HA; treatment of cells with hyaluronidase enables very late antigen-4 (VLA-4) integrin-mediated adhesion [35], a well-known proinflammatory signal.

Recently, it has been observed that peripheral blood monocytes are stimulated through their binding to HA cables but that this interaction induces the expression of growth factors and matrix components rather than proinflammatory mediators (C.A. de la Motte, unpublished). This indicates that HA cables do not just provide a physical retention mechanism but also convey the

message to the bound leukocyte that it must help heal. Significantly, the clearance of HA from inflamed tissue, which is a prerequisite to permanent matrix restoration, is also mediated by CD44⁺ monocytic cells [37]. Although numerous previous reports have demonstrated that HA has proinflammatory effects on leukocytes and other types of stromal cells, the data are not inconsistent with our opinion. Most of the proinflammatory observations have involved smaller molecular weight or purified fragments of HA that signal at least in part through Toll-like receptor 4 (TLR4) receptors [38,39]; this is in contrast to the large molecular weight cross-linked cable structures discussed here, which probably signal through CD44. In this regard, cross-linked HA structures might be more resistant to degradation, providing another important limiting step in inflammation.

Formation and activity of HA fibrils

In addition to the HA cables described earlier there are also likely to be other HA–protein complexes that are proadhesive for leukocytes. For example, it has been found that the pre-incubation of HA with TSG-6 enhances or induces the binding of HA to lymphocyte cell lines and that this is probably due to the formation of HA–TSG-6 fibrils (Figure 1d) that can activate CD44 through receptor clustering [10]. Although this structure is clearly proadhesive, its functional role is not yet clear. Given the other properties of TSG-6 [19,28,29] it seems likely that these fibrils will be anti-inflammatory, where it can be envisaged that their formation at sites of inflammation and release into the microcirculation could inhibit the adhesive interactions of circulating lymphocytes with the vascular endothelium; TSG-6–HA complexes were found to be effective competitors of CD44-mediated cell attachment and rolling on immobilized HA *in vitro* [10]. However, further work is clearly required to determine the structure of these fibrils and their precise role in inflammation.

Concluding remarks

Over recent years a diverse range of cross-linked HA structures have been identified that appear to have a key role in inflammation. Although it is well-established that leukocytes can bind to HA, the purpose of this interaction has been unclear. Previous literature supported a proinflammatory role for HA but here we suggest a protective or ‘counter-inflammatory’ role for the highly cross-linked HA structures. The two notions are not necessarily exclusive. We are now beginning to appreciate that HA is an information rich system and, depending upon its degree of cross-linking, its size and the nature of the bound proteins, this simple sugar polymer might achieve different functions. The finding that a single protein, such as TSG-6, can support multiple cross-linking mechanisms (Figure 1) suggests that so far we have uncovered just the tip of this particular iceberg and that we should expect to find many more ways to organize HA and, thus, modulate its function (Box 2).

Acknowledgements

We are indebted to Caroline Milner for her critical review of the manuscript and to the Arthritis Research Campaign (grants 16119 and

16539; A.J.D.) and the National Institute of Health (DK58867 and DK57756; C.d.l.M.) for their support for our research.

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