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SCIENCE DIRECT
European Journal of Cell Biology 85 (2006) 699–715
European Journal of Cell Biology
www.elsevier.de/ejcb

Hyaluronan fragments: An information-rich system

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Received 7 April 2006; received in revised form 5 May 2006; accepted 5 May 2006

Abstract

Hyaluronan is a straight chain, glycosaminoglycan polymer of the extracellular matrix composed of repeating units of the disaccharide [-D-glucuronic acid-β1,3-*N*-acetyl-D-glucosamine-β1,4-]$_n$. Hyaluronan is synthesized in mammals by at least three synthases with products of varying chain lengths. It has an extraordinary high rate of turnover with polymers being funneled through three catabolic pathways. At the cellular level, it is degraded progressively by a series of enzymatic reactions that generate polymers of decreasing sizes. Despite their exceedingly simple primary structure, hyaluronan fragments have extraordinarily wide-ranging and often opposing biological functions. There are large hyaluronan polymers that are space-filling, anti-angiogenic, immunosuppressive, and that impede differentiation, possibly by suppressing cell–cell interactions, or ligand access to cell surface receptors. Hyaluronan chains, which can reach 2×10^4 kDa in size, are involved in ovulation, embryogenesis, protection of epithelial layer integrity, wound repair, and regeneration. Smaller polysaccharide fragments are inflammatory, immuno-stimulatory and angiogenic. They can also compete with larger hyaluronan polymers for receptors. Low-molecular-size polymers appear to function as endogenous "danger signals", while even smaller fragments can ameliorate these effects. Tetrasaccharides, for example, are anti-apoptotic and inducers of heat shock proteins. Various fragments trigger different signal transduction pathways. Particular hyaluronan polysaccharides are also generated by malignant cells in order to co-opt normal cellular functions. How the small hyaluronan fragments are generated is unknown, nor is it established whether the enzymes of hyaluronan synthesis and degradation are involved in maintaining proper polymer sizes and concentration. The vast range of activities of hyaluronan polymers is reviewed here, in order to determine if patterns can be detected that would provide insight into their production and regulation.

Keywords: Hyaluronan; Hyaluronidase; Oligosaccharides; Glycosaminoglycans; Extracellular matrix

Abbreviations: ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; ICAM-1, intercellular adhesion molecule-1; HARE, HA receptor for endocytosis; HA, hyaluronic acid, hyaluronan; HAS, HA synthase; Hsp, heat shock protein; Hyal, hyaluronidase; GAG, glycosaminoglycan; LuCa-1, lung cancer-1; LYVE, lymphatic vessel endothelial HA receptor; MDR, multi-drug resistance; MMP, matrix metalloproteinase; NHE1, Na^+–H^+ exchanger 1; PAI-1, plasminogen activator inhibitor-1; RHAMM, receptor for HA-mediated motility; TGF-β, transforming growth factor-β; TSG-6, tumor necrosis factor-alpha stimulated gene-6; TLR, toll-like receptors.

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doi:10.1016/j.ejcb.2006.05.009

Introduction

Hyaluronan (HA) occurs typically as a high-molecular-size polymer of the extracellular matrix (ECM) of up to 2×10^4 kDa, composed of repeating disaccharides of *N*-acetyl-glucosamine and glucuronic acid, connected exclusively by β-linkages (Meyer and Palmer, 1934; Fraser et al., 1997; Lee and Spicer, 2000). In marked contrast with all other glycosaminoglycans (GAGs), HA is synthesized, not in the Golgi apparatus, but on the cytoplasmic surface of the plasma membrane (Prehm, 1984). A number of HA synthases (HAS) are involved in HA synthesis (Weigel et al., 1997; Sugiyama et al., 1998). The polymer is transported out of the cell, possibly by way of a multi-drug resistance (MDR) transporter system (Prehm and Schumacher, 2004; Misra et al., 2005). Because of its enormous size, HA is transported out of the cell as it is being synthesized. This simple polymer does not undergo sulfation or epimerization, as do all other GAGs. These post-synthetic modifications endow other GAGs with complex patterns that are involved in a vast array of functions. However, HA is also involved in widely varying biological activities without benefit of such distinguishing modifications.

Hyaluronan is degraded through three different pathways, though the hyaluronidase (Hyal) enzyme family appear to be prominent in HA catabolism. It is not known whether the enzymes of synthesis or degradation are involved in generating and maintaining specific HA polymer sizes. Polymer size alone appears to confer specific functions on HA fragments. Location and polymer concentration, as well as HA size-specific binding proteins are other variables (Day and Prestwich, 2002). However, a recent plethora of references suggest that size alone is an essential element.

HA fragments of defined sizes are being used increasingly to study specific biological activities, by using them in vitro. The number of such studies is increasing rapidly. The time is right, perhaps, to review these studies, in an attempt to understand how these HA fragments are generated and regulated.

Caveats

There are several serious problems intrinsic to the study of shortened HA polymers that are tabulated here.

1. In many studies, sizes of oligosaccharides are not measured accurately. Also, an effect may be attributed not to the major population, but to minor oligosaccharide components. Techniques for the precise measure and evaluation of HA oligomer preparations are now available (Lee and Cowman, 1994; Mahoney et al., 2001; Jing and DeAngelis, 2004). A list of current suppliers for HA, some of which provide polymers of defined sizes, is presented in Table 1.
2. Another problem is non-HA contamination. Some of the alleged effects of HA preparations result from even minor contaminants. There are reports that the inflammatory effects of HA oligosaccharides is the result of DNA contamination (Filion and Phillips, 2001; Hu et al., 2005). Highly purified HA extracted from scar retains a collagenous component (Burd et al., 1989). In fact, HA from all animal sources is associated with some protein (Burd, 2004), including low levels of peptide growth factors such as transforming growth factor-β (TGF-β) (Locci et al., 1995), problems that could occur with HA from any animal source. Hyaluronan of bacterial origin presents other problems. Removal of endotoxin and other pyrogens is particularly difficult from such sources.
3. All of the evidence on the biological effects of HA fragments is based on their being added exogenously to cell culture systems. Some effects may result from HA fragments synthesized and utilized intracellularly. For HA fragments that confer paracrine effects, exogenous addition may not mimic effects

Table 1. Companies that provide high-molecular-size HA or HA polymers of defined length

Name, location	Web site
Anika Therapeutics, Woburn, MA 01801, USA	www.anikatherapeutics.com
Amersham-Pharmacia Biotech., Piscataway, NJ 08844, USA (now GE Healthcare)	www.amershambiosciences.com
Fidia, SpA., Padua I-35129, Italy	www.fidiapharma.it
Genzyme, Cambridge, MA 02142, USA	www.genzyme.com
Glycoscience Labs., Inc., Tokyo 113-8549, Japan	www.glycoscience.jp
Hyalose LLC, Oklahoma City, OK 73104, USA	www.hyalose.com
ICN Biomedicals, Inc., Costa Mesa, CA 92676, USA	www.icnbiomed.com
Lifecore Biomedical, Inc., Chaska, MN 55318, USA	www.lifecore.com
Seikagaku Kogyo Co., Tokyo 103-0023, Japan	www.seikagaku.co.jp
Sigma-Aldrich, St. Louis, MO 63178, USA	www.sigmaaldrich.com
Wako Pure Chemicals, Osaka 540-8605, Japan	www.wako-chem.co.jp

of HA fragments generated in situ. Pericellular HA fragments generated in vivo may be decorated with a unique profile of HA-binding proteins that are not provided in fragments added exogenously. Effects may also depend on cellular uptake, but there are no references documenting that specific receptors exist for the variably sized HA chains. Receptor uptake may also require appropriate HA-binding proteins for ligand recognition.

4. The conformational diversity of HA, particularly in the small oligosaccharide range, is highly dependent on pH, temperature, salt concentration, and specific cations. Short HA chains favor intramolecular interactions over intermolecular condensations (Cowman and Matsuoka, 2005). Anomalous migration of HA fragments smaller than eight saccharides (Kinoshita and Kakehi, 2005) may result from structures fixed by intramolecular hydrogen bonding between carboxylic acid and amide groups (Scott et al., 1984). All such considerations may have an effect on apparent effects of HA preparations.
5. There is the phenomenon of cooperativity, in which multiple binding sites for receptors can exist on such a long chain of repeating disaccharides. The presence of one ligand will change the affinity for the second, etc. Thus, binding effects are non-linear and difficult to quantitate. Such cooperativity is well recognized in enzymology and receptor–ligand biology, but, to-date, no specific references are available for such a phenomenon in HA biology.
6. The presence of a glucuronic acid or *N*-acetylglucosamine at either the reducing or non-reducing end may provide the signal for apparent size-specific activities. Such structural information is not usually taken into account, but the time is now at hand when such fine structural details are necessary.
7. Intracellular forms of HA, free, within membranous vesicles, as well as participating in cable formations, are well documented (Evanko and Wight, 1999; Evanko et al., 1999, 2004; Tammi et al., 2001; Hascall et al., 2004). However, the size and functions of such materials are unknown. Therefore they are not included in this review.
8. There are no reports documenting the ability of cells or tissues to release small HA oligosaccharides. In addition, it is difficult to imagine that low-molecular-size HA fragments can exist in the intercellular space in a freely diffusible form. Such oligosaccharides may be bound and protected by HA-binding proteins. A wide variety of HA-binding proteins, also referred to as hyaladherins, have been documented. They can occur as extracellular components of the matrix, including proteoglycans such as aggrecan and versican. Other HA fragments confined to the intracellular compartment may also be protected by binding proteins.
9. Short HA fragments may be made directly by biosynthesis. However, there is no documentation of short HA fragments generated by the HAS enzymes. These synthases are membrane proteins that lose activity when solubilized, and are very difficult to study under physiological conditions.
10. Hyal activities can be modulated in order to maintain proper HA polymer size from the high-molecular-mass precursors. However, there is to date no supporting evidence that these enzymes perform such functions.
11. There are no plausible mechanisms to explain how extracellular HA internalized and delivered to lysosomes can yield oligosaccharides. Specifically, there are no transporters for the small HA fragments out of the lysosomes, and no mechanism that protect them from the very active lysosomal exo-β-glycosidases, β-*N*-acetylglucosaminidase and β-glucuronidase.
12. The ability of cells to respond to HA fragments of different sizes varies with cell type. Polymers of up to 500 saccharides can induce an inflammatory response in monocytes (Horton et al., 1999a, b; Noble, 2002), while Langerhans-type dendritic cells respond to much smaller oligosaccharides, in the 4–6 size range (Termeer et al., 2000; Taylor et al., 2004).

HA synthesis

HAS are glycosyl transferases that occur in vertebrates, bacteria, and algal viruses (DeAngelis, 1999a). There are four HAS genes in most vertebrate genomes, with only three in mammals, expressing enzymes with different properties. They have distinct expression patterns controlled in part by various growth factors and cytokines (Sugiyama et al., 1998; Kennedy et al., 2000; Recklies et al., 2001; Itano et al., 1999, 2004). Expression of the HAS genes also appear to be tissue- and cell-specific (Itano and Kimata, 1996, 2002; Weigel et al., 1997). Even though these enzymes catalyze the same reaction, they differ in the size of their reaction products (Hamaguchi et al., 1999).

However, such experiments are conducted with transfection models in cultured cells, and it is difficult to extrapolate such results into the in vivo situation. HAS do not require primers; however, in vitro addition of HA tetramers to a bacteria-derived HAS system from *Pasteurella multocida* stimulates biosynthesis 20- to 60-fold, compared to synthesis without primers (DeAngelis, 1999b).

Catabolism of HA

The degradation of HA occurs in a step-wise process (Roden et al., 1989; Lepperdinger et al., 2004), but

details have defied explication. HA turnover in the body occurs through three separate pathways. The relative contribution of each of these pathways to total turnover is unknown.

1. There is local cellular turnover that includes binding, internalization, and degradation within cells. Binding is by the predominant HA receptors, CD44 (Lesley et al., 2000; Ponta et al., 2003) and receptor for HA-mediated motility (RHAMM) (Zhang et al., 1998; Cheung et al., 1999; Lynn et al., 2001).
2. The second pathway is at the tissue level. HA is released from tissue matrices, drained into the vasculature and lymphatics, with final steps that include liver, kidney, and possibly spleen. This pathway involves unique receptors such as HA receptor for endocytosis (HARE) (Zhou et al., 2000), also known as stabilin 2 (Politz et al., 2002; Falkowski et al., 2003), lymphatic vessel endothelial HA receptor (LYVE)-1 (Banerji et al., 1999), and layilin (Bono et al., 2005).
3. Scission of HA can occur by free radicals under oxidative conditions, and is promoted by divalent cations (Myint et al., 1987; Deguine et al., 1998; Uchiyama et al., 1990; Saari et al., 1993; Soltes et al., 2005). Free radicals and Hyals may have their activities coordinated under certain pathologic situations. There is a vast literature describing the scission of HA by free radicals, too extensive to be reviewed here. What is impressive is the lack of studies relating them to physiological situations using cultured cells or cell-free extracts.

However, it is only the first of these three pathways that is the focus here.

The cellular pathway for HA catabolism

The degradation of HA occurs in cells by a series of coordinated enzymatic reactions. A recently formulated catabolic scheme (Stern, 2003, 2004), proposes that the high-molecular-mass polymer is cleaved progressively by a series of enzymes in which the product of one reaction becomes the substrate for the subsequent one. These successive enzymatic events generate HA fragments of ever-decreasing size. It is reasonable to assume that these same enzymes can conduct some of the business of supplying and maintaining size-specific fragments. However, there is no evidence for such a function.

Two Hyals, Hyal1 and Hyal2, are involved predominantly in catabolism in somatic tissues. Degradation begins when extracellular high-molecular-mass HA polymers of the ECM are tethered to the cell surface through the combined action of CD44 (Culty et al., 1992) and Hyal2 (Bourguignon et al., 2004). Receptors other than CD44 may be involved, perhaps on a tissue-specific basis. In some cells, binding occurs with the assistance of Na^+–H^+ exchanger 1 (NHE1) (Bourguignon et al., 2004). It has not been documented whether or not most cells utilize this NHE1 mechanism.

Hyal2 is a glycosylphosphatidylinositol (GPI)-linked enzyme attached to the external surface of the plasma membrane (Lepperdinger et al., 2001; Rai et al., 2001). The enzyme makes the initial cleavage in high-molecular-mass HA, generating 20 kDa-sized products of approximately 100 saccharides (Lepperdinger et al., 1998). The HA fragments are delivered to early endosomes, and to lysosomes where fragmentation continues through the action of acid-active Hyal1, generating predominantly tetrasaccharides. Such fragments, when added to in vitro cultured systems induce heat shock proteins (Hsps) and suppress apoptosis (Xu et al., 2002). But it must be reiterated that such observations are made from tetrasaccharides added exogenously to cultured cells. There is no evidence to date that such oligosaccharides generated intracellularly have such an effect.

Additionally, despite all these details, it remains unclear at what point the fragmentation of HA switches from an extracellular or cell surface process to an endosomal or lysosomal process.

HA polymers of varying sizes

HA polymers occur in a variety of sizes that have a vast array of properties, some of which appear to be contradictory. The very large HA polymers are extracellular, are space-occupying and have an array of regulatory and structural functions. The small polymer fragments are angiogenic, inflammatory, and immunostimulatory. In general these short oligosaccharides tend to be involved in the body's alarm system, transmitting various modes of "danger signals" (Powell and Horton, 2005). However, some of the even smaller HA oligosaccharides appear to ameliorate the effects of these stress signals. The biology of all sizes of HA chains is reviewed below, reviewing the function of high-molecular-mass HA, and then examining the role of fragmented chains in various biological systems.

High-molecular-size HA (4×10^2–2×10^4 kDa; 2×10^3–10^5 sugars)

The very-high-molecular-size HA polymers are among the largest of matrix molecules. Their apparent size is even greater when the solvent volume of surrounding water is considered. It is also one of the

most highly charged of molecules, accounting in part for its unusual properties (Lee and Spicer, 2000; Toole, 2000). High-molecular-size HA can function as a lubricant, as a shock absorber, as in the fluid within the joint capsule, and a space-occupying material, as in the vitreous of the vertebrate eye, and in Wharton's jelly, where it may suppress compression of the umbilical cord. Such large HA molecules also function in organizing the ECM (Laurent and Fraser, 1992).

In pathologic situations, such as in shock, septicemia, massive trauma, post-surgery, and blood loss, and in burn patients (Berg et al., 1988; Onarheim et al., 1991), circulating high-molecular-mass HA increases, perhaps as a naturally occurring intravascular volume expander.

Inside-out matters

A major difference may exist between high-molecular-size HA in vivo and such HA chains after they have been extricated. Very little is known of the actual properties of the high-molecular-size HA within the narrow confines of the ECM or the restricted volume of the intercellular space. When HA undergoes aqueous extraction from major sources, such as from rooster comb, joint fluid, Wharton's jelly or from bacterial capsules, the extraction fluid has very high elastovasticity. This refers to the rubber-like qualities of the polymer solution (Balazs, 2004). Such HA is in a random coil conformation. However, HA is unlikely to be in such a conformation in vivo, and little is known about the actual state HA is in within tight tissue spaces. It is probably much more structured, and probably has many unknown functions that are lost following extraction (Balazs, 2004).

Forms and functions

High-molecular-size HA can attain a length of 10^5 saccharides (2×10^4 kDa), depending on tissue source and on physiological conditions (Laurent and Fraser, 1992). The polymer is able to incorporate into its solvent domain a large volume of water that is more than 1000 times greater than the volume of the original material (Granger et al., 1984). These are space-filling molecules that hydrate tissues, are able to exclude other molecules and cells, and that are anti-angiogenic (Feinberg and Beebe, 1983; Deed et al., 1997).

They are also anti-inflammatory and immunosuppressive (Delmage et al., 1986; McBride and Bard, 1979). The immunosuppressive effect derives in part from the ability of high-molecular-size HA to coat cell surfaces preventing ligand access to surface receptors. The high concentrations of HA in the fetal circulation (Decker et al., 1989) and amniotic fluid (Dahl et al., 1983) can account for much of the immuno-suppression in the developing fetus. High-molecular-size HA is intimately involved with the entire process of ovulation, fertilization, and embryogenesis. It also inhibits phagocytosis by monocytes, macrophages, and PMNs (Forrester and Balazs, 1980).

High-molecular-mass HA promotes cell quiescence and supports tissue integrity. High-molecular-size HA causes cell cycle arrest, a process mediated by transmembrane CD44 together with the intracellular protein, Merlin (Morrison et al., 2001). They also protect cells against injury, maintaining epithelial cell integrity, protecting against apoptosis through an NF-κB-mediated mechanism (Jiang et al., 2005). It has been proposed that high-molecular-mass HA mimics or re-enforces cell surface HA.

High-molecular-size HA, in general promotes tissue integrity and quiescence, while HA breakdown products signal that injury has occurred. High-molecular-size HA, even at sites of inflammation may continue to have an anti-inflammatory effect. An unusual form of high-molecular-size HA occurs at sites of inflammation that originates intracellularly. These "stress cables" arise from the peri-Golgi, with interweaving strands extruded from multiple cells. They become cross-linked, and complexed into cables and fibrils at sites of inflammation, and to which inflammatory cells bind. These are well documented in inflammatory bowel disorders, Crohn's disease and ulcerative colitis (de la Motte et al., 2003). These HA cables serve as a "fly paper" for inflammatory cells, where they are maintained in an inactive state. The cables and fibrils also sequester pro-inflammatory mediators, thus suppressing the entire inflammatory process (Day and de la Motte, 2005). This "super" high-molecular-size cross-linked HA modulates the inflammatory process, to provide a protective shield, and to maintain tissue integrity. As a form of high-molecular-size HA, it continues to provide an anti-inflammatory function.

High-molecular-size HA also accumulates around demyelinated lesions in the nervous system, particularly around the lesions of multiple sclerosis. Oligodendrocyte progenitors that normally mature into myelin-forming oligodendrocytes, are prevented from doing so in the presence of the high-molecular-mass HA, thus maintaining the demyelinated foci that are characteristic of multiple sclerosis (Back et al., 2005).

In experimental models of embryogenesis, high-molecular-size HA can have varying effects. Chick embryo skeletal myoblasts cultured on plastic proliferate, fuse, and begin to synthesize muscle-specific actin and myosin. These same cells cultured on high-molecular-size HA will proliferate, but will not fuse, nor produce actin or myosin (Kujawa et al., 1986b). Apparently, suppression of muscle differentiation

occurs when culturing cells on a film of high-molecular-size HA. This is in marked contrast with the process of chondrogenesis. HA bonded to cell-culture surfaces stimulates chick embryo chondrogenesis (Kujawa and Caplan, 1986), while a partially degraded substratum of HA, digested briefly with Hyal, fails to support such differentiation (Kujawa et al., 1986a).

Similarly, high-molecular-size HA suppresses epidermal differentiation in organotypic cultures of keratinocytes (Passi et al., 2004), a differentiation that can occur only after HA degradation. These examples parallel the well-documented ability of high-molecular-size HA to suppress development in a number of model systems of embryogenesis, while degradation of HA triggers the onset of programs of differentiation (Toole, 1991).

HA fragments

Smaller fragments of HA polymers are involved in a variety of normal and pathological processes. HA fragments have sizes that overlap in the functions they perform. It would seem to be more expedient henceforth, to review their physiological roles and their participation in various physiological and pathological processes.

A partial list of HA fragments and their assigned biological functions is presented in Table 2, correlated with molecular size.

Angiogenesis, and wound healing

Wound healing is an example of the precise regulation required of HA fragmentation. In the earliest phase of wound healing, there is a sharp increase in HA after injury, a result of a combination of increased synthesis and impaired clearance. In these very first stages, high-molecular-size HA accumulates with the ability to bind fibrinogen, a reaction intrinsic to clot formation (Frost and Weigel, 1990). This initial HA is a product of platelets (de la Motte et al., submitted), with contribu-

Table 2. Sizes of HA with key functions (partial list)

Size (saccharides)	Function	References
High-molecular-mass HA > 1000–5000	Suppression of angiogenesis	Feinberg and Beebe (1983)
	Immune suppression	McBride and Bard (1979), Delmage et al. (1986)
	Inhibition of phagocytosis	Forrester and Balazs (1980)
	Suppression of HA synthesis	Lueke and Prehm (1999)
HA fragments		
~1000	Induction of inflammatory chemokines	Noble et al. (1993)
	Stimulation of PAI-1	Horton et al. (2000)
	Stimulation of urokinase	Horton et al. (2000)
10–40	Induction of CD44 cleavage	Sugahara et al. (2003)
	Promotion of tumor cell migration	Sugahara et al. (2003)
8–32	Stimulation of angiogenesis	West et al. (1985), Sattar et al. (1994), Slevin et al. (1998, 2002)
	Stimulation of tumor neovascularization	Rooney et al. (1995)
~15	Suppression of smooth muscle cell proliferation	Evanko et al. (1999)
12	Endothelial cell differentiation	Takahashi et al. (2005)
	Up-regulation of PTEN in tumor cells	Ghatak et al. (2002)
10	Displacement of matrix HA on oocyte surface	Camaioni et al. (1993)
	Displacement of proteoglycans from cell surface	Solursh et al. (1980)
6	Suppression of HA cable formation	de la Motte et al. (2003)
	Induction of NO and MMPs in chondrocytes	Knudson and Knudson (2004a, b)
	Induction of HAS2 in chondrocytes	Knudson and Knudson (2004a, b)
4–6	Induction of cytokine synthesis in dendritic cells	Termeer et al. (2000, 2002), Taylor et al. (2004)
	Transcription of MMPs	Fieber et al. (2004)
4	Up-regulation of Hsp 72 expression	Xu et al. (2002)
	Suppression of apoptosis	Xu et al. (2002)
	Induction of chemotaxis	R. Savani, personal communication
	Up-regulation of heat shock factor-1	Xu et al. (2002)
	Up-regulation of Fas expression	Fujii et al. (2001)
	Suppression of proteoglycan sulfation	Solursh et al. (1980)

tions by the blood stream. The initial large HA polymer is anti-angiogenic and immunosuppressive, as aforementioned, and opens up tissue spaces, facilitating PMN access to the wound site for removal of dead tissue, debris and bacteria. Edema, one of the cardinal signs of inflammation and wound repair, is attributed to HA. The PMNs, the first line of defense, then begin to disappear from the wound site.

The next stage of wound healing is the inflammatory stage. HA of lower molecular size accumulates during this phase. The mononuclear cells, including monocytes and lymphocytes, now appear at the wound site. Expression of inflammatory cytokines is induced by HA fragments in the 1000–1250 saccharide range (200–250 kDa). The function of this size of fragments was first examined in in vitro studies (Noble et al., 1993, 1996; Noble, 2002; Hodge-Dufour et al., 1997; Horton et al., 1999a, b; McKee et al., 1996, 1997). Similar results have been shown with renal tubular epithelial cells (Beck-Schimmer et al., 1998), cancer cells (Fitzgerald et al., 2000) and eosinophils (Ohkawara et al., 2000).

The inflammatory stage of wound healing is followed and overlaps partially with an angiogenic response. The first report of HA oligomer involvement in angiogenesis appeared in 1985, in which HA fragments limited to the 6–20 size range were shown to be angiogenic (West et al., 1985, West and Kumar, 1989; Kumar et al., 1989; Rooney et al., 1995; Horton et al., 1998, 2000). These HA fragments are not only mitogenic for endothelial cells (Slevin et al., 1998, 2002), but also enhance endothelial cell migration and induce multiple signaling pathways (Sattar et al., 1994; Slevin et al., 2002). Such HA fragments induce tyrosine kinase cascades (Lokeshwar and Selzer, 2000).

Subsequent to the angiogenic response is the proliferation of fibroblasts, involved in the final stage of the repair process. HA fragments similar to those involved in the angiogenic response, in the 6–20 range of saccharides, stimulate fibroblast proliferation and synthesis of collagen (Rooney et al., 1993).

HA fragments of a smaller size, in the 4–6 saccharide range, stimulate cytokine production by dendritic cells, the antigen-presenting cells of the immune system (Termeer et al., 2000, 2002, 2003). Such fragments also identify areas of wounding through toll-like receptors (TLRs) on endothelial cells (Taylor et al., 2004). These small HA fragments function through particularly TLR-2 and -4 (Jiang et al. 2005). Such fragments of HA polymers also induce metallo-elastases in macrophages (Horton et al., 1999b), and induce plasminogen activator inhibitor-1 (PAI-1) and inhibit urokinase activity in mouse alveolar macrophages following acute injury (Horton et al., 1999a, 2000). They synergize with interferon-γ to induce C-X-C chemokines Mig, and interferon-inducible protein-10 (Horton et al., 1998). These fragments also enhance IL-8 expression (Mascarenhas et al., 2004), and are able to induce nitric-oxide synthase (McKee et al., 1997; Rockey et al., 1998).

Tumor cells and dendritic Langerhans cells of skin share a number of properties. Both cell types can penetrate surrounding tissues, enter the lymphatic systems, and travel to regional lymph nodes. They can also enter the blood stream and travel to distant sites.

The response, restricted to HA fragments in the 4–6 saccharide range, involves the NF-κB pathway. The identity of the receptor for this response is not known, though it has been established that the TLR-4 on tumor cells, and the CD44 and RHAMM receptors are not involved (Fieber et al., 2004). This suggests that there are receptors for HA that remain to be identified.

Specific profiles of Hyals induced at sites of inflammation may be involved in the generation of such small HA oligosaccharides (Weigel et al., 1986; Sampson et al., 1992). Alternatively, or perhaps in tandem, reactive oxygen species that occur with inflammation, and which are also observed at sites of malignancy, may be involved in the generation of such materials (Moseley et al., 1997; Agren et al., 1997).

Potent mechanisms for the later clearing of HA breakdown products in tissues are an important prerequisite for resolution of injury and continued promotion of the repair process. This has been particularly well demonstrated following experimental lung injury (Teder et al., 2002; Jiang et al., 2005).

Cancer biology

HA deposition is up-regulated in most malignancies. While high-molecular-mass HA is found in most normal biological processes, much lower weight material is readily detected in cancers (Kumar et al., 1989; Lokeshwar et al., 1997), where it facilitates tumor cell motility and invasion.

HA oligosaccharides of a certain size range induce proteolytic cleavage of CD44 on the surface of cancer cells, and promote tumor cell migration in a CD44- and dose-dependent manner (Sugahara et al., 2003, 2004), re-enforcing the concept that HA fragments facilitate cancer progression. Tumor cells can also secrete constitutively abundant amounts of Hyal activity, generating HA fragments in the 10–40 saccharide range, enhancing cleavage of their own CD44 and their own motility. Such an autocrine mechanism can promote malignant progression in the absence of external stimulation (Sugahara et al., 2005).

The size-range of HA fragments that induce cleavage of CD44 in cancer cells have been carefully investigated. Added exogenously to cultured pancreatic cancer cells, 6–14 saccharides induce maximal cleavage in a dose-dependent manner. The cleaved CD44 is released into

the circulation. This may be part of a strategy for the cancer cell to become independent of CD44-related controls, by providing a circulating form of the molecule that competes with cell surface CD44. Increased CD44 cleavage has been documented in gliomas, breast, colon, and ovarian cancers, and in non-small cell carcinomas of the lung (Okamoto et al., 2002).

Highly invasive bladder cancers produce HA fragments in the 30–50 saccharide range, the sizes that are angiogenic for endothelial cells (Lokeshwar et al., 1997), though larger in size than those reported earlier. This is perhaps the mechanism for their invasiveness, and serves as an example of how malignancies can commandeer normal physiological functions for their own purposes.

The 35 saccharide size fragments of HA activate tumor cell integrins, enhancing cell binding to intercellular adhesion molecule-1 (ICAM-1) (Fujisaki et al., 1999). By contrast, the HA fragments ranging from 6 to 24 saccharides inhibit B16F10 melanoma cell proliferation in vitro, as well as the formation of tumors from subcutaneously injected cells in vivo (Zeng et al., 1998).

Very small HA oligosaccharides also have unique biological activities. HA oligosaccharides in the 6–7 saccharide range inhibit a variety of tumors. In vitro, they inhibit anchorage-independent growth of tumor cells (Ghatak et al., 2002). Small HA fragments, in the 4–6 saccharide range, enhance expression of matrix metalloproteinases (MMPs) in cancer cells, specifically MMP-9 and MMP-13 (Fieber et al., 2004).

Thus, the oligosaccharides of HA can either promote or inhibit tumor progression. The confusion and inconsistencies that abound can be attributed to the adage that different tumors do different things, and the same tumor can do different things at different times. Instability of the tumor genome, and the constant Darwinian selection process of tumor metastases underscore the resilience and ingenuity of malignant cells in their ability to survive and thrive. This applies apparently also to the widely differing ability to generate different sizes of HA fragments.

Chondrogenesis

HA oligosaccharides have a key role in chondrogenesis, in the expression of the cartilage-specific phenotype from mesenchymal precursors. Understanding such interactions is pivotal in tissue engineering.

Hexasaccharides of HA induce activation of a specific profile of transcription factors in chondrocytes that is not observed in other tissues (Ohno et al., 2005a). Enhanced expression of key genes involved in cartilage remodeling occurs, including MMP-3 and type II collagen. By contrast, high-molecular-weight HA suppresses such signaling.

However, the biology of chondrogenesis in relation to HA differs substantially from other processes. As mentioned above, myoblasts from chick embryo skeletal muscle cultured on plastic dishes will grow, fuse, and begin synthesizing actin and myosin, but fail to do so on HA-coated dishes (Kujawa et al., 1986b), whereas chick embryo mesenchyme cells cultured on HA are stimulated to undergo chondrogenesis (Kujawa and Caplan, 1986).

Isolated articular chondrocytes increase their expression of CD44 and MMPs 1, 3, and 9 following treatment by neutral PH-20 Hyal (Ohno-Nakahara et al., 2004; Ohno et al., 2005a). The fragments of HA generated by such Hyal treatment may be the basis of the effect. This is inferred by the enhanced expression of MMP-3 in chondrocytes when HA oligosaccharides are added to such cultures (Ohno et al., 2005b).

The pioneering work of Solursh et al. (1980) documents anomalous effects that are still not resolved. Decasaccharides of HA added to cultures of chick embryo limb chondrocytes displace new proteoglycans from the cell surface. However tetrasaccharides inhibit their sulfation, but are not sufficient to cause displacement.

The in vivo effects of HA fragments are beginning to accumulate. Intra-articular HA therapy in osteoarthritis is molecular weight dependent. Long-term beneficial effects of HA occur with 500 kDa size (2500 saccharides), while polymers four times that size are ineffective (Ghosh and Guidolin, 2002). The same size HA fragments inhibit MMP-3 synthesis induced by the inflammatory reagent, IL-1beta, in cultured human osteoarthritis chondrocytes (Monfort et al., 2005). HA fragments also activate nitric oxide synthase and the production of nitric oxide in normal articular chondrocytes (Iacob and Knudson, 2006), as in other tissues.

Infection

Very little investigation has been carried out on the role of HA polymers on infectious processes. Just as malignancies have commandeered normal HA functions for their own progression, similar techniques may have evolved by organisms for successful infections.

Aspects of HA metabolism have been shown to participate in infection with bacteria (Markowitz et al., 1959), mycobacteria (Aoki et al., 2004), *Leishmania* (Rao et al., 1999), sheep retroviruses (Rai et al., 2001), a porcine virus (Bratanich and Blanchetot, 2006), hepatitis B (Lara-Pezzi et al., 2001), and HIV (Guo and Hildreth, 1995). It can be anticipated that various sized fragments of HA polymers participate in the multi-step processes of infections. To date, only one example has been identified. The sequestration of *Plasmodium*

Table 3. Size-specific HA oligomers in signal transduction pathways

Molecular size (saccharides)	Signaling molecules	References
~4	IL-12, TNFα Up-regulation of Fas expression	Termeer et al. (2002) Fujii et al. (2001)
4	Erk, JNK, p38 stimulation	M. Tammi and R. Tammi, personal communication
6	Activation of NF-κB in chondrocytes	Knudson and Knudson (2004a)
~12	Up-regulation of PTEN in tumor cells	Ghatak et al. (2002)
6–20	Inhibition of anchorage-independent growth through suppression of PI 3 kinase	Ghatak et al. (2002)
~34	FAK, PI 3 kinase	Fujita et al. (2002)
Not determined	Activation of NF-κB	Fitzgerald et al. (2000)

falciparum-infected red blood cells in the placenta is inhibited by 12-mers of HA (Chai et al., 2001). Further exploration of the role of HA fragments in various infections promises to be a productive area.

Signal transduction

Several signal transduction pathways are initiated by various sizes of HA fragments binding to cell surface HA receptors such as CD44 and RHAMM. Table 3 provides a sample of only some of the transduction pathways that have been documented.

Angiogenic oligosaccharides of HA induce tyrosine kinases in endothelial cells, and activate several cytoplasmic signaling transduction pathways such as Raf-1 kinase, MAP kinase, and extracellular signaling kinases such as ERK-1, all of which result in proliferation (Slevin et al., 1998). HA-CD44 interactions stimulate ERK signaling and transcriptional activation in ovarian cancers (Bourguignon et al., 2005), as well as promoting drug resistance in head and neck cancers through phospholipase C-mediated Ca^{2+} signaling (Wang and Bourguignon, 2006). There is, as well, phosphorylation of the CD44 receptor, increased levels of protein kinase C, and translocation of phospholipases within the plasma membranes (Slevin et al., 2002).

Oligosaccharides of HA in the 6–20 range regulate Erb2 phosphorylation and signaling in cancer cells (Ghatak et al., 2005). The ability of small oligosaccharides such as hexamers to inhibit tumor growth can be attributed to PTEN, a phosphatase that degrades PIP3, the product of PI 3 kinase action, inhibiting growth by inducing pro-apoptotic mediators (Ghatak et al., 2002).

Hyaluronan oligosaccharides in the 4–6 saccharide range also activate an NF-κB/I-κB alpha auto-regulatory loop (Noble et al., 1996), inducing transcription of metalloproteases MMP-9 and -13 (Horton et al., 1999b; Fieber et al., 2004). The induction of nitric-oxide synthase by such saccharides also occurs through a nuclear NF-κB-dependent mechanism (McKee et al., 1997).

Such low-molecular-size HA fragments are also important regulators of microglia at the site of ischemic brain damage (Wang et al., 2004), where c-Jun N-terminal kinase and p38 mitogen-activated protein kinase induce nitric oxide synthase expression.

HA fragments modulate effects of the HA receptor, CD44

Fragments of HA can function at the cell surface by multiple mechanisms. High-molecular-mass HA chains inhibit their own elongation when bound to the plasma membrane-associated HAS. However, activation of the synthase can occur by HA oligosaccharides displacing these large nascent chains (Lueke and Prehm, 1999). CD44 appears to be involved in this process, presumably by keeping the growing chain in the vicinity of the synthase. An HA fragment of 20–30 saccharide size can bind to a variant of CD44 that induces proliferation of endothelial cells (Lokeshwar et al., 1996).

Tissues also differ in the manner in which HA is compartmentalized. In keratinocyte cultures, HA is present on cell surfaces in two equal pools, in patches or diffusely spread. The HA in patches is bound to CD44, and can be displaced by decasaccharides, but not by hexasaccharides (Tammi et al., 1998), a phenomenon that does not occur in the diffuse HA pool. This may indicate that the diffuse pool reflects HA in the process of being synthesized and extruded by the HAS, and not displaceable by HA decasaccharides. This arrangement is characteristic of keratinocytes in which the

extracellular HA plays an important role in physiology and maturation.

Size-specific binding of HA fragments to hyaladherins

HA fragments can bind to HA-binding proteins or hyaladherins (Toole, 1990; Knudson and Knudson, 1993). Such binding has an array of functions, from intracellular effects, such as regulators of the cell cycle (Grammatikakis et al., 1995) or as splicing factors (Deb and Datta, 1996). Extracellular effects are provided by binding to cell surface receptors such as RHAMM and CD44, or to extracellular proteoglycans such as aggrecan and versican. Association with specific HA-binding proteins provides the structural integrity of many tissues by way of their extracellular matrices, including cartilage, brain, and the walls of blood vessels (Day and Prestwich, 2002; Seyfried et al., 2005b).

Variations occur in the minimum size of HA oligosaccharides that bind to HA-binding proteins. The HA chain takes on various secondary and tertiary structures that are in part dependent on polymer size (Scott et al., 1984; Scott and Heatley, 2002). The earliest among these studies (Hardingham and Muir, 1973; Hascall and Heinegård, 1974) demonstrated that a 10-mer is the minimum size HA oligomer able to bind strongly to the proteoglycan, aggrecan. The avidity of binding to CD44 increases with oligomer size up to 38 sugars (Lesley et al., 2000).

Specific lengths of HA fragments also stabilize or organize arrays of hyaladherins by supporting or inhibiting various combinations of such proteins. One of the earliest demonstrations of such an inhibitory function was a study in 1985. A decasaccharide is able to inhibit the interaction of HA with hyaluronectin, a versican-like HA-binding protein from brain (Bertrand and Delpech, 1985).

The most appropriate explanation may be that specific HA lengths can fill the groove provided by binding proteins. Alternatively, size-specific HA oligosaccharides have structural conformations that provide protein access, or conversely, hyaladherin binding stabilizes a particular HA conformation. Most likely, it is reciprocity between all such interactions that provide the final tertiary spatial arrangements (Day and de la Motte, 2005). Some of these relationships between HA oligomer size and hyaladherins are summarized in Table 4.

Anomalies among the smallest HA oligosaccharides

Hexasaccharides of HA inhibit endothelial migration and formation of capillary-like tubules (Banerjee and Toole, 1992), indicating that subsequent products of HA degradation have the capacity to inhibit earlier stages in angiogenesis and wound healing.

HA hexasaccharides inhibit the formation of pericellular coats on cultured chondrocytes (Knudson and Knudson, 1991; Knudson et al., 1996), while high-molecular-size HA stimulates chondrogenesis. Hexasaccharides of HA may actually induce chondrolysis (Knudson et al., 2000).

Tetrasaccharides when added to cultured cells, induce expression of Hsps. The tetrasaccharides are also anti-apoptotic, suppressing cell death in cultures undergoing hyperthermia or when cells are serum-starved (Xu et al., 2002). Such properties appear to be components of survival pathways, generated in response to "danger signals" induced by larger HA fragments.

Table 4. Minimum size of HA oligosaccharides that bind HA-binding proteins

Molecular size (saccharides)	Proteins	References
6	HABP1	Deb et al. (2002)
	CD44 and TSG-6 link module	Kohda et al. (1996)
	Chondrocyte CD44	Knudson (1993)
	Smooth muscle cell CD44	de la Motte et al. (2003)
8	Heavy chain of inter-α-trypsin inhibitor	Mukhopadhyay et al. (2004)
	TSG-6	Kahmann et al. (2000)
8–10	SHAP	Yoneda et al. (1990)
10	Aggrecan	Hascall and Heinegård (1974)
	Versican	Seyfried et al. (2005b)
	Link protein	Seyfried et al. (2005b)
	Keratinocyte CD44 (CD44E)	Tammi et al. (1998)
50	Link protein plus aggrecan	Kimura et al. (1979)

Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the PI 3 kinase/Akt cell survival pathway (Ghatak et al., 2002). HA oligosaccharides in the 6–7 saccharide range inhibit a variety of tumors. In vitro, they inhibit anchorage independent growth of tumor cells (Ghatak et al., 2002). HA hexasaccharides thus can inhibit tumor growth, counteracting the effects of larger fragments that stimulate tumor cell motility, angiogenesis, and cancer progression.

Thus, another generalization can be posited. The smallest products of the HA catabolic cascade can turn about and suppress the action of larger predecessors, and thereby mollifying their effects.

Conclusion

HA polymers occur that are of varying sizes. The metabolic pathways for HA synthesis and degradation are highly ordered, composed of carefully controlled reactions that rely on regulation of individual enzyme activities. How such regulation is accomplished, and whether or not these pathways are coordinated with the maintenance of size-specific HA fragments is unknown.

From the evolutionary perspective, it is postulated that the HA-rich glycocalyx (Hedman et al., 1979), or pericellular matrix, preceded the intercellular ECM. The first polymer carbohydrate conjugates of the pericellular matrix may have been strictly structural entities, and were probably not informative. But in the course of evolution, the HA polymers may have undergone the ability to become degraded following various insults to the cell. These fragments began to act as informative molecules, eliciting various danger/alert signaling pathways within the cell, functioning as survival mechanisms for the organism. At present, oligosaccharides form highly specific recognition sites on cells and proteins. These informative carbohydrates may have evolved from such early sugar polymer fragments, with the added virtue that they do not require genetic templates, and with an information density greater than that of nucleic acids and proteins.

Currently, HA polymer fragments have widely differing size-specific biological activities, and are involved in many essential processes. Chemico-enzymatic synthesis of monodisperse HA polymers (Jing and DeAngelis, 2004), and biologically active fluorescence labeled HA oligosaccharides are now available that will facilitate rapid progress in this field (Seyfried et al., 2005a) that will make it possible to test several of the hypotheses presented here.

HA is not just "goo" (Toole, 2000) as initially presumed, when it was thought to function only as a voluminous inert space-filler, as a highly expandable excelsior. With the realization that HA has a vast repertoire of functions, it has also been referred to as a "multifunctional megaDalton stealth molecule" (Lee and Spicer, 2000).

Identifying the participation of specific enzymatic and non-enzymatic reactions in the overall scheme of the generation and maintenance of HA polymers will make it possible, perhaps, to design specific inhibitors for these reactions. Structural studies of the Hyals (Jedrzejas and Stern, 2005; Stern and Jedrzejas, 2006), are currently being used to facilitate design of small molecular inhibitors that could serve as paradigms for such a general approach.

Hyaluronic acid (HA) has come along way since its initial description (Meyer and Palmer, 1934), including being re-baptized as hyaluronan (Balazs et al., 1986). We ought to consider renaming it once again, with an appellation that reflects more accurately its many subtleties. Highly ironic acid is among the possibilities.

Acknowledgments

The authors acknowledge informational conversations with Drs. Endre Balazs, Lilly Bourguignon, Vincent Hascall, Peter Prehm, Carl Verkoelen, and Mr. Leif Tellmann, and are grateful for their many useful comments, and encouragement.

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