PERSPECTIVE ARTICLE

Hyaluronan in wound healing: Rediscovering a major player

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ABSTRACT

Wound healing involves a series of carefully modulated steps, from initial injury and blood clot to the final reconstituted tissue or scar. A dynamic reciprocity exists throughout between the wound, blood elements, extracellular matrix, and cells that participate in healing. Multiple cytokines and signal transduction pathways regulate these reactions. A major component throughout most of the process is hyaluronan, a straight-chain carbohydrate extracellular matrix polymer. Hyaluronan occurs in multiple forms, chain length being the only distinguishing characteristic between them. Levels of hyaluronan in its high–molecular-weight form are prominent in the earliest stages of wound repair. Progressively more fragmented forms occur in a manner not previously appreciated. We outline here steps in the wound healing cascade in which hyaluronan participates, as well as providing a review of its metabolism. Although described by necessity in a series of quantum steps, the healing process is constituted by a smooth continuum of overlapping reactions. The prevalence of hyaluronan in the wound (initially termed "hexosamine-containing mucopolysaccharide"), particularly in its early stages, was pointed out over half a century ago by the Harvard surgeon J. Engelbert Dunphy. It appears we are now returning to where we started.

Wound healing is a series of complex reactions initiated by the disruption of tissue architecture. The goal of the process is to restore the integrity of the injured tissue. Though variations in wound repair exist dependent on the host as well as on location and severity of the wound, the process is essentially the same for all wounds. It is one of the most intricate of biological processes, involving multiple synchronized biological pathways that occur in a series of complex and partially overlapping steps.

The response to wounding begins with tissue disruption, the tearing of blood vessels, and the deposition of the platelet-rich clot, followed by an influx of acute inflammatory cells. In the absence of pathobiology, the injury is resolved either by the regeneration of the original tissue or by deposition of a fibrotic scar.

One of the components present throughout the entire process is hyaluronan, or hyaluronic acid (HA).[1–3] It plays a large number of widely differing roles considering its simple, unadorned molecular structure. HA normally occurs at low concentrations in the bloodstream. At the wound site, however, HA levels increase quickly, contributed initially by platelets, but also by injured endothelial cells. Following peak level around the initial wound site, HA becomes degraded in a series of sequential catalytic steps modulated not only by the hyaluronidase enzymes but also by reactive oxygen species (ROS). As HA is being fragmented, HA polymers orchestrate specific functions that are often size-dependent.[4]

An early increase in hexosamine-containing mucopolysaccharide in wounds was first pointed out over half a century ago by the Harvard surgeon J. Engelbert Dunphy.[5,6] A major component of that unidentified "hexosamine-containing mucopolysaccharide" was certainly HA. We are only now beginning to appreciate this prescient observation. In wound healing in adults, HA levels increase rapidly, reaching a maximum on day 3 and then decreasing,[5–9] as documented originally in the work of Dunphy and Udupa.[5,6] A figure from that original article,[6] is provided (Figure 1). The tensile strength of the wound is also indicated, following closely the deposition of collagen. This is certainly collagen Type 1, though type-specific collagens were not known at that time.

Many metabolic pathways consist of a series of enzymes, the product of each enzyme providing a substrate for the subsequent reaction. Such cascades characterize numerous

ECM	Extracellular matrix
EGF	Epidermal growth factor
GAG	Glycosaminoglycan
HA	Hyaluronan, hyaluronic acid
HARE	HA receptor for endocytosis
HAS	HA synthase
HMW	High molecular weight
LMW	Low molecular weight
LYVE-1	Lymphatic vessel endothelial receptor-1
MMP	Matrix metalloproteinase
NHE-1	Na^+-H^+ exchange enzyme 1
RHAMM	Receptor for HA-mediated motility
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

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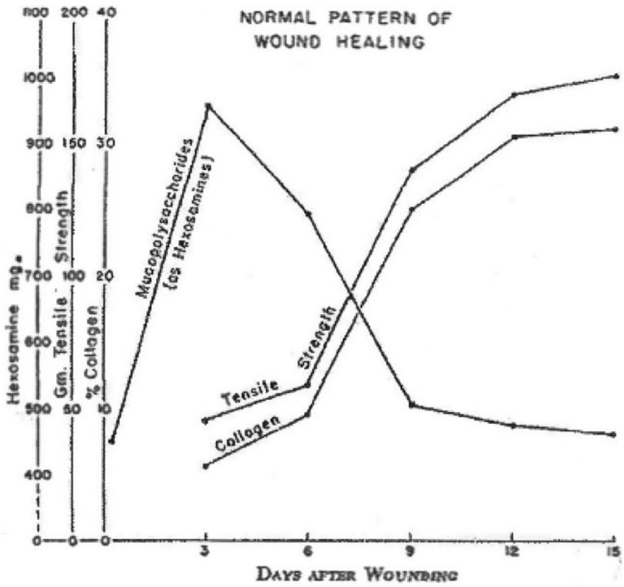

Figure 1. A scheme of wound healing taken from a reference by J. E. Dunphy and K. N. Udupa[6] demonstrating levels of mucopolysaccharide deposition (measured as hexosamines) as a function of time following wounding. We can now identify that material as having been predominantly hyaluronan. The tensile strength of the wound is also indicated, which follows closely the levels of collagen deposition. The latter is now known to be mostly type I collagen. The prescient insight formulated nearly 60 years ago by these surgeons and early investigators of wound healing can now be appreciated. Reprinted with permission from the *New England Journal of Medicine* (http://www.nejm.org/doi/full/10.1056/NEJM195511172532002).

biological cycles but are particularly relevant for wound healing. One such cascade involves the various forms of HA in wound healing. In this article, we provide an overview of HA metabolism, as well as an account of the currently understood role of HA in wound repair. It would be tempting to invoke a cascade of ever-decreasing HA fragment sizes as wound healing progresses. However, there is currently no direct evidence for the existence of such a pathway. This may be partly due to the continuous addition of newly synthesized high molecular weight (HMW) HA in the course of the healing process. Metabolic pathways are typically thought of as a linear stream of reactions. In the case of HA, it may become necessary to formulate the process in three dimensions.

Although great strides have been made in the understanding of wound repair at the cellular and molecular levels, current limitations in wound care make it clear that new concepts are needed. Treatment of pathological situations such as excessive fibrotic reactions, contractures, hypertrophic scars, keloids, gastrointestinal adhesions, chronic ulcers, and wound dehiscence remain major clinical obstacles in wound management. We propose to bring attention to the much-overlooked participation of HA in wound healing. Knowing the multiple roles of HA will increase understanding of wound repair and perhaps lead to new treatment modalities.

In addition to the pioneering work of Dunphy and collaborators, HA in wound healing is reviewed periodically.[10,11] Because of rapid progress, in part facilitated by the Human Genome Project, new information has become available, warranting a fresh review of this important subject.

HYALURONAN METABOLISM

A brief review of current concepts in HA metabolism is provided here, in order to place the discussion in perspective. In addition, a highly hypothetical scheme of HA metabolism has been presented recently.[12]

Hyaluronan

HA is a straight-chain glycosaminoglycan (GAG) carbohydrate polymer that is a major component of the extracellular matrix (ECM).[1–3] It is particularly prominent during wound repair, embryogenesis, and whenever rapid tissue turnover and repair occur. HA is structurally an exceedingly simple molecule. It consists of strictly repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine joined by β-linkages. The latter are relatively impervious to degradation except by specific enzymes, in marked contrast to α-linked carbohydrate polymers such as starch and glycogen, which are easily degraded by ubiquitous enzymes. The β-linked sugars tend to have structural functions, while the α-linked sugars serve mainly as foodstuff. The contrast between cellulose and glycogen, each being a polymer of glucose, is an example of the distinction between the two types of linkages.

HA can exist in a number of forms. It may be free, bound to HA-binding proteins known as hyaladherins,[13] or intercalated into complex structures such as in the ECM. The presence of HA surrounded by multiple aggrecans in a bottlebrush configuration in the structure of cartilage is an example of such a complex structure.

HA has important hygroscopic, rheological, and viscoelastic properties that fluctuate with changes in temperature, pH, ionic environment, and binding partners. However, these properties are also highly dependent on chain length. HA can reach over 10^7 Da in molecular mass (HMW-HA), but also exists in multiple smaller forms, referred to as low molecular weight HA (LMW-HA).

HA has other unique properties. It is the only GAG that is not linked to a core protein or synthesized by way of the Golgi pathway. It is also the only GAG that does not undergo postsynthetic modifications such as sulfation, epimerization, or insertion of α-linkages.

High molecular weight hyaluronan molecules

HA has immunosuppressive effects in its HMW form.[3,14,15] Large HA molecules protect against lymphocyte-mediated cytolysis,[14] suppress septic responses to lipopolysaccharides,[16] maintain immune tolerance,[17] induce production of immunosuppressive macrophages,[18] and, in general, modulate the immune response.[19] They also reduce expression of inflammatory cytokines.[20] The HMW-HA molecules are thus not only immunosuppressive but also antiangiogenic[21] and anti-inflammatory. The HMW form of HA additionally has intrinsic antiaging and anticancer effects.[22]

The HMW-HA species support cell integrity in a number of other ways. They cause cell cycle arrest, mediated by transmembrane association between cluster of differentiation 44 (CD44) and the intracellular protein merlin.[23] They also protect against apoptosis by way of a mechanism mediated by nuclear factor kappa-B (NF-κB).[3,24] Thus, HMW-HA is widely expressed in normal healthy tissues.

Low molecular weight hyaluronan fragments

By contrast, short or LMW HA fragments are highly angiogenic,[25] immunostimulatory,[24] and inflammatory.[3] The presence of the small HA forms is a reflection of tissues under stress.[26] Even the smallest fragment, the tetrasaccharide, has specific functions, with an ability to induce heat shock proteins and suppress apoptosis.[27] The very smallest fragments apparently have the ability to ameliorate the intensity of the reactions induced by the small to intermediate-size fragments of HA. These small tetrameric to hexameric polysaccharides identify tissue injury through Toll-like receptors (TLRs). They also have the ability to inhibit the growth of tumor cells.[28]

HA appears to be an information-rich polymer system based purely on fragment size.[4] Another major concept is that components of the ECM on which cells rest, and particularly HA, have not only structural roles but also regulatory functions.

Intracellular HA

There is an intracellular form of HA,[29] whose role in inflammation and wound healing is uncertain. However, important observations have been made in cultured cells. Production of this form of HA is an intracellular stress response, consistent with the external HA fragments that also reflect cell stress. In dividing cells cultured in high glucose, the intracellular HA, in association with a complex of binding proteins including cyclin D3 and a marker for autophagy, is extruded into cablelike structures to which monocytes adhere.[30] Perhaps these cells adherent to the HA cables are kept in abeyance to be released in a controlled manner during the inflammatory reaction.

Hyaluronan synthesis

As the production of HA, unlike that of all other GAGs, does not require protein synthesis, its deposition occurs rapidly following wounding. It is synthesized by three HA synthases (HAS1, HAS2, and HAS3), all membrane-embedded enzymes, the genes for which occur at three chromosomal locations. They produce different sized HA polymers and are under separate control.[31,32] HA is secreted through the plasma membrane as it is being synthesized. Otherwise, the enormous size of the polymer would destroy the cell. HAS2 is the most important synthase, the only one associated with embryonic lethality upon genetic deletion.[33]

A variety of growth factors, including fibroblast growth factor, transforming growth factors alpha and beta (TGF-α and TGF-β), platelet-derived growth factor, and epidermal growth factor (EGF), stimulate HA synthesis by enhancing mRNA for specific HA synthases.[34,35]

Hyaluronan catabolism

HA turns over extremely rapidly, with a half-life in the bloodstream of 2–5 minutes.[36] A 70-kg individual has approximately 15 g of HA, a third of which turns over daily.[37] It is not clear why the body expends so much energy to produce the polymer, only to rapidly degrade it. Most of the degradation of HA occurs through the family of enzymes termed the hyaluronidases. This class of enzymes was long neglected because assays for their activities were not available. Unlike the prokaryotic hyaluronidases, those from eukaryotes catalyze a hydrolytic reaction that cannot be followed by spectrophotometry. Techniques had to be developed that permitted their analysis.[38] Since then, a large amount of information has accumulated.

There are six hyaluronidase-like sequences in the human genome: two sets of three genes clustered closely together on chromosome arm 3p and another three on 7q. The gene products have been extensively reviewed,[39] as has their evolution.[40]

Hyaluronidases 1 and 2

Of the products of the hyaluronidase gene family, there are only two that are important in HA turnover in somatic tissues, hyaluronidases 1 and 2 (HYAL1 and HYAL2).[41] Of these two, HYAL2 is the first one that HA encounters. The HYAL2 molecule is linked to the external plasma membrane surface of the cell by a glycosylphosphatidylinositol link embedded in clathrin pits. It tethers HA together with CD44 in association with Na^+–H^+ exchange enzyme 1 (NHE-1).[42] The NHE-1 enzyme creates acidic foci on the cell surface. This cluster of molecules coordinates the cleavage of the HA polymer into 20-kDa fragments, corresponding to approximately 50 disaccharides. Endocytosis of such HA fragments then occurs.

The endocytosed HA is delivered to early endosomes and then to lysosomes, where the acid-active HYAL1 proceeds to degrade the HA. The predominant digestion products are tetrasaccharides, molecules of the right size to fit precisely into the active site of the HYAL1 enzyme.[39] Additional lysosomal exoglycosidase enzymes, β-glucuronidase and β-*N*-acetyl-glucosaminidase, degrade the fragments to monosaccharides. These single sugars are then able to exit the lysosome into the cytoplasm, where they participate in further metabolic reactions. As 5 g of HA turns over daily in the average individual, this is obviously an active pathway.

Hyaluronan receptors

A complex series of HA receptors occur on cell surfaces, some specific for HA, others with additional ligands of which many have not yet been identified. A spectrum of signaling pathways are induced by these variously sized HA ligands.[3,11,43] However, correlation between the precise sizes of HA fragments and those of the various receptors is only now beginning to be addressed.[44]

CD44

CD44 is the predominant receptor for HA,[45] though HA is not its only ligand. It is a transmembrane glycoprotein consisting of 10 constant exons in the human, with 10 variant exons inserted in various combinations at a single extramembrane site. The constant isoform, referred to as CD44S, is comprised of the invariant exons only (1–5 and 16–20). It remains expressed on the surface of cells, while many variant-containing molecules also occur intracellularly.

An enormous number of splice variants can be generated, carrying various exons.[46] An important goal would be to document the specific CD44 variant molecules occurring at each stage of wound healing. A confounding issue is that other ligands also bind to CD44, such as collagens, osteopontin, and matrix metalloproteinases (MMPs).

Many of the functions of HA are carried out in conjunction with or while HA is bound to CD44. CD44 is also critical for clearing HA degradation products from injured areas. Macrophage CD44 is a negative regulator in lung inflammation.[47] Blocking CD44 results in impaired clearance and delays in wound healing.[48] CD44 thus stimulates inflammation but can also moderate the inflammatory response.

The important concept regarding the interactions of HA with receptors such as CD44 is that the same receptor can have opposing functions depending on the stage of wound healing. This may depend on the particular CD44 splice variants being expressed.

RHAMM

The HA receptor RHAMM (receptor for HA-mediated motility), or CD168, directs cell movement, forming links with cytoskeletal proteins and activating protein kinases, which results in locomotion.[49,50] The precise roles of RHAMM in inflammation and wound healing are not as clear, but a RHAMM-mimetic peptide blocked HA signaling and reduced inflammation and fibrogenesis in an excisional skin wound model.[51] RHAMM has functions in addition to HA binding: Intracellular RHAMM associates with microtubules while regulating mitosis.[52]

Toll-like receptors 2 and 4

TLRs 2 and 4 have very small inflammatory HA fragments as their ligands.[53–55] They are a group of ancient, highly conserved proteins that recognize molecules associated with severe threats. They provide the ability to sense invasion by pathogenic organisms and other such threats to survival. These TLR molecules signal expression of a number of inflammatory genes, including chemokine production in macrophages.[24]

HARE and LYVE-1

HARE (HA receptor for endocytosis), also known as stabilin 2, mediates systemic clearance of HA.[56] This receptor occurs on the inner surface of endothelial cells in both vascular and lymphatic channels. LYVE-1 (lymphatic vessel endothelial receptor 1), however, occurs only in lymphatic endothelium.[57]

Systemic clearance of HA by HARE is in response to NF-κB–mediated gene expression. This occurs, however, within a very narrow size range—with HA fragments of 40 to 400 kDa.[58] Thus, HARE can monitor bodywide HA turnover, though this has not been examined directly. The participation of LYVE-1 under such conditions has also not been investigated.

Involvement of HARE and LYVE-1 in the local wound healing process has not yet been documented, but it would be of intrinsic interest to establish whether expression of these receptors undergoes major changes during massive wounding, major surgery, sepsis, profound blood loss, or other stress-inducing injuries. It is very likely that there is a degree of coordination between local wound healing and the systemic clearance of HA.

STAGES OF WOUND HEALING

Formation of the initial clot and platelet plug

Disruption of tissue architecture, bleeding, and the transition of liquid blood to the solid platelet-rich clot initiate the process of wound healing. Platelets contain large amounts of HA within their cytoplasm, as do megakaryocytes.[59] This HA is of a large molecular size. It is postulated that the megakaryocyte is a large cell precisely because of its HA content and its associated voluminous water domain. No other hematopoietic bone marrow cells contain HA.[59]

Platelets also contain HYAL2 in a membranelike pattern. HYAL2 can also be identified in the megakaryocyte. The HYAL2 of the platelet cuts the HA into inflammatory-sized polymers. The platelet, however, unlike all other cells, lacks HYAL1 and does not have the ability to make further cuts. This may account, in part, for why platelets are inflammatory.[59]

It also seems likely that the HYAL2 reaction must be held in abeyance for a short period following formation of the platelet plug. The HA molecules accumulating at the wound site participate in the local edema, a function which is most efficiently performed by HMW-HA.

Fibrinogen is deposited by platelets during the formation of the initial clot. Fibrinogen is an HA-binding protein and, together with its fibrin product, helps to maintain a local concentration of HA. The HMW-HA forms the architectural matrix for deposition of the clotted fibrin.[60]

An untested hypothesis is that fibrinogen or fibrin may be a hyaluronidase inhibitor. This may account for the initial abeyance of HYAL2 activity when platelets become activated or release their contents. This model for the earliest stage of wound healing—that HA and fibrin interact to form a three-dimensional matrix that has both structural and regulatory functions—was proposed over 30 years ago.[60–62]

Increased vascular permeability and edema in early wound healing

Edema, or "tumor," is one of the five cardinal signs of inflammation. HA is a major component of edema fluid, filling and expanding the tissue surrounding the fresh wound.

HA has a remarkable charge at neutral pH; it is probably the most negatively charged anion in biology. The water of solvation of HA is actually quite small, but because of its large negative charge, there is an enormous water domain that accompanies the molecule. This expansion of tissue represents the edema of early wound healing and the "tumor" of the inflammatory response. Massive accumulation of HA is documented in the edema fluid observed in animal models of inflammation.[63,64] HA is capable of expanding its solvent domain up to 10,000 times its actual polymer volume.[65] The space thus created facilitates infiltration of the first-recruited inflammatory cells, the neutrophils. The inflammatory-sized HA fragments constitute one of the signals to recruit these cells. These cells phagocytose debris, remove dead tissue, and sterilize the space opened up by the HA-induced edema.

Increased vascular permeability participates in the edema formation—plasma proteins constitute another component of the edema fluid, particularly in its later stages. The HA plays a crucial role in the maintenance of vascular integrity. It modulates the endothelial glycocalyx. The caveolin-enriched microdomains interact with endothelial HA-binding proteins. The increased hyaluronidase activity and ROS break down HMW-HA to LMW fragments that damage the endothelial glycocalyx. HA fragments activate specific HA-binding proteins that promote actin cytoskeletal reorganization and inhibition of endothelial cell–cell contacts.[66,67] By contrast, HMW HA promotes endothelial integrity and actually inhibits vascular leakiness.[68] A recently identified player in this scenario is HA-binding protein 2 (HABP2).[69] HABP2 regulates vascular integrity by Rho kinase signaling.

Initial inflammatory cell response

The first responders are the acute inflammatory cells, the polymorphonuclear leukocytes. They are recruited from the blood stream and from bone marrow. These cells constitute the innate immune response, the first line of defense in microbial infection. It is well established that HA deposition precedes this leukocyte response.[70] The recruitment occurs through the binding of HA,[58] with CD44 modulating the cells' appearance. Enhanced synthesis of HA by disrupted or injured endothelial cells may be one source of the HA involved in the recruiting mechanism.[71] Neutrophils have a short half-life of 6–7 hours.[72] They then disappear rapidly from the wound.

The myeloperoxidase enzyme reaction associated with neutrophils in early inflammation is also an HA degradation mechanism. Free radicals are generated, particularly ROS.[73] This may be a major catabolic mechanism, but the relative proportions of HA degraded by enzymatic and free radical-based catabolism are unknown. They would, however, be straightforward to determine. Hyaluronidase-catalyzed breakdown generates HA fragments that are identical in structure to the parent polymer, while ROS-based degradation yields HA polymers with oxidized termini. Determining the ratio between these two classes of molecule would be straightforward, but so far, this has not been attempted.

Myeloperoxidase is the most abundant protein in neutrophils, constituting more than 5% of their dry weight. The iron-containing heme enzyme is the reason that pus and mucus are tinged with green.[74]

Mononuclear infiltrate

Lymphocytes and monocytes, together referred to as mononuclear cells, arrive at the wound site as neutrophils disappear. This constitutes the chronic inflammatory infiltrate. Monocytes also contain the myeloperoxidase enzyme, but to only one-third the level of neutrophils. The enzyme is lost as these cells mature into tissue macrophages. Some free-radical cleavage of HA continues during the chronic inflammatory phase.

The TLR2 and TLR4 receptors responding to small HA fragments induce macrophage inflammatory gene expression. In cultured human macrophages, HA induces expression of tumor necrosis factor alpha (TNF-α),[75] as well as many other chemokines involved in the chronic phase of inflammation.[76,77]

It is the monocyte/macrophage that is the master cell of the entire wound healing response. If the wound is sterile, wound healing can proceed in the absence of neutrophils or lymphocytes. However, the wound healing process stops immediately in the absence of macrophages. Likewise, HA is the "keystone" molecule in the inflammatory milieu and in immune regulation of the inflammatory response.[78] The HA is the mediator of the cross-talk between the wound ECM and the incoming inflammatory cells, and it is the monocyte that is the key cell.

Angiogenesis

The HMW-HA polymers suppress angiogenesis with their ability to inhibit early response genes such as *c-fos*, *c-jun*, and *Krox-20* in endothelial cells.[79] The HA synthesized by disrupted endothelial cells, however, rapidly becomes cleaved. The LMW fragmented HA moieties are highly angiogenic,[25,80] ensuring a blood supply for the healing area. Myriad signaling pathways, involving both proliferation and cell migration, are induced by the HA fragments.[81,82]

The initial step in angiogenesis involves MMPs dissolving the basal lamina of existing vessels. This permits exit and growth of cells toward the area of healing. Monocytes and macrophages cooperate with progenitor endothelial cells in the process of neovascularization, particularly in spatial organization and localization.[83] Expression of vascular endothelial growth factor is also controlled in part by fragmented HA.[84] Both CD44- and RHAMM-receptor–mediated signaling pathways are involved in this angiogenic response.[85,86]

Development of myofibroblasts and deposition of granulation tissue

Granulation tissue—the complex of endothelial cells, fibroblasts, myofibroblasts, and inflammatory cells—is the most complex and most dynamic tissue of wound healing. A certain population of fibroblasts are destined to become myofibroblasts.[87] They begin to express smooth-muscle actin and myosin. Granulation tissue is rich in HA and CD44 throughout.[88] There is also a constant HA-mediated cross-talk between fibroblasts and myofibroblasts that is only beginning to be understood.

Though HA levels are high, there is a decreased rate of net turnover of HA as fibroblasts differentiate into myofibroblasts, suggesting that there is some unknown hyaluronidase-inhibitory activity associated with granulation tissue.

HA orchestrates the appearance and maintenance of the myofibroblast phenotype. It does this through TGF-β1–dependent steps.[89,90] Organization of a fibroblast HA-rich pericellular coat precedes differentiation into the myofibroblast.[91] TGF-β1–dependent induction of the HA synthase enzyme HAS2 and the hyaladherin TSG-6 (TNF-α–stimulated gene 6) provides for the pericellular HA coat assembly. Additionally, the monocyte–macrophage series take on the appearance of epithelioid cells during the course of granulation tissue formation.

A panoply of cells occur in granulation tissue, accompanied by multiple cytokines, laying the groundwork for the subsequent fibrosis.[92] Understanding the role of each in greater detail may make it possible to modify the fibrotic reaction.

Fibroblast invasion and proliferation

Fibroblasts are major players in the final stages of the healing process. Invasion and proliferation by fibroblasts is a prerequisite for the deposition of collagens. They are the essential cells for the structure of the wound patch. Fibronectin, a glycoprotein found in the ECM as well as in high concentrations in the circulation, binds collagen and fibrin. Fibroblast invasion into the fibrin matrix is dependent on fibronectin,[93] which, coupled with HA, stimulates wound healing.[94] Thus, HA can be perceived, together with fibronectin, as a guiding runway for fibroblast migration, providing an ECM for wound closure.

There are historical arguments that some of the macrophages of the wound can develop into fibroblasts,[83] but this is not universally accepted. HA production is stimulated in fibroblasts by TGF-β and Smad signaling. Smads are intracellular proteins that transduce extracellular signals from TGF-β to the nucleus, where they activate gene transcription. Fibroblasts are stimulated to divide, and HA production is up-regulated.[95] This is an autocrine, self-stimulating system, as the HA is also mitogenic for the fibroblasts.

HA is constantly being produced in a HMW form and must be continuously cleaved for all the stages of wound healing, including the concluding fibroblast proliferation phase. The maximum HA size for such stimulation is 70 kDa.[96] Native HMW HA, by contrast, inhibits proliferation under these conditions in vitro. It is for this reason that an HA-based wound healing pathway may perhaps have to be formulated in a nonlinear pattern.

Deposition of type III and type I collagens

Deposition of collagens by fibroblasts follows the granulation stage of wound healing. The fibroblasts proliferate as angiogenesis slows. Type III collagen, the soft malleable collagen, is deposited first—a temporary patch that does not provide tensile strength to the wound.

Cultured dermal fibroblasts normally synthesize type I and type III collagens simultaneously in an approximate ratio of 3:1 to 4:1. HA, however, stimulates specifically the production of type III collagen.[97] Interestingly, both native HMW-HA and polymers of 12 saccharide units are able to enhance type III collagen synthesis.

A different scenario is also feasible—that an entirely different population of fibroblasts are responsible for the type III and type I collagens. The fibroblasts of granulation tissue synthesize mostly type III, and the fibroblasts of the maturing wound deposit predominantly type I, suggesting that a shift in cell type may be responsible for the switch from predominantly type III collagen to type I.

Another hypothesis is that fetal-like fibroblasts may be commandeered from the HA-rich bone marrow during wound healing.[98] This would also explain the clinical observation that irradiated areas do not heal well. The vasculature in a previously irradiated area is compromised to such an extent that bone marrow cells may not have the ability to migrate efficiently into the wound site.

Reepithelialization

One half of the 15 g of HA in the human body is contained in skin. Even there, the turnover is rapid, with a half-life of 2–3 days.[36] The HMW-HA is abundant in the extracellular spaces between keratinocytes throughout the epidermis, as is CD44.[88] It is particularly abundant in the basal layer, where it protects proliferating cells, promotes mitosis, and stimulates migration of newly divided cells up from their basal origin.

HA is also a component of the basal lamina upon which keratinocytes rest. They are dependent on this position for cell division. The function and precise structural role of HA within basement membranes has not been determined, but it is well known that proper tissue repair and regeneration is dependent on an intact basal lamina scaffolding. The HA content of basal lamina structures decreases with age, and in various basement membranes of the eye, it is not detectable after age 50.[99] This may account in part for the decreasing quality of wound repair with aging.

In the healing wound, histologically, HA faces the wound margin, together with CD44. This complex regulates keratinocyte proliferation during the process of reepithelialization, as well as sustaining local HA homeostasis.[100] Additionally, the heparin-binding form of EGF activates HA synthesis, also facilitating keratinocyte repair during epithelialization.[101]

An interesting observation, well known to surgeons, is that if granulation tissue in the wound bed grows up above the surface of the skin (proud flesh), the epithelium is not able to grow and cover the area. This then requires debridement of the excess tissue. What prevents epithelial growth is not known.

Final repair and remodeling

Normally, the HA is cleared from the wound site by day 10 following injury. The tensile strength of the wound is entirely dependent on type I collagen and correlates with the time when stitches can be removed. Type I collagen now predominates in the fibrous scar, as type III collagen is being removed. A collagenolytic MMP that specifically degrades type III collagen and spares type I must exist on theoretical grounds, but no MMP with such an activity has ever been identified. A testable, but to date untested, hypothesis is that the simultaneous presence of HA directs substrate-specificity of MMP activities.

The final mature scar continues to be remodeled, a process that can continue for months and years. The final scar decreases in size and, in most cases, finally becomes indiscernible.

OTHER ASPECTS OF HYALURONAN METABOLISM IN WOUND HEALING

The lactate effect

The initial wound disrupts or compromises the blood supply to that tissue area, causing anoxia. This in turn generates lactate, the product of anaerobic metabolism. The synthesis of HA by fibroblasts and by endothelial cells is greatly enhanced in the presence of lactate.[102,103] This is a very important phenomenon when dealing with the low oxygen levels that occur especially at wound margins. Lactate also up-regulates expression of CD44 and alters splice variants in that highly variable molecule. Sequence analyses of promoter regions of genes for CD44, caveolin-1, and HYAL1 and HYAL2 reveal multiple AP-1 and ets-1 response elements.[102] RNA from lactate-treated fibroblasts analyzed by reverse transcriptase–polymerase chain reaction reveals increased transcripts of *c-fos*, *c-jun*, *c-ets*, *HYAL1* and *HYAL2*, *CD44*, and caveolin 1. These are all lactate-activated genes critical for the wound healing response. A similar response occurs with tumor progression and is known as the Warburg effect.[102]

In addition, lactate has an effect on local pH. The lowered pH that occurs in the presence of lactate increases the availability of oxygen, which is particularly important at the wound margin. This is a result of the Bohr effect, the decreased ability of hemoglobin to bind oxygen at lower pH. This increase in available oxygen minimizes cell damage and necrosis at wound margins.

A curious compensatory effect occurs in diabetes. Fibroblasts from wounds of diabetic patients produce 30% more lactate, and thus more HA, than wounds from age-matched controls.[103,104] Even fibroblasts from uninjured sites produce more lactate than controls. Fibroblasts from diabetic patients show higher levels of CD44 expression, greater sensitivity to lactate, and significant increases in HMW-HA. All of these observations may relate to the chronic ulcers observed in diabetes, a decreased ability to fragment wound HA, and a compromised quality of wound healing. It is thus also possible to conjecture that the markedly increased size of infants born to diabetic mothers is due to higher levels of lactate and HA and an associated increase in tissue edema fluid.

Hyaluronan, growth factors, and cross-talk between extracellular matrix and cells

The growth factors of wounds are extraordinarily potent. This is best exemplified by the observation that in chickens, tumors caused by Rous sarcoma virus occur only at the site of virus inoculation, even though there may be millions of plaque-forming units per milliliter of blood. It is only at the site of the wound caused by the needle puncture that a tumor grows.[105]

Modulation of the wound healing response

Because the inflammation associated with the wound healing response can be overzealous, reactions have evolved to ameliorate the intensity of the reaction and to make it self-limiting. Several of these involve HA. CD44 binding is integral to HA in inflammation, but simultaneously, it is critical for the process of clearing inflammation, as has been elegantly characterized in inflammation of the lung.[48] Free radicals and ROS can degrade HA, but HA is also a sink for these radicals. The HA fragments act as scavengers for free radicals, protecting the cells of granulation tissue, in particular, from ROS damage.[106,107]

HA also acts in the negative feedback loop with other constituents of inflammation. TNF-α expressed in the course of inflammation stimulates production of TSG-6. TSG-6 is an HA-binding protein that forms a stable complex with the circulating protease inhibitor inter-α inhibitor (IαI) that promotes plasmin-inhibitory activity. Plasmin is part of the proteolytic cascade of MMPs and other proteases that cause tissue damage during inflammation. In this way, the TSG-6–IαI–HA complex limits inflammation and stabilizes granulation tissue as the wound matures. This parallels the elaboration of TSG-6 that limits the severity of inflammatory arthritis.[108,109]

The superoxide dismutases (SODs) also have an ameliorating role in wound inflammation.[85] The SODs are present in the ECM, inside cells, and also in mitochondria that catalyze the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide. Thus, they are important antioxidant defense mechanisms. They dampen the oxidative burst associated with the myeloperoxidase reaction. Extracellular SOD has a polycationic matrix-binding domain that binds to HA. SOD can inhibit inflammation in part by preventing superoxide-mediated fragmentation of HA.

It appears that nature has invested the body with opposing mechanisms that help to restrict the intensity of inflammation, a potentially highly destructive system that must be constantly monitored. It is also evident that these factors that modulate wound repair are a potential source of pharmacological agents, many of which remain to be identified.

Scar formation, adhesions, and other pathologies of wound healing

Most individuals heal with minimum scarring, but certain patients heal with excessive scar tissue. There is a continuum, with hypertrophic scar formers reflecting one extreme end of the spectrum. Some of this variation has a genetic basis, but the causes of excessive fibrosis are complex and involve multiple cellular mechanisms.

There are myriad inflammatory cytokines that can contribute to excessive scarring. The system appears to be degenerate—the same effect can be performed by multiple cytokines. The process of wound healing is so vital to the survival of the organism that nature has supplied multiple failsafe mechanisms. This makes it difficult experimentally to factor out the players involved.

One form of excessive scar is the keloid, a tissue reaction distinctly different from hypertrophic scar formation. The HA content of keloids is far below that of ordinary scars, as determined by histochemical observations and from studies of keloid-derived fibroblasts.[110] The sizes of the HA chains produced in the cultured fibroblast system are of normal length, suggesting that it is not an excess of hyaluronidase activity that is responsible for the diminished levels of HA. HAS and HYAL activities are also reduced in keloids, supporting the earlier observations.[111] Hypertrophic scars also have decreased HA deposition, particularly in the papillary dermis, but intermediate between that of normal scars and that of keloids.[112]

Hyaluronidase inhibitors

The identification and characterization of hyaluronidase inhibitors, despite their importance, is virtually an untouched area of human biology. Attempts have been made to summarize this field.[113] The lack of information is in part due to a lack of rapid and reliable assays for detecting hyaluronidase-inhibitory activity, a problem that is now being resolved.[114,115]

HA levels rise very rapidly in the bloodstream and in other tissues in response to extreme stress. They increase with remarkable speed following extensive burns,[116–119] massive blood loss, major surgical procedures, infections, and septic shock.[120,121] It may be, with its enormous associated cloud of solvent water, that HA is an emergency intravascular volume expander—a survival mechanism under extreme conditions. For instant response to an emergency situation, it is faster to inhibit HA degradation than to resort to increased rates of HA synthesis. The latter is inherently a slower process. Hyaluronidase inhibitors are among the acute-phase proteins made by the liver under such emergency conditions. The existence of one such inhibitor has been established.[122]

Deficiency in vitamin C compromises effective wound healing. Lack of hydroxylation of the prolines and lysines in collagen is presumed to be the predominant mechanism. However, vitamin C is also a hyaluronidase inhibitor and stimulates net deposition of HA, as well as other GAGs, in fibroblast cultures.[123] A fatty-acid derivative of vitamin C has been shown to be a very potent inhibitor of hyaluronidase.[124,125] It may be that one of vitamin C's major functions in wound healing is as a hyaluronidase inhibitor; this is a hypothesis that requires further analysis.

A deficiency of vitamin C also compromises the quality of wound healing. Ascorbate has several functions in the healing process. It is a required cofactor for the hydroxylation reaction for the prolines and lysines of collagen. It also has an inhibitory effect on HA, promoting its deposition, in part, by inhibition of hyaluronidase activity. Vitamin C has the ability to stimulate GAG deposition in general.[123]

The hyaladherin TSG-6 exerts a potent anti-inflammatory effect. The complex of TSG-6, IαI, and HA appears to function as a negative-feedback control on the inflammatory reaction, stabilizing the healing process and doing so in part by its hyaluronidase inhibitor activity.[108,109,122] The TSG-6–IαI complex provides a two-pronged effect on wound healing, the TSG-6 component functioning as an anti-inflammatory molecule and the IαI portion serving as a hyaluronidase inhibitor.

Myriad LMW hyaluronidase inhibitors come from the field of ethnopharmacology. Many of the herbs and plant extracts used since ancient times as wound salves have hyaluronidase-inhibitory activity. Most of these, however, are of low inhibitory activity.

Systemic and local effects of wound healing

The effect of a local wound, particularly a massive wound, on systemic HA metabolism is not well understood. The turnover of HA occurs not only locally, but also systemically in lymph nodes, liver, and kidneys.[126–128] Ligations of either the hepatic or renal arteries cause an instantaneous increase in circulating HA.[126] The systemic effect on expression of the vascular and lymphatic HA receptors LYVE-1 and HARE following wound healing on a large scale, such as occurs following major trauma or extensive surgery, should be investigated.

It is well known among surgeons that previous wounds shorten the time for healing of subsequent incisions. The systemic effect involved in this mysterious phenomenon deserves investigation; in all probability, it involves HA metabolism and the cytokines and growth factors it induces. Of particular interest is that this "wound healing memory" continues for a period of time.

A related observation is that in HA-related wound healing experiments conducted with Dr. Nancy Byl, former chair of the Department of Physical Therapy and Rehabilitation at the University of California, San Francisco, data from control sites on the contralateral side of a wounded animal were consistently intermediate between those from the experimental sites and excisions taken from naïve animals[129,130] (some observations unpublished). Circulating wound healing factors do exist, and they require identification.

Fetal wound healing

It is well recognized that the fetus heals without scar formation. There is an extensive literature describing this phenomenon.[7–9,131] This scarless wound healing continues until some time late in the second trimester, at which point wound healing becomes adultlike. HA is invoked as a mechanism that supports this scarless repair.[8]

Amniotic fluid and embryonic tissues are rich in HA. In many ways, the progressive steps of wound healing recapitulate embryogenic development. The high concentrations of HA in the early phases of wound repair parallel the HA-rich milieu of fetal development. This limits the deposition of the ECM and of collagen in particular. HA delays tissue differentiation during embryogenesis, thus preventing fibrosis and scar formation in the fetus and in the early phases of wound healing. Application of amniotic fluid in experimental animal wounds facilitates earlier wound closure and decreases levels of scarring. However, this is associated with decreased tensile strength.[129]

TGF-β3, prominent during embryogenesis and early development, is also up-regulated by HA.[116] HA is abundant in the fetus, as well as in amniotic fluid. The presence of type III collagen early in the fibrotic stage of wound healing resembles the collagen profile of the fetus. The presence of HA and the persistence of type III in the fetal environment may explain the scarless healing found therein, as discussed below. This also reinforces the concept that adult wound healing recapitulates the successive stages in development.

Interleukin 10 has recently been identified as a key factor in the HA production and the scarless healing of the midterm fetus. Its decreasing levels in gestation parallel the onset of scar formation, as observed in fetal surgery.[132]

Wound healing in the aged

Wounds in the elderly do not heal well. Histochemically, HA levels in skin appear to be decreased, with very little detectable after age 60.[133] However, biochemical data contradict this. The levels of HA remain constant throughout life. The HA content in skin becomes progressively more tightly tissue-associated and unavailable for metabolism and is thus unavailable for detection by staining procedures,[134] as binding proteins cover on a continuous basis the site used for detection by histochemical and immunohistochemical techniques.

Decreased wound healing with age is attributed in part to compromised circulation. In the elderly, chronic wounds that fail to heal, particularly in the lower extremities, are major clinical problems. Deterioration in wound healing with aging is partially due to flaws in HA metabolism[135] and, in particular, a decreased ability to process HA.[136] A study of the size distribution of HA in rat skin as a function of age documents an abundance of HMW-HA in old animals, perhaps reflecting an inability to generate lower–molecular-size fragments.[137] The suppression of ability to generate such small fragments would compromise the wound healing process.

There are also age-related decreases in the HA organization of the pericellular coat of fibroblasts as they differentiate into myofibroblasts, a key step in the deposition of wound granulation tissue.[91] Aging fibroblasts also resist diifferentiation into myofibroblasts because of impaired HA-dependent EGF receptor signaling.[138]

Aged keratinocytes also appear to compromise the wound healing phenomenon.[139] HA interaction with CD44 promotes keratinocyte function and can overcome age-related epidermal dysfunction.

Diabetic wounds

The dysregulation of HA metabolism is a feature of diabetes and may underlie the poor wound healing and its many complications observed in diabetics. Increased glucose level is considered a main cause of this phenomenon. Advanced glycation products and the enrichment of ECM with HMW-HA under the action of high glucose level occur in vascular smooth muscle cells, skin fibroblasts, and endothelial and mesangial cells. This effect accelerates the proliferation of vascular smooth muscle cells and fibroblasts, promoting the transformation of acute wounds into chronic ulcers.

Inhibition of collagen fibril formation and subsequent cross-linking by glucose can be demonstrated in vitro.[140] Collagen that is not cross-linked is unstable and more susceptible to collagenolytic attack. Interference with collagen cross-linking and more rapid degradation can explain the decreased amounts of interstitial collagen and the poor healing of wounds associated with diabetes, as well as the dehiscence that can occur with wounds following surgery of diabetic patients, particularly abdominal wounds.

Steroids and wound healing

Wound healing is notoriously poor in patients on steroids, in particular cortisones. Both topical and systemic treatments carry such risks. Both decreased synthesis and increased degradation of HA occur, as has been shown in tissue and organ culture systems.[141] Topical treatment of skin with glucocorticoids rapidly reduces HA levels.[142] Glucocorticoids have the specific effect of suppressing HAS2 activity.[143] The effect of steroids in diminishing the HA content of tissue is the probable basis of the associated poor wound healing. As half the HA of the body occurs in skin, the solvent water of HA accounts for skin moisture; that steroids decrease HA levels probably accounts for the atrophic appearance of steroid-treated skin.

Exogenous application of hyaluronan to wounds

A major focus of wound treatments in the past has been the application of collagens, with highly variable results. All collagens are xenogenic, with broad differences between species. The advantage of HA-based materials, however, is that HA is a highly conserved molecule and, in purified form, is identical between all species and even between phyla.

There is an extensive literature describing the application of exogenous HA to improve wound healing.[130,144,145] In burn patients, application of HA significantly shortens the time to reepithelialization.[146] In experimental rat models of wound healing, adding HA solutions to superficial skin wounds stimulates epithelial migration and hastens the healing process.[147] In otolaryngology, healing of perforated tympanic membranes is speeded by the application of HA.[148] High levels of HA are expressed in the skin of the *Hoxbl13* knockout mouse, which enhances wound healing.[149]

New technologies for investigating hyaluronan in wound healing

The precise size of HA polymers and how they are recognized and utilized by the cells of wound healing need to be further defined. Fortunately, size-specific HA fragments are now commercially available for testing in the various stages of wound healing. In addition, genetically modified mice with conditional gene deletions are available for each of the major players in the healing scenario: the HA synthases, the various hyaluronidases, and the spectrum of HA receptors and HA-binding proteins. Hyaluronidase inhibitors are becoming recognized, and a specific inhibitor of HA synthesis, 4-methylumbelliferone, is now available. Mannose, another inhibitor of HA synthesis, inhibits dermal fibroblast invasion and prevents the leukocyte binding to HA that occurs in cells treated with inflammatory mediators such as interleukin 1β. Mannose also reduces HA in subcutaneous sponge granulation tissue, a model for skin wounds, and suppresses leukocyte recruitment. Mannose thus suppresses wounding-induced inflammation by attenuating HA synthesis.[150] These are all reagents and techniques currently available that can be used to probe the steps in wound healing. They can now be assembled in experimental models, testing the sequential steps in the process, in order to understand in greater depth the participation of HA in wound healing.

CONCLUSION

We continue to travel along the trajectory initiated by the Harvard surgeon Dr. J. Engelbert Dunphy, the first to point out that acid mucopolysaccharides were prominent in the matrix of healing wounds, particularly in the early phases of the process. Many parameters of HA metabolism still need to be investigated before we understand fully this dynamic process and how aspects of it can be utilized to improve wound care. Understanding the multiple ways in which HA functions may provide both direct and indirect cues to the subtle balancing act that occurs during the process of wound healing.

The major conclusion is that HA is present through all the steps of wound healing. It is not only a product of inflammation but, more importantly, a promoter of inflammation and of the entire process of wound repair. It is a sentinel for tissue damage through changes in polymer size and drives both the inflammatory and reparative processes of wound healing.

The human heals with great velocity, with the ability to rapidly deposit copious amounts of a fibrotic scar. Excessive scarring in humans may reflect an ancient survival mechanism. We heal faster and more aggressively than even our closest primate relatives. Recognizing and understanding the role of HA in the various steps of healing will provide a mechanism for pharmaceutically improving wound repair and even facilitating a move toward healing with less scarring.

A tabulation of some of the steps in wound healing in which HA participates is presented in Table 1. Many of these

Table 1. Tabulation of hyaluronan participation in wound healing

Day	Hyaluronan participation in wound healing
0	Clot formation. HA binds to fibrinogen, loosening the clot while also stabilizing it. HA pours into the area from platelets as well as from wounded endothelial cells.
0.1	HMW-HA comprises much of the edema fluid, the "tumor" component of the inflammatory reaction, constituting the swelling of early wound healing. Anoxia at the wound stimulates lactate production that enhances local HA synthesis.
	The lowered pH resulting from lactate production releases oxygen more easily from hemoglobin, known as the Bohr effect. This is particularly important at wound margins and for survival of tissue.
0.2	Platelet hyaluronidase (HYAL2) is released from inhibition by an unknown inhibitor.
	Platelet hyaluronidase generates intermediate-sized HA fragments that attract polymorphonuclear leukocytes.
	The spaces created by edema facilitate entry of polymorphonuclear leukocytes and their removal of dead tissue, debris, and bacteria.
	HMW-HA limits intensity of the myeloperoxidase reaction and free-radical injury of tissues. This dampens the intensity of the inflammatory response.
1	The attraction of mononuclear cells into the wound may be induced by further HA fragmentation.
	TLR2 and TLR4 respond to small HA fragments, inducing macrophage inflammatory gene expression, including that of TNF-α and other chemokines.
3–5	Signaling by HA fragments induces mitosis, sprouting, and tropism of endothelial cells, providing angiogenesis and neovascularization.
	Stimulation of MMPs by HA helps to regulate angiogenesis.
	Expression of vascular endothelial growth factor is controlled in part by HA.
	Organization of a fibroblast HA-rich pericellular coat precedes differentiation of fibroblasts into myofibroblasts.
	Granulation tissue is formed with a matrix rich in HA. This provides cushioning of the wound and structural organization of the evolving matrix.
6	Migration and proliferation of fibroblasts are stimulated by HA.
	The HA also stimulates preferential synthesis of collagen type III by fibroblasts, as well as other extracellular matrix components.
	HA, together with fibronectin, provides a guiding runway for fibroblast migration and an ECM for wound closure.
8	Preferential degradation of collagen III occurs with sparing of type I collagen.
9–14	Continued deposition of collagen type I occurs with growing tensile strength of the wound, perhaps initiated by decreasing levels of HA. This constitutes much of the fibrous connective tissue of the wound.
	In the repairing wound, HA faces the wound margin, together with CD44. This complex regulates keratinocyte proliferation and migration during the process of reepitheliazation

This is only a partial list of putative HA-dependent reactions. This is a highly speculative formulation of HA reactions, but provides multiple testable hypotheses. The size range of the HA polymers and the subsequent fragments that participate in these reactions have not been determined. The multiple variant exons of CD44 expressed in the stages of wound healing have not been established. Conceivably, antibodies against individual variant exons will make it possible to control with great specificity particular steps in the wound healing response. No MMP that preferentially degrades collagen type III has been identified, but theoretically, an MMP in the presence of HA may take on such specificity. The time span indicated above is idealized. Fascial wounds with an abundant blood supply heal more quickly, sometimes within 5 days, while wounds of the lower extremity, particularly in the elderly, take much longer to heal, occasionally several weeks.

are highly conjectural, but they are eminently testable. The goal here has been to stimulate interest in basic wound healing research.

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