Hyaluronan in Tissue Injury and Repair

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Abstract

A hallmark of tissue injury and repair is the turnover of extracellular matrix components. This review focuses on the role of the glycosaminoglycan hyaluronan in tissue injury and repair. Both the synthesis and degradation of extracellular matrix are critical contributors to tissue repair and remodeling. Fragmented hyaluronan accumulates during tissue injury and functions in ways distinct from the native polymer. There is accumulating evidence that hyaluronan degradation products can stimulate the expression of inflammatory genes by a variety of immune cells at the injury site. CD44 is the major cell-surface hyaluronan receptor and is required to clear hyaluronan degradation products produced during lung injury; impaired clearance of hyaluronan results in persistent inflammation. However, hyaluronan fragment stimulation of inflammatory gene expression is not dependent on CD44 in inflammatory macrophages. Instead, hyaluronan fragments utilize both Toll-like receptor (TLR) 4 and TLR2 to stimulate inflammatory genes in macrophages. Hyaluronan also is present on the cell surface of lung alveolar epithelial cells and provides protection against tissue damage by interacting with TLR2 and TLR4 on these parenchymal cells. The simple repeating structure of hyaluronan appears to be involved in a number of important aspects of noninfectious tissue injury and repair that are dependent on the size and location of the polymer as well as the interacting cells. Thus, the interactions between the endogenous matrix component hyaluronan and its signaling receptors initiate inflammatory responses, maintain structural cell integrity, and promote recovery from tissue injury.

Contents

INTRODUCTION

The successful repair of tissue injury requires a well-coordinated host response to limit the extent of tissue damage. The innate response to an insult, the component of the host response that is in place prior to the insult, serves as the first line of defense against injury and initiates the inflammatory response. The host must also generate signals to minimize the extent of structural cell damage. The ultimate outcome of the host depends on the balance between the containment of injury,

maintenance of structural cell integrity, and activation of repair mechanisms. The mechanisms that regulate the host response to noninfectious tissue injury are poorly understood. This review focuses on recent work that investigates the interactions between extracellular matrix (ECM) and innate immune receptors in regulating noninfectious lung injury and repair.

The ECM plays an important role in regulating the host response to lung injury. Accumulation of ECM can be seen during tissue injury following a variety of insults such as those that occur in the adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis, chronic persistent asthma, and bronchiolitis obliterans syndrome (reviewed in Noble & Jiang 2006). Moreover, ECM components are degraded to smaller species (Noble & Jiang 2006). Collagen degradation products can be measured in bronchoalveolar lavage (BAL) fluid in the patients with ARDS and correlate inversely with survival, suggesting that matrix remodeling may play a much more important role in determining the outcome of lung injury than previously recognized (Chesnutt et al. 1997). Following the initial insult, there is an influx of inflammatory cells in surviving patients, a fibroproliferative phase of ARDS that develops approximately 10–14 days after the onset of this syndrome (Meduri 1995). The cellular events that are associated with the fibroproliferative phase of ARDS are not well understood, but pathologic studies have suggested that there is a significant component of ECM turnover, with the deposition of fibrin degradation products, collagen, fibronectin, and proteoglycans such as versican and the glycosaminoglycan hyaluronan (HA) (Bensadoun et al. 1996).

Hyaluronan Turnover in Tissue Injury, Inflammation, and Fibrosis

HA is a normal constituent of basement membrane and makes up approximately 10% of the proteoglycan content of the lung (Hance & Crustal 1975). HA turnover is not limited to lung injury and pulmonary diseases; degradation of HA also occurs in liver injury (George & Stern 2004), kidney injury (Wuthrich 1999), and brain injury (Al'Qteishat et al. 2006). The measurement of serum HA can be a useful tool in the diagnosis of liver diseases such as cirrhosis (Lindqvist 1997).

HYALURONAN

HA first was isolated from the vitreous humor of bovine eyes (Meyer & Palmer 1934) and later from umbilical cord and many other sources such as rooster comb. The first suggestions that HA might have a role in disease pathogenesis arose from observations on HA isolated from synovial fluid of patients with inflammatory arthritis (Ragan & Meyer 1949). HA is distributed widely in nature, such as in the cell wall of *Streptococcus* groups A (Lowther & Rogers 1955) and C (Maclennan 1956) and of *Pasteurella multocida* (Carter 1972). In mammals, HA is abundant in heart valves, skin, skeletal tissues, the vitreous of the eye, the umbilical cord, and synovial fluid (Fraser et al. 1997). A significant property of HA is its capacity to bind huge amounts of water (1000-fold of its own weight). Therefore, HA functions as a biological lubricant in joints, reducing friction during movement and providing resiliency under static conditions (Engstrom-Laurent 1997). HA belongs to a family of glycosaminoglycans that also includes chondroitins, heparin, heparan sulfate, and keratan sulfate, and interacts with proteins and functions in growth, development, inflammation, and immune responses.

Hyaluronan Structure

Twenty years after the initial discovery of HA, Meyer's laboratory determined the exact chemical structure of HA, a nonsulfated, high-molecular-weight glycosaminoglycan composed of repeating polymeric disaccharides D-glucuronic acid and *N*-acetyl-D-glucosamine linked by a glucuronidic $\beta(1\rightarrow3)$ bond (Weissman & Meyer 1954). The

ECM: extracellular matrix

ARDS: adult respiratory distress syndrome

BAL: bronchoalveolar lavage

HA: hyaluronan

Structure of
hyaluronan.
Hyaluronan is
composed of
repeating units of
D-glucuronic acid-
β(1→3)-*N*-acetyl-
D-glucosamine.
The disaccharide
units are linked by
a β(1→4) bond
(*red*). Two
disaccharide units
are shown. GlcA,
D-glucuronic acid;
GlcNAc, *N*-acetyl-
D-glucosamine.
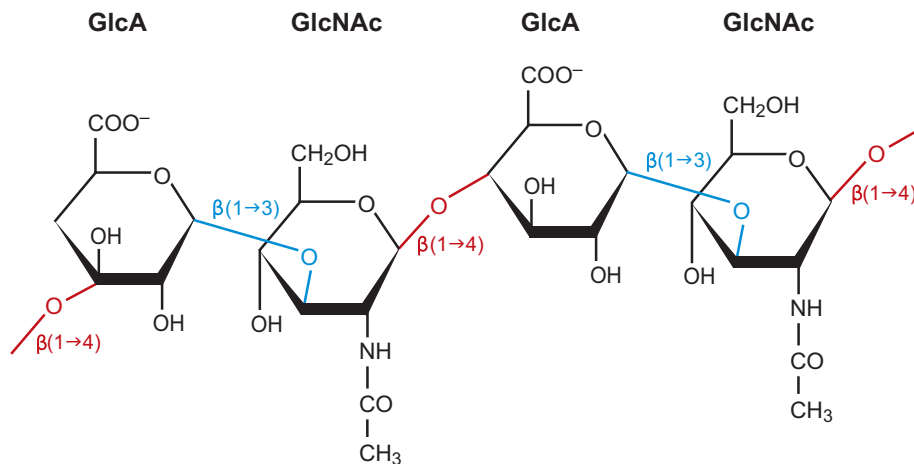
disaccharide units are then linearly poly-
merized by hexosaminidic β(1→4) linkages
(**Figure 1**). The number of repeat disac-
charides in a completed HA molecule can
reach 10,000 or more, a molecular weight of
∼4000 kDa.

HYALURONAN SYNTHASES

HA is synthesized by membrane-bound
synthases (HASs) at the inner surface of
the plasma membrane, and the chains
are extruded through pore-like structures
into the extracellular space (Watanabe &
Yamaguchi 1996). The amino acid sequence
of HAS1 shows significant homology to the
hasA gene product of *Streptococcus pyogenes*,
a glycosaminoglycan synthase from *Xeno-
pus laevis*, and a murine HAS (Watanabe &
Yamaguchi 1996). Because HAS1, HAS2, and
HAS3 are located on different autosomes
(Spicer et al. 1997), the HAS gene fam-
ily may have arisen comparatively early in
vertebrate evolution by sequential duplica-
tion of an ancestral HAS gene. Expression
of mammalian HASs led to HA biosynthesis
in transfected mammalian cells (Itano et al.
1999, Spicer & McDonald 1998). The HAS1
protein alone is able to synthesize HA, and
different amino acid residues on the cyto-
plasmic central loop domain are involved
in transferring *N*-acetyl-D-glucosamine and
D-glucuronic acid residues. HAS3 synthesizes

HA with a molecular weight of 1×10^5 to
1×10^6 Da; this mass is less than HA syn-
thesized by HAS1 and HAS2, which have
molecular weights of 2×10^5 to approximately
2×10^6 Da (Itano et al. 1999). Furthermore,
comparisons of HA secreted into the cul-
ture media by stable HAS transfectants show
that HAS1 and HAS3 generated HA with
broad size distributions (molecular weights of
2×10^5 to approximately 2×10^6 Da), whereas
HAS2 generated HA with a broad but ex-
tremely large size (average molecular weight
of $>2 \times 10^6$ Da) (Itano et al. 1999). Subse-
quent studies suggested that all three HAS
enzymes drive the biosynthesis and release
of high-molecular-weight HA (1×10^6 Da)
(Spicer & Tien 2004).

Hyaluronan Synthase 2
and Development

Whereas HAS2 is exclusively expressed in
some tissues, its expression pattern overlaps
and/or complements that of HAS1 and HAS3
in others. Targeted deletion of HAS2 has
been the major development in the field,
yielding insights into the in vivo functions of
HA (Camenisch et al. 2000). There are major
abnormalities in heart and blood vessel de-
velopment, resulting in an embryonic lethal
phenotype (Camenisch et al. 2000, 2002).
HAS2-null embryos at embryonic day (E)
9.5 completely lack endocardial cushions

HAS: hyaluronan
synthase

(Camenisch et al. 2000), resembling the features in naturally occurring versican knockouts. These studies suggest that HA-ECM interactions play a significant role in cardiac development. Elucidating the role for HAS2 in lung disease has been difficult because of the embryonic lethal phenotype in the HAS2-deficient mice.

Regulation of Hyaluronan Synthase by Exogenous Stimuli

Stimuli such as growth factors and cytokines can regulate the expression of HAS isoforms in vitro. Recombinant tumor necrosis factor (TNF), lymphotoxin, and interferons stimulate HA production and increase cellular HAS activity by normal human lung fibroblasts (Elias et al. 1988). IL-1β and TNF-α induce HAS-2 mRNA, and fluticasone and salmeterol attenuate IL-1β- and TNF-α-induced HAS2, suggesting that enhanced synthesis of HA by the proinflammatory cytokines can be abrogated by specific corticosteroid and β2 blocker combinations shown to be effective in the treatment of asthma (Wilkinson et al. 2004). Epidermal growth factor induces HAS2 expression and HAS2-dependent HA synthesis in rat epidermal keratinocytes (Pienimaki et al. 2001). TGF-β activates HAS1, leading to an increase in HAS activity, through the p38 MAPK and MEK pathway but not the JNK pathway (Stuhlmeier & Pollaschek 2004). Conversely, transforming growth factor-β (TGF-β) suppresses HAS3 mRNA (Stuhlmeier & Pollaschek 2004). Stimulation of mesothelial cells with platelet-derived growth factor-BB induces HAS activity (Heldin et al. 1992) and an upregulation of HAS2 mRNA (Jacobson et al. 2000). TGF-β reduces HAS2 mRNA slightly but does not significantly affect the expression of mRNAs for HAS1 and HAS3 in mesothelial cells (Jacobson et al. 2000). Interestingly, there is a natural antisense mRNA of HAS2 (HASNT, for HAS2 antisense) in human and mouse (Chao & Spicer 2005). HASNT is transcribed from the opposite strand of the HAS2 gene locus and is represented by several independently expressed sequence tags in human. The natural antisense mRNAs of HAS2 are able to regulate HAS2 mRNA levels and HA biosynthesis and may have a regulatory role in the control of HAS2, HA biosynthesis, and HA-dependent cell functions in vivo (Chao & Spicer 2005).

Upregulation of HASs has been found in tissue injury (Al'Qteishat et al. 2006, Li et al. 2000, Tammi et al. 2005, Yung et al. 2000), consistent with the findings that HA accumulates during a number of injuries. For example, HAS2 mRNAs are increased in rats after radiation-induced lung injury (Li et al. 2000). Ventilation-induced low-molecular-weight HA production is dependent on de novo synthesis of HA through HAS3 by fibroblasts and plays a role in the inflammatory response of ventilator-induced lung injury (Bai et al. 2005). Similarly, epidermal HA is significantly increased after epidermal trauma in adult mice caused by tape stripping (Tammi et al. 2005). The HA response is associated with a strong induction of HAS2 and HAS3 mRNA (Tammi et al. 2005). Increased accumulation of HA and increased HAS expression have been noticed in autoimmune (Feusi et al. 1999) and mechanical renal injury (Yung et al. 2000).

HYALURONAN DEGRADATION

Hyaluronidases (i.e., hyaluronoglucosaminidases, or HYALs) hydrolyze the hexosaminidic β(1→4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in HA. These enzymes also hydrolyze β(1→4) glycosidic linkages between N-acetyl-galactosamine or N-acetylgalactosamine sulfate and glucuronic acid in chondroitin, chondroitin 4- and 6-sulfates, and dermatan. Some bacteria, such as *Staphylococcus aureus*, *S. pyogenes*, and *Clostridium perfringens*, produce hyaluronidases as a means for greater bacterial mobility through the body's tissues and as an antigenic disguise that prevents recognition of bacteria by

HYAL:
hyaluronoglucosaminidase

phagocytes (Kreil 1995). In human, there are six hyaluronidases identified thus far: hyaluronidase 1–4, PH-20, and HYALP1.

The HYAL1 gene encodes a hyaluronidase found in the major parenchymal organs such as liver, kidney, spleen, and heart. HYAL1 is also present in human serum (Csoka et al. 2001) and urine (Csoka et al. 1997). The enzyme intracellularly degrades HA, is active at an acidic pH, and is the major hyaluronidase in plasma (Csoka et al. 2001). Mutations in the HYAL1 gene are associated with mucopolysaccharidosis type IX, or hyaluronidase deficiency (Natowicz et al. 1996).

HYAL2 was initially thought to be a lysosomal enzyme (Lepperdinger et al. 1998) and was later identified as a glycosylphosphatidylinositol-anchored cell-surface receptor (Rai et al. 2001) in all mouse tissue types except brain. HYAL2 has very low hyaluronidase activity compared with serum hyaluronidase HYAL1 (Rai et al. 2001), and HYAL2 hyaluronidase activity has a pH optimum of less than 4 (Lepperdinger et al. 1998). Also in contrast to HYAL1, the HYAL2 enzyme hydrolyzes only HA of high molecular weight, yielding intermediate-sized HA fragments of approximately 20 kDa, which are further hydrolyzed to small oligosaccharides by PH-20 (Lepperdinger et al. 1998). HYAL2 serves as a receptor for Jaagsiekte sheep retrovirus (Rai et al. 2001).

HYAL3 transcripts show strongest expression in testis and bone marrow but relatively weak expression in other organs (Csoka et al. 2001). The role of HYAL3 in the degradation of HA is not clear, and there are no studies that demonstrate hyaluronidase activity of HYAL3 to date.

The testicular enzyme PH-20 is encoded by the SPAM1 (sperm adhesion molecule 1) gene (Lathrop et al. 1990). This glycosylphosphatidylinositol-anchored enzyme is located on the human sperm surface and inner acrosomal membrane. During most mammalian fertilization events, hyaluronidase is released by the acrosome of the sperm cell after it has reached the oocyte. Upon contact with the egg, PH-20 hydrolyzes the HA present in its outermost, HA-rich cumulus layer surrounding the oocyte and the HA in the zona pellucida, thus enabling conception (Cherr et al. 1996). PH-20 hyaluronidase is active at neutral pH. The majority of enzyme activity in acrosome-intact sperm extracts occurs at neutral pH, whereas the soluble hyaluronidase activity released at the acrosome reaction is active predominantly in acid (Cherr et al. 1996).

Reactive oxygen species accumulate at sites of tissue injury and may provide a mechanism for generating HA fragments in vivo. Reactive oxygen species degrade ECM components such as collagen, laminin, and HA in vitro (Bates et al. 1984). This may further exaggerate the inflammation state at the sites of tissue injury because the fragmented HA in turn augments inflammatory responses. However, HA has the capacity to absorb reactive oxygen species, playing an active role in protecting articular tissues by scavenging reactive oxygen species (Sato et al. 1988). It is unknown if active HA fragments are generated by this process.

Expression and Regulation of Hyaluronidases in Health and Disease

Expression and activity of hyaluronidases have long been noticed in diseases such as rheumatoid arthritis (Regan & Meyer 1950) and periodontal disease (Hershon 1971). Patients with advanced scleroderma have decreased serum Hyal-1 activity and elevated circulating levels of HA (Neudecker et al. 2004). In one study, six patients with bone or connective tissue abnormalities had lower levels of Hyal-1 activity than did healthy donors (Fiszer-Szafarz et al. 2005).

HYALURONAN-BINDING PROTEINS

HA covalently binds to a variety of proteins (HABPs), also referred to as hyaladherins

HABPs:
hyaluronan-binding proteins, also called hyaladherins

(Toole 1990). These include receptors such as CD44, RHAMM (receptor for hyaluronan-mediated motility expressed protein), and LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1). Some hyaladherins are associated with cell membranes, whereas others are found in the extracellular matrix. Structurally, link module (Kohda et al. 1996) and the B(X$_7$)B motif (Yang et al. 1994) are thought to constitute the HA-binding region. The solution structure of the link module from human TNF-α-stimulated gene-6 (TSG-6) was determined and found to consist of two alpha helices and two antiparallel beta sheets arranged around a large hydrophobic core (Kohda et al. 1996). This module defines the consensus fold for the link-domain superfamily (Kohda et al. 1996). In addition, Goetinck and associates identified that the sites for interaction with HA are in the tandemly repeated sequences of link protein and that there are four potential sites available for that interaction (Goetinck et al. 1987). Turley and colleagues determined that the B(X$_7$)B motif (where B is arginine or lysine and X is any nonacidic amino acid) is a minimal binding requirement for HA in RHAMM, CD44, and link protein (Yang et al. 1994) and is observed in many HABPs.

Human cartilage link protein (CRTL1) is 354 amino acid residues long (Osborne-Lawrence et al. 1990). Although proteoglycan link protein genes have been excluded as the cause of disease in the families of patients with pseudoachondroplasia (Hecht et al. 1992), mice lacking link protein do develop dwarfism and craniofacial abnormalities (Watanabe & Yamaguchi 1999), suggesting that HA–link protein interactions are important for the formation of proteoglycan aggregates and normal organization of hypertrophic chondrocytes. Furthermore, the cartilage-specific transgene expression of link protein completely prevents the perinatal mortality in link protein–deficient mice and rescues the skeletal abnormalities (Czipri et al. 2003).

CD44 is the major cell-surface HABP (Aruffo et al. 1990). CD44 is a polymorphic type I transmembrane glycoprotein whose diversity is determined by differential splicing of at least 10 variable exons encoding a segment of the extracellular domain, termed exons v1–10, and by cell-type-specific glycosylation (Lesley et al. 1993). Most cells express the standard isoform, which is an 85-kDa protein that undergoes posttranslational modification (Lesley et al. 1993). Most cells—including stromal cells such as fibroblasts and smooth muscle cells, epithelial cells, and immune cells such as neutrophils, macrophages, and lymphocytes—all express CD44 (Sherman et al. 1994). HA-CD44 interactions may play an important role in development, inflammation, T cell recruitment and activation, and tumor growth and metastasis (Lesley et al. 1993). Although glycosaminoglycan side chains associated with some CD44 isoforms can bind a subset of heparin-binding growth factors, cytokines, and ECM proteins such as fibronectin, most of the functions ascribed to CD44 thus far can be attributed to its ability to bind and internalize HA (Sherman et al. 1994).

Mice with a targeted deletion of standard CD44 and all isoforms develop normally (Schmits et al. 1997). Studies have suggested an important role for CD44 in inflammatory states such as rheumatoid arthritis (Mikecz et al. 1995) and the extravasation of T cells to sites of tissue inflammation (DeGrendele et al. 1997). CD44 may have an important role in the recruitment of inflammatory cells in allergen-induced airway inflammation in mice (Katoh et al. 2003). More recently, CD44 has been suggested to play a critical role in regulating chronic inflammation (Hayer et al. 2005), suggesting that CD44 may have a critical role in regulating macrophage activation independently of interactions with HA.

RHAMM binds to biotinylated HA (Hardwick et al. 1992). It is believed to be an HA receptor involved in cell locomotion (Hardwick et al. 1992). Transfection experiments in fibroblasts suggested that RHAMM plays a role in Ras-dependent oncogenesis (Hall et al. 1995), but this role has been

RHAMM: receptor for hyaluronan-mediated motility expressed protein

LYVE-1: lymphatic vessel endothelial hyaluronan receptor-1

challenged by others (Hofmann et al. 1998), reflecting the complexity of the interactions between HA and HABPs during biological and pathological conditions. Nevertheless, RHAMM-HA interactions have an important role in tissue injury and repair (Zaman et al. 2005).

LYVE-1 was cloned as a lymph-specific HA receptor on the lymph vessel wall (Banerji et al. 1999). It is a type I integral membrane glycoprotein, contains a putative link module, and binds both soluble and immobilized HA (Banerji et al. 1999). Although it has been believed to be lymph specific, LYVE-1 is also present in normal hepatic blood sinusoidal endothelial cells in mice and humans (Mouta Carreira et al. 2001). LYVE-1 plays a role in the transport of HA from tissue to lymph by uptaking HA via lymphatic endothelial cells. Despite the implied importance of this molecule, LYVE-1-deficient mice are grossly normal (Gale et al. 2006, Huang et al. 2006). One study did not observe obvious alterations in lymphatic vessel ultrastructure or function or any apparent changes in secondary lymphoid tissue structure or cellularity in LYVE-1-deficient mice (Gale et al. 2006). This suggests that LYVE-1 is not obligatory for normal lymphatic development and function and that either compensatory receptors exist or LYVE-1 has a more specific role than previously envisaged (Gale et al. 2006). However, a second study identified morphological and functional alterations of lymphatic capillary vessels in certain tissues, marked by constitutively increased interstitial-lymphatic flow and a lack of typical irregularly shaped lumens in LYVE-1-deficient mice (Huang et al. 2006).

HARE (hyaluronan receptor for endocytosis, also called stabilin-2) was identified from sinusoidal endothelial cells of liver, lymph node, and spleen (Zhou et al. 2000). The HARE protein features multiple $B(X_7)B$ HA-binding motifs, several fasciclin-like adhesion domains, and 18–20 epidermal growth factor domains (Politz et al. 2002). HARE protein binds to HA (Harris et al. 2007), and a blocking antibody blocks this binding (Zhou et al. 2000).

HYALURONAN AS A SIGNALING MOLECULE

Role of Hyaluronan Fragment Size

In its native state, such as in normal synovial fluid, HA exists as a high-molecular-weight polymer, usually in excess of 10^6 Da. However, under inflammatory conditions HA is more polydisperse, with a preponderance of lower-molecular-weight forms (Saari & Konttinen 1989). West and colleagues showed that oligosaccharides derived from high-molecular-weight HA have distinct functions compared with the larger molecular forms of HA (West et al. 1985). HA oligomers of 8–16 disaccharides stimulated angiogenesis in vivo and endothelial proliferation in vitro; in contrast, native, high-molecular-weight HA had no effect (West et al. 1985). Fragmented HA containing 6 mers to 40 mers enhanced CD44 cleavage by tumor cells, whereas large polymer HA failed to enhance CD44 cleavage (Sugahara et al. 2003). A 6.9-kDa HA (36 mers) also promoted tumor cell motility in a CD44-dependent manner (Sugahara et al. 2003). Enhanced expression of matrix metalloproteinase (MMP)-9 and MMP-13 was induced in lung carcinoma cells by only small HA fragments containing 6 mers to 40 mers but not by HA preparations with molecular weight greater than 600 kDa (Fieber et al. 2004). Similarly, Termeer and colleagues demonstrated that HA oligomers of tetra- and hexasaccharide size, but not higher-molecular-weight HA species, induced immunophenotypic maturation of human monocyte-derived dendritic cells (Termeer et al. 2000).

We have found that lower-molecular-weight forms of HA (<500 kDa, many preparations in 100–250 kDa), but not the native form (>1000 kDa), induce inflammatory responses in inflammatory but not resident macrophages (Hodge-Dufour et al. 1997;

a

b
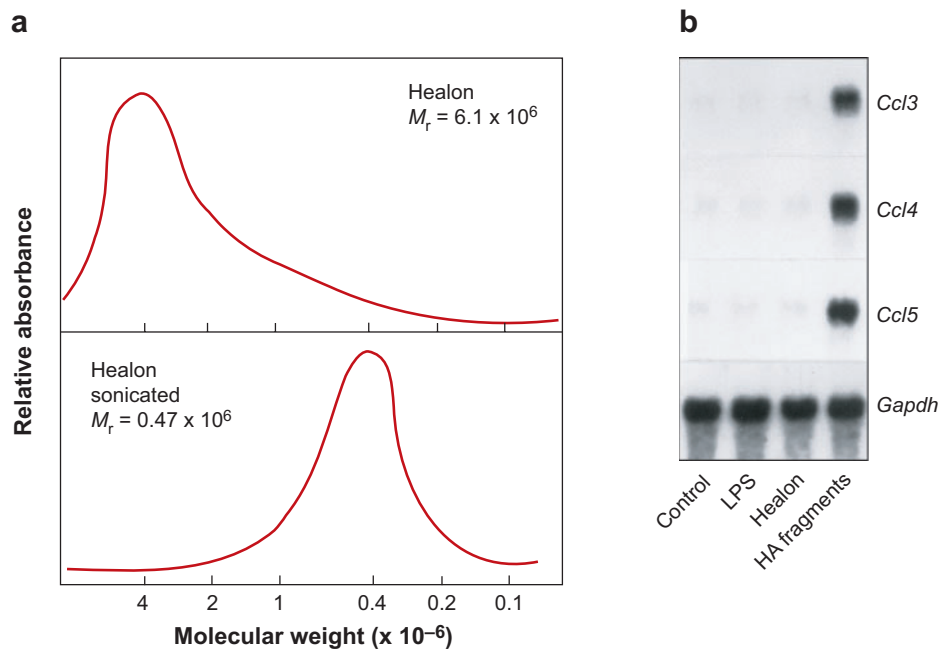
Size of hyaluronan (HA) fragments. (*a*) Size distribution of HA preparations. The molecular weight of purified Healon HA (*top panel*) and of HA fragments generated by sonicating purified Healon HA for 2 min (*bottom panel*). (*b*) HA fragments but not high-molecular-weight HA induce chemokine gene expression in MH-S cells demonstrated by Northern analysis. MH-S cells were stimulated with medium alone (lane 1), lipopolysaccharide (LPS) (lane 2), Healon HA (lane 3), or the sonication-generated HA fragments (lane 4), for 4 h at 37°C (the LPS inhibitor polymixin B was included in all treatments). Adapted from McKee et al. (1996). *Ccl*, chemokine (C-C motif) ligand; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

Horton et al. 1998a,b, 1999; Jiang et al. 2005; McKee et al. 1996, 1997; Noble et al. 1996) (**Figure 2**). Subsequently, a number of laboratories have found similar results with a variety of cell types, supporting the concept that HA fragments potentially generated at sites of inflammation, but not native high-molecular-weight HA, can stimulate the production of inflammatory mediators by many cell types. For example, fragmented HA ($0.08–0.8 \times 10^6$ Da), but neither purified high-molecular-weight HA (Healon) nor HA hexamers, markedly increased monocyte chemoattractant protein-1 (MCP-1) mRNA and protein expression (Beck-Schimmer et al. 1998) and intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) steady-state mRNA

and cell-surface expression by murine kidney tubular epithelial cells (Oertli et al. 1998).

Noninfectious Model of Lung Injury and Repair

Intratracheal administration of bleomycin is a commonly used murine model of noninfectious epithelial cell injury in sensitive strains such as C57Bl/6 mice (Adamson & Bowden 1974). Bleomycin is an antitumor antibiotic produced by fungi and has two main structural components: a bithiazole component that partially intercalates into the DNA helix, parting the strands, and pyrimidine and imidazole structures, which bind iron and oxygen, forming an activated complex capable of releasing damaging oxidants in

close proximity to the polynucleotide chains of DNA. In addition, bleomycin is capable of causing cell damage independently of its effects on DNA breakage by the induction of lipid peroxidation. Bleomycin is used in the treatment of many malignant tumors, such as germ cell tumors, lymphomas, head and neck tumors, and Kaposi's sarcomas. However, bleomycin has significant pulmonary toxicity, limiting its clinical usage. Nevertheless, intratracheal installation of bleomycin in mice has been an excellent animal model to study lung injury and repair (Adamson & Bowden 1974). A number of laboratories have used the bleomycin model to investigate extensively the role of ECM turnover (Olman et al. 1996). As shown in **Figure 3**, following lung injury, there is an influx of inflammatory cells, and peak lung injury is between days 5–9 in rats and mice (Nettelbladt et al. 1989). Concurrently, the inflammatory

cells and stromal cells at the injury site produce chemokines, cytokines, and other inflammatory/immunoregulatory substances. Interestingly, maximal HA content in the lung occurs at the time of this peak injury (Nettelbladt et al. 1989). The accumulation of HA occurs in both the alveolar and interstitial spaces and has been proposed as an important mechanism for edema formation because of the hydrophilic properties of HA (Nettelbladt et al. 1989). The lung has a tremendous capacity to clear HA that appears to commence at the time of peak injury such that over the ensuing 7–10 days HA levels return to near-baseline levels. Collagen deposition then begins in earnest once the HA content is restored to these levels. In addition, work from many laboratories as well as from our own laboratory has shown that HA species of lower molecular weight accumulate in the lung following bleomycin lung injury (Nettelbladt et al. 1989, Teder et al. 2002). These studies suggest that the HA breakdown products following lung injury may have important roles in the host response to exogenous injury.

HA fragments (0.22×10^6 Da) were found in BAL fluid in the bleomycin model of lung injury (Nettelbladt et al. 1989). HA synthesis and degradation appear to be an important response to tissue injury. Lower-molecular-weight HA species accumulate in wild-type mice after bleomycin-induced lung injury, and clearance is dependent upon hematopoietic CD44 (Teder et al. 2002). Four distinct molecular weight peaks in CD44$^{-/-}$ mice were detected, with an accumulation of smaller fragments not observed in the wild-type mice after bleomycin treatment, suggesting that CD44 plays a critical role in HA homeostasis following lung injury. In particular, CD44 appears to be critical to clear low-molecular-weight HA from sites of tissue injury. Another model of acute lung injury utilized high tidal volume ventilation, and investigators found that two low-molecular-weight peaks (at 180 kDa and 370 kDa) and one high-molecular-weight peak (at
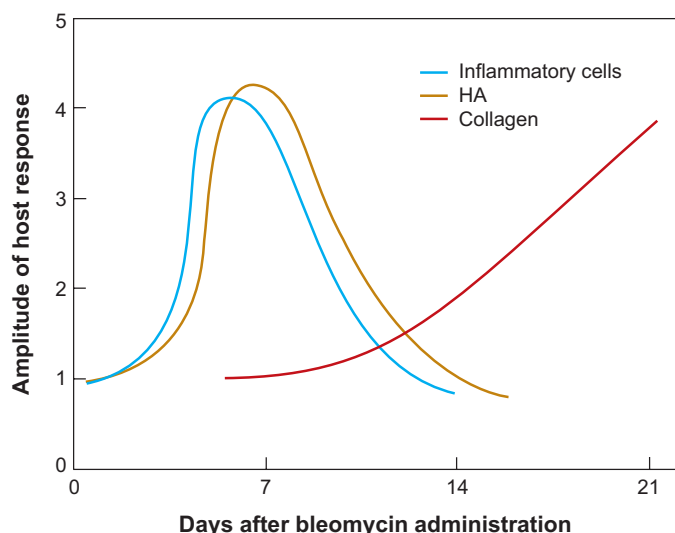
Figure 3

Bleomycin lung injury model. Bleomycin was given intratracheally to mice or rats to induce lung injury. In rats and mice, an influx of inflammatory cells peaks between days 5–9 after bleomycin. Simultaneously, hyaluronan (HA) content accumulates and also peaks at days 5–9 after bleomycin injury. Inflammatory cells and stromal cells at the injury site release significant amounts of chemokines and cytokines (not shown). Collagen deposition then begins once the HA content is restored to near-baseline levels. The amplitude of the response is represented in arbitrary units.

3100 kDa) of HA accumulated compared with control nonventilated animals, in which only the high-molecular-weight (3100 kDa) form accumulated (Bai et al. 2005, Mascarenhas et al. 2004). A low-molecular-weight (3–10 disaccharide) form of HA can be detected in postmortem tissue and in serum of patients at 1, 3, 7 and 14 days (peaking at 7 days) after ischemic stroke (Al'Qteishat et al. 2006).

The accumulation of low-molecular-weight HA fragments occurs by a variety of mechanisms, including depolymerization by reactive oxygen species, enzymatic cleavage, and de novo biosynthesis (Saari 1991, Sampson et al. 1992). Degradation by hyaluronidases may generate HA fragments as well because these enzymes are intracellular glycosidases that require low pH and can be released upon cell death at sites of inflammation. Additionally, lung fibroblasts release hyaluronidase after stimulation with interferon and TNF-α (Sampson et al. 1992). However, the precise mechanisms for generating HA fragments in vivo remain unknown.

Hyaluronan-Inducible Genes

There is increasing evidence that HA functions as more than a structural scaffold and is a dynamic molecule that influences cell behavior. One of the mechanisms of HA regulation of cell behavior is through the induction of a program of gene expression. More recently, a number of laboratories have shown that HA can induce the expression of genes in fibroblasts, kidney epithelial cells, eosinophils, and cells derived from amniotic membranes (Fitzgerald et al. 2000, Oliferenko et al. 2000, Termeer et al. 2000). Several studies have suggested that CD44 mediates this signaling (Oertli et al. 1998). Work from our own laboratory has suggested that HA fragments in the 200-kDa range induce in macrophages the expression of a number of inflammatory mediators, including chemokines, cytokines, growth factors, proteases, and nitric oxide (Horton et al. 1998a,b, 1999; McKee et al. 1996, 1997; Noble et al. 1993, 1996). Other laboratories

(Fitzgerald et al. 2000, Oliferenko et al. 2000, Termeer et al. 2000) have obtained similar results. We have provided data that CD44 is important in mediating HA induction of chemokine expression in macrophages by partially inhibiting HA fragment signaling in the presence of anti-CD44 antibodies (Hodge-Dufour et al. 1997). However, HA signaling still occurs in the absence of the standard form of CD44, suggesting that CD44 is not essential, other receptors are involved, or lack of CD44 can be compensated for in the CD44-deficient animal (Khaldoyanidi et al. 1999). CD44 may also have a role in fibroblast migration and survival (Henke et al. 1996). These studies support a concept in which the modified matrix regulates inflammation and repair processes during tissue injury.

However, high-molecular-weight HA prevents liver injury by reducing proinflammatory cytokines in a T cell–mediated injury model (Nakamura et al. 2004). High-molecular-weight HA downregulates aggrecanase-2 as well as TNF-α, IL-8, and inducible nitric oxide synthase in fibroblast-like synoviocytes, suggesting that high-molecular-mass HA may have a structure-modifying effect and an anti-inflammatory effect for osteoarthritis (Wang et al. 2006). High-molecular-weight fragmented HA that may arise at sites of tissue injury may have different effects on cell behavior.

CD44-Dependent Signaling Pathway

Numerous studies suggest that HA fragments may signal through a CD44-dependent tyrosine kinase pathway. For example, HA fragment–induced ERK1/2 (extracellular signal–related kinase 1 and 2) activation and proliferation of endothelial cells are CD44 receptor mediated (Slevin et al. 1998). Exogenous HA enhances hematopoiesis through two different HA receptors on bone marrow macrophage–like cells. One of the receptors, CD44, links to a pathway activating p38 mitogen-activated protein (MAP) kinase, whereas the other, as-yet-unknown receptor

induces Erk activity (Khaldoyanidi et al. 1999). HA-CD44 interaction with one of the Rho signaling components serves as a signal integrator by modulating Cdc42 cytoskeletal function, mediating Elk-1-specific transcriptional activation, and coordinating cross talk between a membrane receptor CD44 and a nuclear-hormone-receptor signaling pathway during ovarian cancer progression (Bourguignon et al. 2005). The cumulative interpretation of these data is that the role of CD44 in regulating HA interactions depends on the cell type.

Rho-Dependent GTPase Pathway

The interaction of CD44 with a RhoA-specific guanine nucleotide exchange factor (GEF) in human head and neck squamous carcinoma cells suggests that CD44 interaction with leukemia-associated RhoGEF (LARG) and epidermal growth factor receptor (EGFR) plays a pivotal role in Rho/Ras coactivation, phospholipase Cε (PLCε)-Ca^{2+} signaling, and Raf/ERK upregulation, which are required for calcium/calmodulin-modulated kinase II (CaMKII)-mediated cytoskeleton function and in head and neck squamous cell carcinoma progression (Bourguignon et al. 2006). In addition, a previously unrecognized pathway for cell migration and invasion that is HA dependent and involves Ras activation has been described and may account for the developmental phenotype observed in CD44-null mice (Camenisch et al. 2000).

HYALURONAN AS AN IMMUNE REGULATOR

Monocytes, Dendritic Cells, and Macrophages

There are several reports demonstrating that HA fragments can influence dendritic cell maturation. Goldstein and colleagues showed that 135-kDa fragments of HA induce dendritic cell maturation and initiate alloimmunity (Tesar et al. 2006). Activation of dendritic cells with HA enhances their ability to stimulate allogeneic and antigen-specific T cells markedly (Do et al. 2004). CD44 expression on T cells but not dendritic cells plays a critical role in antigen-specific T cell responsiveness (Do et al. 2004). Langerhans cells are skin-specific members of the dendritic cell family and crucial for the initiation of cutaneous immune responses. Systemic, local, or topical administration of HA blocking peptide prevented hapten-induced Langerhans cell migration from the epidermis and inhibited the hapten-induced maturation of Langerhans cells in vivo (Mummert et al. 2000). Small HA fragments of tetra- and hexasaccharide size increase dendritic cell production of the cytokines interleukin (IL)-1β, TNF-α, and IL-12 as well as their allostimulatory capacity (Termeer et al. 2000). These small HA fragments induce dendritic cell maturation independently of CD44 or RHAMM and are dependent upon Toll-like receptor (TLR) 4 (Termeer et al. 2002).

T Cells

The interaction of cell-surface HA on T cells with CD44 is manifested by polarization, spreading, and colocalization of cell-surface CD44 with a rearranged actin cytoskeleton. Thus, cytokines and chemokines present in the vicinities of blood vessel walls or present intravascularly in tissues where immune reactions take place can rapidly activate the CD44 molecules expressed on T cells (Ariel et al. 2000). Naive splenic T cells did not bind fluoresceinated HA constitutively. HA binding requires the activation of splenic T cells by a CD44-specific monoclonal antibody (Lesley et al. 1992). Lesley and colleagues suggested that CD44 functions associated with HA binding involve a regulated process (Lesley et al. 1992).

Eosinophils

HA stimulates the growth of CD34$^+$ progenitor cells into specifically differentiated,

mature eosinophils (Hamann et al. 1995). Both TNF-α (with fibronectin) and HA contribute to long-term eosinophil survival in vivo by enhancing granulocyte macrophage colony stimulating factor (GM-CSF) production in asthma conditions (Esnault & Malter 2003).

Fibroblasts

HA stimulates IL-1β, TNF-α, and IL-8 production in human uterine fibroblasts (Kobayashi & Terao 1997). However, cytokines, in particular TNF-α, can regulate HA production by human lung fibroblasts (Sampson et al. 1992). Interestingly, HA purified from stretch-induced fibroblasts caused a significant dose-dependent increase in IL-8 production both in static and stretched epithelial cells, indicating that de novo synthesis of low-molecular-weight HA is induced in lung fibroblasts by stretch and may play a role in augmenting the induction of proinflammatory cytokines in ventilator-induced lung injury (Mascarenhas et al. 2004). The production of low-molecular-weight HA during ventilator-induced lung injury is dependent on de novo synthesis through HAS3 (Bai et al. 2005).

Endothelial Cells

Both cell-surface and intracellular HA receptors have been isolated from rat liver endothelial cells (Raja et al. 1988). HA expressed by dermal endothelium in acute graft-versus-host disease supports lymphocyte adherence under flow conditions, and CD44-HA interactions contribute to lymphocyte recruitment to skin in acute graft-versus-host disease (Milinkovic et al. 2004). Human pulmonary endothelial cells and lung microvessel endothelial cells bind HA (Lokeshwar & Selzer 2000). Both HA and HA fragments (10–15 disaccharide units) induce tyrosine phosphorylation of p125(FAK), paxillin, and p42/44 ERK in primary pulmonary endothelial cells and lung microvessel endothelial cells (Lokeshwar & Selzer 2000). HA-induced proliferation of endothelial cells is CD44 receptor mediated and accompanied by early-response-gene activation (Slevin et al. 1998).

HARE/stabilin-2 is found on sinusoidal endothelial cells of liver, lymph node, and spleen (Zhou et al. 2000). HARE/stabilin-2, a transmembrane protein, binds to HA, advanced glycation end product–modified proteins, and oxidized lipids. HARE mediates the systemic clearance of HA from the circulatory and lymphatic systems (Zhou et al. 2000). HARE/stabilin-2 (not stabilin-1) is associated with clathrin and adaptor protein-2 (Hansen et al. 2005). Systemic clearance of HA by HARE/stabilin-2 is through a clathrin-dependent, coated pit–mediated uptake (Zhou et al. 2000).

HA stimulates the locomotion of adult bovine aortic smooth muscle cells. Bovine aortic smooth muscle cells and their association with the cell monolayer increased following wounding injury, which is dependent on the presence of RHAMM (Savani et al. 1995).

HYALURONAN SIGNALING THROUGH TOLL-LIKE RECEPTORS

Independence of CD44

CD44 is the major cell-surface receptor for HA, and HA binds to CD44 in many cell types (Aruffo et al. 1990). Although some of the antibody experiments suggest that anti-CD44 antibodies partially inhibit HA fragment signaling in macrophages (Hodge-Dufour et al. 1997), other studies suggest that anti-CD44 antibodies have no effect on macrophage metalloelastase (MME) expression (Horton et al. 1999). With the aid of CD44-deficient mice, studies have demonstrated that HA is able to stimulate chemokine production in the absence of CD44, suggesting that CD44 is not required to mediate HA signaling (Fieber et al. 2004, Jiang et al. 2005, Khaldoyanidi et al. 1999). Exaggerated inflammation

associated with the failure of HA clearance in CD44-deficient mice after bleomycin administration induces lung injury, suggesting that HA degradation products are still able to induce chemokine and cytokine expression in vivo (Teder et al. 2002). Similarly, the HA-induced activation of dendritic cells is independent of CD44 (Do et al. 2004). These studies demonstrated that, although CD44 is the major cell-surface receptor and mediates HA binding, it is not required to mediate HA fragment signaling in certain cell types.

Hyaluronan Structure Has the Features of Pathogen-Associated Molecular Patterns

HA is a component of the cell coat of groups A and C of *Streptococcus* (Lowther & Rogers 1955, Maclennan 1956) and *P. multocida* (Carter 1972). The repeating disaccharide structure of HA has features of pathogen-associated molecular patterns (PAMPs). Many PAMPs on pathogens utilize TLRs to initiate innate immune responses. These structural features suggest that HA modified by the inflammatory milieu may attain functions consistent with that of a PAMP.

Toll-Like Receptors and Ligands

The innate immune system uses TLRs to recognize microbes and initiate host defense (Kopp & Medzhitov 1999). Ten TLR genes in humans and 13 genes in mice have been identified so far. TLR4 has been unequivocally identified as the transmembrane signaling protein required for LPS signal transduction in macrophages (Takeuchi et al. 1999). TLR2 is the mediator of macrophage recognition of mycobacteria (Underhill et al. 1999) and gram-positive organisms (Takeuchi et al. 1999). TLR9 mediates macrophage activation by bacterial DNA CpG motifs (Hemmi et al. 2000), whereas TLR5 and TLR7 recognize bacterial flagellin (Hayashi et al. 2001) and single-stranded RNA (Diebold et al. 2004), respectively.

Signaling Pathway

TLR family receptors share a structural similarity as well as a similar signaling pathway with IL-1 receptors (Barton & Medzhitov 2003). Upon ligand binding to TLR, the adaptor molecule MyD88 is recruited to the TLR complex as a dimer (Medzhitov et al. 1998). Binding of MyD88 promotes association with IL-1 receptor–associated kinase-4 (IRAK-4) and IRAK-1 (for reviews, see Akira 2006 and Miggin & O'Neill 2006). TNF-associated factor 6 (TRAF6) is recruited to IRAK-1. The IRAK-4/IRAK-1/TRAF6 complex dissociates from the receptor and then interacts with another complex consisting of TGF-β-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), and TAB2 (for reviews, see Akira 2006 and Miggin & O'Neill 2006). TAK1 is subsequently activated in the cytoplasm, leading to the activation of IκB kinase kinases (IKKs) (for reviews, see Akira 2006 and Miggin & O'Neill 2006). IKK activation leads to the phosphorylation and degradation of IκB and consequent release of nuclear factor-κB (NF-κB). Once translocated into the nucleus, NF-κB activates inflammatory chemokines and cytokines.

In addition to MyD88, other Toll/interleukin-1-receptor (TIR)-domain-containing adaptor proteins have been identified and characterized. For example, TIRAP (TIR-domain-containing adaptor protein), TRIF (TIR-domain-containing adaptor protein inducing interferon-β), and TRAM (TRIF-related adaptor molecule) mediate MyD88-independent induction of interferons, which in turn activates the expression of interferon-inducible genes such as *CXCL10* (for reviews, see Akira 2006 and Miggin & O'Neill 2006). In particular, TLR4 is subsumed by a MyD88-independent pathway (Yamamoto et al. 2003). In addition, some TLRs appear to require dimerization for signaling. This raises the possibility that more than one TLR is involved in responsiveness to a single pathogen.

Demonstration of Hyaluronan Signaling Through Toll-Like Receptors

To investigate the potential role for TLRs in mediating HA signaling, we utilized elicited peritoneal macrophages from mice deficient in either MyD88, TLR1, TLR2, TLR3, TLR4, TLR5, or TLR9. Stimulation of chemokine gene expression by HA fragments was abolished in the MyD88-deficient macrophages (Jiang et al. 2005). Chemokine macrophage inflammatory protein 2 (MIP-2) expression was reduced but remained present in both TLR2- and TLR4-deficient macrophages. We reasoned that HA signaling may require both TLR2 and TLR4. To this end, TLR4- and TLR2-deficient mice were then crossed to generate TLR2 and TLR4 double-deficient (TLR2$^{-/-}$TLR4$^{-/-}$) mice. HA fragment–induced chemokine and cytokine expression was completely abolished in TLR2$^{-/-}$TLR4$^{-/-}$ peritoneal macrophages (Jiang et al. 2005). These data suggest that the polymeric properties of HA mimic cell-wall components from both gram-positive and gram-negative organisms. In contrast, targeted deletion of TLR1, TLR3, TLR5, or TLR9 had no effect on HA fragment–induced chemokine expression. Caveats in all in vitro studies invoking TLRs include the need to exclude contaminating TLR agonists. Although great lengths can be taken to exclude contaminating substances (Jiang et al. 2005, Scheibner et al. 2006), concerns regarding the interpretation of in vitro studies with HA fragments still remain, particularly those from commercial sources (Scheibner et al. 2006, Tsan & Gao 2004).

To determine if these findings were relevant to acute lung injury in patients, we examined circulating HA fragments produced in vivo and purified from the serum of patients with acute lung injury. The human HA degradation products were of similar molecular weight (peak at 200 kDa) to the in vitro–generated HA degradation products and stimulated chemokine production in wild-type macrophages but not in either TLR2$^{-/-}$TLR4$^{-/-}$ or MyD88$^{-/-}$ peritoneal macrophages (Jiang et al. 2005).

Dendritic Cell Activation by Hyaluronan Through Toll-Like Receptors

HA oligosaccharides induce maturation of dendritic cells via TLR4 (Termeer et al. 2002). HA oligosaccharide treatment results in distinct phosphorylation of p38/p42/44 MAP kinases, nuclear translocation of NF-κB, and TNF-α production (Termeer et al. 2002). Priming of alloimmunity by HA-activated dendritic cells is dependent on signaling via TIRAP, a TLR adaptor downstream of TLR2 and TLR4 (Tesar et al. 2006). However, this effect is independent of alternate TLR adaptors, MyD88, or TRIF (Tesar et al. 2006). We further demonstrated that HA accumulates during skin transplant rejection and suggested that fragments of HA can act as innate immune agonists that activate alloimmunity (Tesar et al. 2006). Horton and colleagues reported similar findings, using a commercial source of HA fragments, although they suggested a primary role for TLR2 (Scheibner et al. 2006).

Endogenous Ligands for Toll-Like Receptors

Several studies have suggested that glycosaminoglycan degradation products can signal through TLRs. Oligosaccharides of HA activate dendritic cells and endothelial cells via TLR4 (Taylor et al. 2004, Termeer et al. 2002). HA fragments signal through TLR2 and TLR4 in macrophages (Jiang et al. 2005, Tesar et al. 2006). The maturation of dendritic cells by soluble heparan sulfate occurs through TLR4 (Johnson et al. 2002). In addition, numerous reports suggest that many of the other endogenous ligands for TLRs do not have the features of PAMPs (for reviews,

see Jiang et al. 2006 and Tsan & Gao 2004). Several inflammatory proteins or peptides, such as heat shock protein 60 (hsp60) family chaperones, have been suggested as ligands for TLR2 and/or TLR4 in macrophages and B lymphocytes (Ohashi et al. 2000). Stimulation of neutrophils, monocytes, or macrophages by high mobility group box 1 (HMGB1) requires both TLR2 and TLR4, resulting in the enhanced expression of proinflammatory cytokines (Park et al. 2004). Murine β-defensin 2 acts directly on immature dendritic cells as an endogenous ligand for TLR4 to induce dendritic cell maturation (Biragyn et al. 2002). The presence of the endogenous TLR ligands during tissue injury mimics that of the PAMPs in pathogens. Many immunostimulants, including defensins, cathelicidin, eosinophil-derived neurotoxin, and HMGB1, have been termed alarmins to recognize their role: serving as early warning signals to activate innate and adaptive immune systems (Yang & Oppenheim 2004). S100 proteins (S100A8, S100A9, and S100A12) are found at high concentrations in inflamed tissue, where neutrophils and monocytes belong to the most abundant cell types, and exhibit proinflammatory effects in vitro at concentrations found at sites of inflammation in vivo (Foell et al. 2007).

However, the view of endogenous TLR ligands faces some skepticism owing largely to the agonist contamination issue; most reagents have been prepared in microbial systems (Tsan & Gao 2004). Biochemical analysis to demonstrate direct binding between TLR and endogenous ligands is essential for this concept to receive wider acceptance. Data from in vivo studies (Jiang et al. 2005, Schaefer et al. 2005), in which ligand contamination is less likely to contribute to TLR signaling, support the concept of endogenous ligands for TLRs. For example, Schaefer and associates demonstrated in a set of elegant in vivo experiments that biglycan signals through TLR4 and TLR2 (Schaefer et al. 2005).

TOLL-LIKE RECEPTOR–HYALURONAN INTERACTIONS AND TISSUE INJURY

Toll-Like Receptor Deficiency and Lung Injury

Our discovery that both TLR2 and TLR4 are required for macrophages to express inflammatory genes in response to HA fragments suggested that TLR2/TLR4 deficiency should lead to an impaired inflammatory response to noninfectious lung injury. To test this hypothesis, we challenged TLR2$^{-/-}$TLR4$^{-/-}$ mice with noninfectious bleomycin-induced lung injury. TLR2$^{-/-}$TLR4$^{-/-}$ mice developed a reduced inflammatory response to lung injury, with a decrease in transepithelial neutrophil migration and reduced expression of MIP-2 (Jiang et al. 2005). However, these mice had a higher mortality rate than did wild-type mice. This surprising finding led us to inspect the degree of tissue injury and epithelial cell integrity. We found evidence to suggest increased tissue injury and epithelial cell apoptosis (Jiang et al. 2005). Thus, TLR2/TLR4 deficiencies appear to protect the host from acute inflammation but may impede epithelial cell repair processes important in tissue injury recovery.

Hyaluronan-Cell Interactions in Lung Injury

We then tested to determine if preventing HA action with an HA-blocking peptide could produce a similar phenotype as in TLR2$^{-/-}$TLR4$^{-/-}$ mice. An HA blocking peptide has been effective in vivo in inhibiting HA-cell interactions (Mummert et al. 2000, Taylor et al. 2004). Inhibition of HA binding with the peptide in vivo recapitulated the phenotype observed in the TLR2$^{-/-}$TLR4$^{-/-}$ mice after lung injury, enhanced lung injury, increased epithelial cell apoptosis, and decreased transmigration of neutrophils (Jiang

et al. 2005). After the administration of a high dose of bleomycin, increasing production of high-molecular-weight HA by overexpression of HAS2 under direction of the lung-specific CC10 promoter was protective against mortality, lung injury, and epithelial cell apoptosis (Jiang et al. 2005). Collectively, these studies suggested that HA may have several functions in tissue injury and repair, depending on the nature and location of hyaluronan and specific cell interactions.

Hyaluronan–Toll-Like Receptor Interactions on Epithelial Cells in Host Defense

Previous studies in kidney injury models have demonstrated that fragmented HA markedly stimulates monocyte chemoattractant protein-1 (MCP-1) production by renal tubular cells (Beck-Schimmer et al. 1998), triggers cell adhesion molecule expression through a mechanism involving the activation of NF-κB and activating protein-1 (Oertli et al. 1998), and promotes proximal tubule cell migration and reepithelialization through MAP kinase activation (Itano et al. 2004). Quinn and colleagues have shown that low-molecular-weight HA stimulates cytokine IL-8 production by lung epithelial cells, via the JAK2 kinase, not the MEK1/2, pathway (Mascarenhas et al. 2004). Injured epithelial cells may respond to HA fragments by producing chemokines and cytokines to recruit inflammatory cells to the injury sites. To investigate the hypothesis that HA and TLR interactions are important in lung injury and repair processes, we asked if HA on the epithelial cell surface plays a role in lung injury. Isolated lung alveolar epithelial cells have increased rates of apoptosis at baseline and exhibit greater apoptosis in response to bleomycin. The exogenous addition of high-molecular-weight HA is protective against apoptosis (Jiang et al. 2005). We found that bleomycin induces both NF-κB activation and apoptosis in primary lung epithelial cells (Jiang et al.

2005). These data suggest that HA may regulate basal NF-κB activation in epithelial cells. NF-κB regulates apoptosis (Lawrence et al. 2005), and HA fragments can activate NF-κB in macrophages (Noble et al. 1996). Primary epithelial cells from TLR2$^{-/-}$TLR4$^{-/-}$ mice have a significant increase in spontaneous apoptosis relative to wild type (Jiang et al. 2005). Furthermore, we made the remarkable observation that cell-surface HA is severely abrogated in TLR2$^{-/-}$TLR4$^{-/-}$ epithelial cells (Jiang et al. 2005). The mechanism underlying the abolishment of cell-surface HA in TLR2$^{-/-}$TLR4$^{-/-}$ epithelial cells is unknown. These data suggest that epithelial cell-surface HA promotes basal NF-κB activation in a TLR-dependent manner and that this activation has a protective effect against injury (O'Neill 2005).

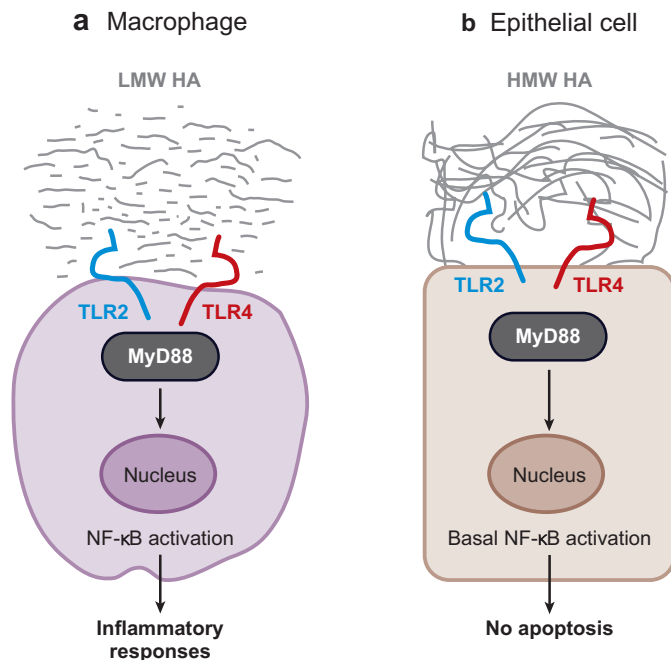## Toll-Like Receptors and Tissue Injury

Recent studies from Medzhitov and colleagues suggest that TLRs may have homeostatic functions in the gut epithelium. These investigators demonstrated that the stimulation of TLR by commensal intestinal flora is critical for protecting against intestinal epithelial injury (Rakoff-Nahoum et al. 2004). This elegant work emphasizes the importance of cross talk between factors involved in innate immunity and cytoprotection in the maintenance of intestinal epithelial homeostasis. Medzhitov and colleagues further demonstrated that the maintenance of intestinal epithelial homeostasis by the TLR recognition of molecular signatures from commensal intestinal bacteria depends on the presence of IL-10 (Rakoff-Nahoum et al. 2006).

Toll-Like Receptors in Noninfectious Tissue Injury

It is becoming clear that TLRs not only play a role in the recognition of pathogens and

in the initiation of immune responses but also have a fundamental role in noninfectious disease pathogenesis (for reviews, see Jiang et al. 2006 and Noble & Jiang 2006). Recently, several studies also examined the expression patterns of TLR2 and/or TLR4 in smoke-induced lung injury and in patients with chronic obstructive pulmonary disease (Karimi et al. 2006). Hemorrhage-induced lung TNF-α production, neutrophil accumulation, and protein permeability, but not NF-κB activation, are dependent on a functional TLR4 (Barsness et al. 2004). TLR4-TLR2 cross talk activates a positive-feedback signal,

leading to alveolar macrophage priming and exaggerated lung inflammation in response to invading pathogens during hemorrhage-induced acute lung injury (Fan et al. 2006). However, a recent study demonstrated that TLR4 has a protective role in oxidant-mediated injury by maintaining appropriate levels of antiapoptotic responses in the face of oxidant stress (Zhang et al. 2005). TLR4 plays a role in maintaining constitutive lung integrity by modulating oxidant generation, providing insights into the development of emphysema (Zhang et al. 2006). These studies are in accord with our study involving a bleomycin-induced lung injury model (Jiang et al. 2005).

CONCLUSIONS

The synthesis and degradation of ECM are fundamental components of tissue injury and repair. HA appears to have extraordinary functions in this biological process. Following tissue injury, HA fragments accumulate. The removal of HA fragments requires the HA receptor CD44, expressed on hematopoietic cells. The failure to remove HA from the lung following injury results in unremitting inflammation. Our work and the work of others have suggested that interactions of HA with innate immune receptors (TLRs) serve two essential functions in host defense against noninfectious lung injury (**Figure 4**). HA degradation products can stimulate inflammatory cells to produce chemokines and cytokines that recruit inflammatory cells to the site of injury to modulate and resolve tissue injury. In addition, cell-surface high-molecular-weight HA limits the extent of lung epithelial cell injury by providing a basal level of NF-κB activation, inhibiting apoptosis, and promoting the repair of parenchymal cell injury through a TLR-dependent mechanism. Future studies to ascertain more directly the role of HA in tissue injury and repair await the generation of cell-specific deletions in HASs.

Figure 4

Hyaluronan (HA) signals through Toll-like receptors TLR2 and TLR4. (*a*) Low-molecular-weight HA (LMW HA) fragments generated during tissue injury signal through TLR2 and TLR4 and an adaptor molecule, MyD88, to stimulate chemokine/cytokine expression in macrophages, leading to inflammatory responses. (*b*) High-molecular-weight hyaluronan (HMW HA) on the cell surface or surrounding epithelial cells signals through to TLR2 and TLR4 and MyD88, providing cells with basal nuclear factor-κB (NF-κB) activation. In turn, the tonic NF-κB activity prevents epithelial cells from undergoing apoptosis upon injury. Thus, HMW HA on epithelial cells provides cells with a survival signal. Adapted from Jiang et al. (2006).

SUMMARY POINTS

1. Extracellular matrix including the glycosaminoglycan hyaluronan (HA) accumulates and is degraded during tissue injury.

2. Fragmented HA stimulates the expression of inflammatory genes by inflammatory cells at the injury site.

3. The HA receptor CD44 is required to clear HA following lung injury, but HA signaling is independent of CD44 in inflammatory macrophages.

4. Signaling of HA fragments requires both TLR4 and TLR2 to stimulate inflammatory cells to produce inflammatory chemokines and cytokines.

5. Disruption of HA-TLR interactions results in exaggerated injury in a noninfectious lung injury model. HA-TLR interactions provide signals that initiate inflammatory responses, maintain epithelial cell integrity, and promote recovery from acute lung injury.

DISCLOSURE STATEMENT

We are not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

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