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Hyaluronan as an Immune Regulator in Human Diseases

PAUL W. NOBLE, **JIURONG LIANG**, and **DIANHUA JIANG**
Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, Duke University School of Medicine, Durham, North Carolina

Abstract

Accumulation and turnover of extracellular matrix components are the hallmarks of tissue injury. Fragmented hyaluronan stimulates the expression of inflammatory genes by a variety of immune cells at the injury site. Hyaluronan binds to a number of cell surface proteins on a variety of cell types. Hyaluronan fragments signal through both Toll-like receptor (TLR) 4 and TLR2 as well as CD44 to stimulate inflammatory genes in inflammatory cells. Hyaluronan is also present on the cell surface of epithelial cells and provides protection against tissue damage by interacting with TLR2 and TLR4 on these parenchymal cells. Hyaluronan and hyaluronan-binding proteins regulate inflammation, tissue injury and repair through regulating inflammatory cell recruitment, release of inflammatory cytokines, and stem cell migration. This review focuses on the role of hyaluronan as an immune regulator in human diseases.

I. HYALURONAN

Extracellular matrix (ECM) plays an essential role in organogenesis, growth, function, and in many human diseases. Hyaluronan (or hyaluronic acid, HA), a major ECM component, is a non-sulfated glycosaminoglycan composed of repeating polymeric disaccharides D-glucuronic acid and *N*-acetyl-D-glucosamine linked by a glucuronidic β(1→3) bond (463,464) (Figure 1. Hyaluronan structure). Hyaluronan forms specific stable tertiary structures in aqueous solution (374). During the last two decades, significant progress has been made in understanding the role of HA in both biological and pathological states, and the mechanisms of HA synthesis and degradation, and the mechanisms of HA regulation of these biological processes. There are several reviews on the roles of HA in different fields, such as in angiogenesis (388), reactive oxygen species (392), cancer (433,434), chondrocytes (200), lung injury (303,438), and in immune regulation (424). The current review will briefly summarize recent advances in understanding HA biology, and will focus on HA as an immune regulator in physiological and pathological conditions.

Hyaluronan is widely distributed from lower organisms such as simple bacteria (241,243) to complex eukaryotes (333). In humans, HA is abundant in the vitreous of the eye (271), the umbilical cord (463), synovial fluid (127,340), heart valves (435), skin (9,182,416), and skeletal tissues (9). HA can be released by many cell types (227,432), although mesenchymal cells are believed to be predominant (433). Importantly, almost all the cell types are responsive to HA stimulation by changing their cell secretory and behavioral properties (177). HA-stem cell interactions have been particularly explored in hematopoietic stem cells (15,193,301), mesenchymal stem cells (43,500,504), and adult multipotent progenitor cells, pointing to the idea that HA and its binding proteins may regulate tissue injury and repair processes through their interaction with a variety of stem cells.

CORRESPONDENCE: Paul W. Noble, MD, Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, Duke University School of Medicine, 106 Research Drive, Durham, North Carolina 27710, USA; Phone, (919) 681-0355; Fax, (919) 684-5266; paul.noble@duke.edu.

Hyaluronan has multiple functions, such as space filling, lubrication of joints, and provision of a matrix through which cells can migrate (433). HA is actively produced during tissue injury, tissue repair, and wound healing (182,390). In addition to providing a framework for ingrowths of blood vessels (388,390) and fibroblasts (234,439), HA also regulates many aspects of molecular mechanisms of tissue repair, such as activation of inflammatory cells to mount an immunological response (149,265,422) and regulation of behavior of epithelial cells (22,165,175,176,505) and fibroblasts (17,157). Elucidation of the role and mechanisms of HA is crucial in aiding the development of novel therapy for many diseases.

II. BIOSYNTHESIS OF HYALURONAN

Hyaluronan is synthesized by membrane-bound synthases on the inner surface of the plasma membrane (332), and the chains are extruded through pore-like structures into the extracellular space (333,455). There are three mammalian hyaluronan synthases (HAS) (159,260,397,460). The enzymes use UDP-α-N-acetyl-D-glucosamine and UDP-α-D-glucuronate as substrates (461) (Figure 2. HA synthase reaction). Based on their similarities and differences, the known HA synthase proteins have been divided into two categories, designated as Class I and Class II (71). The Streptococcal HA synthases and eukaryotic HA synthases are Class I family members, whereas the HA synthase from *Pasteurella multocida* is the only Class II member (71).

Much of our knowledge of the mechanism of HAS has been based on bacterial HA synthases (334,335). Using *Pasteurella multocida* as a model, Prehm proposed that a two-site mechanism in which the reducing end sugar of the growing HA chain would remain covalently bound to a terminal uridine diphosphate (UDP), and the next sugar to be added from the second site would be transferred as the UDP-sugar onto the reducing end sugar to displace its terminal UDP. The HA chain would then be in the second site (334,335).

The major advance in this field was fueled by the cloning of HAS from prokaryotes and mammals (72,73). The DeAngelis group reported the molecular cloning and characterization of the Group A *Streptococcal* gene encoding the protein HasA, as the *S. pyogenes* HAS was later proven to be responsible for HA synthesis (72,73). Expression of these genes in either acapsular Streptococcus strains or *Enteroccus faecalis* conferred the organisms with the ability to synthesize HA and form a capsule, thus demonstrating that HasA is a bona fide HA synthase (72,73). Subsequent cloning of mouse HAS (158,394) and human HAS (383,455) revealed high homology in their protein sequences among humans, mice, frogs, and even bacteria (395,461). The amino acid sequence of human HAS1 shows significant homology to the hasA gene product of Streptococcus pyogenes, a glycosaminoglycan synthase from Xenopus laevis, and a murine HA synthase (455). Genomic location and genomic structure of these HA synthases have been determined (396). Since HAS1, HAS2, and HAS3 are located on different autosomes (396), suggesting that the HAS gene family may have arisen comparatively early in vertebrate evolution by sequential duplication of an ancestral HAS gene.

All the HAS isozymes are highly homologous in their amino acid sequences and have similar hydropathic features, suggesting that they are similarly organized within the membrane. The Weigel group proposed membrane topology for the HAS family proteins (461). Two types of membrane domains are present: transmembrane domains that span the membrane and membrane-associated domains that do not go all the way through the membrane. There are 6 – 8 transmembrane domains and two membrane-associated domains. Over 60% of the whole protein (including the amino and carboxyl termini) are inside the cell. Only about 5% of the protein is exposed to the outside of the cell (136).

In *Xenopus*, xhas1 produces HA with a molecular mass of around 40–200 kDa, while the product formed by xhas2 has a molecular mass above 1000 kDa (206). Expression of mammalian HA synthases led to HA biosynthesis in transfected mammalian cells (160,395). The HAS1 protein alone is able to synthesize HA, and different amino acid residues on the cytoplasmic central loop domain are involved in transferring N-acetyl-D-glucosamine and D-glucuronic acid residues. HAS3 synthesizes HA with a smaller molecular mass than HA synthesized by HAS1 and HAS2 (160). Furthermore, comparisons of HA secreted into the culture media by stable HAS transfectants show that HAS1 and HAS3 generated HA with broad size distributions (molecular masses of 2×10^5 to approximately 2×10^6 Da), whereas HAS2 generated HA with a broad but extremely large size (average molecular mass of $> 2 \times 10^6$ Da) (160). Subsequent studies suggested that all three HAS enzymes drive the biosynthesis and release of high molecular weight HA (1×10^6 Da) (398).

A. Hyaluronan synthase 1 (HAS1)

Human HAS1 gene is located at 19q13.3-q13.4, whereas mouse Has1 is located at Chromosome 17 (396). Human HAS1 protein shows significant homology with the hasA gene product of *Streptococcus pyogenes*, a glycosaminoglycan synthetase (DG42) from *Xenopus laevis*, and a murine HA synthase (395). HAS1 protein is a bona fide synthase since it alone is able to synthesize HA and different amino acid residues on the cytoplasmic central loop domain are involved in transferring N-acetyl-D-glucosamine and D-glucuronic acid residues, respectively (488).

HA accumulation and dysregulated expression of HA synthases have been demonstrated in many diseases in both animal studies and in humans. HA deposition is prominent in MRL-Fas$^{(lpr)}$ mice with renal disease and could be mediated by local synthesis through HAS1 and HAS2. The enhanced synthesis of HA could be promoted by proinflammatory cytokines in vivo (96). Has1 mRNA was expressed predominantly in bone marrow mesenchymal progenitor cells derived from multiple myeloma patients when compared to mesenchymal progenitor cells from normal individuals (43). Bone marrow mesenchymal progenitor cells from myeloma synthesize more HA than those from healthy donors, suggesting that myeloma mesenchymal progenitor cells could be an important component of the myeloma pathophysiology in vivo by their increased expression of extracellular matrix components relevant to plasma cell growth and survival (43). Accumulation of HA is a hallmark of rheumatoid arthritis. In human fibroblast-like synoviocytes, HAS1 expression can be enhanced by TGF-β transcription. The TGF-β-induced transcription is mediated by a p38 MEK dependent, not JNK, pathway. TGF-β treatment leads to an increase in synthase activity and in HA production (402).

B. Hyaluronan synthase 2 (HAS2)

Human hyaluronan synthase 2 is located on chromosome 8, whereas mouse Has2 is on chromosome 15 (396). In an in vitro transfection assay, Kimata and associates demonstrated that all three isoforms of HA synthase exhibited a *de novo*, massive formation of a HA matrix in nontransformed rat 3Y1 embryonic fibroblast cells (157). Hyaluronan and its matrix can modulate contact inhibition of cell growth and migration (157). Inhibition of a phosphatidylinositol 3-kinase pathway resulted in reacquisition of the normal phenotype of HAS2 transfectants, suggesting that the intracellular phosphatidylinositol 3-kinase signaling regulates diminution of contact inhibition induced by formation of the massive HA matrix (157).

Targeted deletion of HAS2 has been a major development in the field, providing in vivo evidence of the functions of HA (45). There are major abnormalities in heart and blood vessel development, resulting in an embryonic lethal phenotype (44,45). HAS2-deficient

embryos at embryonic day 9.5 completely lack endocardial cushions (44,45), consistent with early observations that high content of HA is present in normal human heart valves (435). These defects resemble the features in naturally occurring versican knockouts (276). These studies suggest that HA-ECM interactions play a significant role in cardiac development. Elucidating the role for HAS2 in animal models has been difficult due to the embryonic lethal phenotype in the HAS2-deficient mice.

HAS2 also plays a role in limb development. Overexpression of Has2 in the mesoderm of the chick limb bud in vivo results in the formation of shortened and severely malformed limbs that lack one or more skeletal elements. Skeletal elements in limbs overexpressing Has2 are reduced in length, exhibit abnormal morphology, and are positioned inappropriately (236). In vitro, sustained HA production in micromass cultures of limb mesenchymal cells inhibits formation of precartilage condensations and subsequent chondrogenesis, indicating that proper regulation of HA is essential for formation of the precartilage condensations that trigger cartilage differentiation (236). Conditional inactivation of HAS2 showed a role for hyaluronan in skeletal growth, patterning, chondrocyte maturation and joint formation in the developing limb (258).

Recently, transcription factors Sp1 and Sp3 have been identified as principal mediators of HAS2 constitutive transcription. Sp1 and Sp3 bind to three sites immediately upstream of the HAS2 transcription initiation site and that mutation of the consensus recognition sequences within these sites ablated their transcriptional response. In contrast, NF-Y, CCAAT, and NF-κB binding proteins may not be involved in HAS2 transcription (278). A recent study showed that transgenic expression of Tbx2, a central intermediary of Bmp-Smad signaling, induced Has2 and TGF-β2 expression, facilitating endocardial cushions formation (382).

C. Hyaluronan synthase 3 (HAS3)

The human HAS3 gene is localized to chromosome 16q22.1 and the mouse Has3 to chromosome 8 (396). The HAS3 protein is transmembrane protein. Using green fluorescent protein to tag HA synthases in keratinocytes, HAS2 and HAS3 were found to travel through endoplasmic reticulum, Golgi, plasma membrane, and endocytic vesicles, and a distinct enrichment of plasma membrane HAS was observed in cell protrusions (351). The trafficking of HAS was correlated with its activity, since inhibition of HA synthesis by substrate UDP-glucuronic acid starvation using 4-methyl-umbelliferone prevented HAS access to the plasma membrane (351). Similarly, in cells transfected with green fluorescent protein-tagged Has3, the dorsal surface was decorated by up to 150 slender, 3 to 20-μm-long microvillus-like plasma membrane protrusions, which consisted of filamentous actin, CD44, and lipid raft microdomains, in addition to Has3 (212). Enzymatic activity of HAS was required for the growth of the microvilli. The microvilli induced by HAS3 gradually withered with the introduction of an inhibitor of HA synthesis and rapidly retracted by hyaluronidase digestion, whereas they were independent of HA receptors (212). Keratinocyte growth factor activates keratinocyte migration and stimulates wound healing. At same time, keratinocyte growth factor stimulates epidermal keratinocytes to accumulate intermediate-sized HA in the culture medium and within keratinocytes and leads to a rapid increase of Has2 and Has3 mRNA (188), suggesting that Has2 and Has3 are the targets of keratinocyte growth factor in keratinocytes. Enhanced HA synthesis acts an effector for the migratory response of keratinocytes in wound healing, whereas it may delay keratinocyte terminal differentiation (188).

D. Regulation of HAS expression and activity

In vitro, the expression of HAS isoforms can be regulated by growth factors and cytokines. For example, recombinant TNFα and interferons stimulate HA production by normal human lung fibroblasts (87). IL-1β and TNFα induce HAS-2 mRNA in fibroblasts (472). Epidermal growth factor induces HAS2 expression in rat epidermal keratinocytes (327). TGF-β activates HAS1, leading to an increase in HAS activity (402). Conversely, TGF-β suppresses HAS3 mRNA in human fibroblast-like synoviocytes (402). TGF-β reduces HAS2 mRNA slightly but does not significantly affect the expression of mRNAs for HAS1 and HAS3 in mesothelial cells (164). Stimulation of mesothelial cells with platelet-derived growth factor-BB induces HAS activity (137) and upregulation of HAS2 mRNA (164).

Dysregulation of HA synthases and their activities has been found during tissue injury (235,415,489), 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 (235). 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 (17). Similarly, epidermal HA is significantly increased after epidermal trauma in adult mice caused by tape stripping (415). The HA response is associated with a strong induction of HAS2 and HAS3 mRNA (415). Increased accumulation of HA and increased HAS expression have been noticed in autoimmune (96) and mechanical renal injury (489,490).

III. HA DEGRADATION AND HYALURONIDASES

A. Hyaluronidases

Hyaluronidases (also called hyaluronoglucosaminidases) hydrolyze the hexosaminidic β(1–4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in HA and release HA fragments. Complete digestion of HA with hyaluronidase releases disaccharide D-glucuronic acid-N-acetyl-D-glucosamine. These enzymes also hydrolyze β(1–4) glycosidic linkages between N-acetyl-galactosamine or N-acetylgalactosamine sulfate and glucuronic acid in chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan. Some bacteria, such as *Staphylococcus aureus*, *S. pyogenes*, and *Clostridium perfringens*, produce hyaluronidases as a means for greater bacterial mobility through the host's tissues and as an antigenic disguise that prevents recognition of bacteria by phagocytes (210). In humans, there are six hyaluronidases identified thus far: hyaluronidases 1 – 4, PH-20, and HYALP1 (400).

The human HYAL1 gene is located on 3p21.3-p21.2 (64) and the mouse gene is on chromosome 9 (62). The hyaluronidase 1 gene encodes a hyaluronidase found in the major parenchymal organs such as the liver, kidney, spleen, and heart. HYAL1 is also present in human serum (62) and urine (63). The enzyme intracellularly degrades HA, is active at an acidic pH, and is the major hyaluronidase in plasma (62). Mutations in the HYAL1 gene are associated with mucopolysaccharidosis type IX, or hyaluronidase deficiency (293).

HYAL2 is a glycosylphosphatidylinositol-anchored cell-surface receptor (341) in all mouse tissue types except brain. HYAL2 has very low hyaluronidase activity in comparison to serum hyaluronidase HYAL1 (341), and HYAL2 hyaluronidase activity has a pH optimum of below 4 (229). Furthermore, unlike HYAL1, the HYAL2 enzyme hydrolyzes only HA of high molecular mass, yielding intermediate-sized HA fragments of approximately 20 kDa, which are further hydrolyzed to small oligosaccharides by PH-20 (229). In addition, HYAL2 serves as a receptor for Jaagsiekte sheep retrovirus (341).

HYAL3 transcripts show strongest expression in testis and bone marrow but relatively weak expression in other organs (62). The role of HYAL3 in the degradation of HA is not clear. A recent study showed that Hyal3 overexpressed in cultured cells lacks intrinsic hyaluronidase activity and that Hyal3 may contribute to HA metabolism by augmenting the activity of Hyal1 (138). Hyal3 deficient mice showed a subtle change in the alveolar structure and extracellular matrix thickness in lung-tissues at 12–14 months-of-age, although there was no evidence of HA accumulation, suggesting that HYAL3 may not play a major role in constitutive HA degradation (14). The testicular enzyme PH-20 is encoded by the SPAM1 (sperm adhesion molecule 1) gene (220). This glycosylphosphatidylinositol-anchored enzyme is located on the human sperm surface and inner acrosomal membrane. PH-20 hyaluronidase is active at neutral pH. Mouse HYAL5, not HYALP1, degrades HA (350), involving in penetration of mouse sperm through cumulus mass (198).

Flavonoids, including silybin, apigenin, kaempferol, luteolin, and condensed tannin, were found to be inhibitors of hyaluronidase (216). Kinetic studies of these inhibitors showed that their mode of inhibition was competitive (216). They have been found effective in mice in vivo (215) and in inhibition of hyaluronidase activity (485). They are a useful tool in fertilization research (198). In addition, apigenin has significant anti-inflammatory activity that involves blocking nitric oxide-mediated cyclooxygenase-2 expression and monocyte adherence (226) or involves in vivo effectively blocking TNFα-induced ICAM-1 upregulation (317). Sodium cromoglycate and sodium aurothiomalate are potent inhibitors of hyaluronidases of Naja kaouthia and Calloselasma rhodostoma venoms and they can reduce the systemic and local tissue damage of Naja kaouthia and Calloselasma rhodostoma venoms when injected immediately at the sites of bites (485). Sulphated oligosaccharides such as verbascose, planteose and neomycin showed comparable inhibition on all hyaluronidases, thereby possessing much higher activity than that of the widely accepted hyaluronidase inhibitor apigenin (360).

B. Pathologic

Expression and activity of hyaluronidases have long been noticed in diseases such as rheumatoid arthritis (180,349) and periodontal disease (143). Patients with advanced scleroderma have decreased serum HYAL-1 activity and elevated circulating levels of HA (297). In one study, six patients with bone or connective tissue abnormalities had lower levels of HYAL-1 activity than did healthy donors (100). 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 (21). 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 (364).

Animal studies showed hyaluronidase significantly reduced mortality and reduced “enzymatic” infarct size in gerbils with experimental cerebrovascular accidents (483). With small numbers of patients, Maroko and associates found that hyaluronidase intravenously accelerated the reduction of myocardial ischemic injury in patients with acute myocardial infarction (250) and reduced the frequency of electrocardiographic signs of myocardial necrosis (251). However, the effectiveness of hyaluronidase for patients with acute myocardial infarction was questionable. In a primate model that has a minimal collateral blood supply, hyaluronidase did not significantly reduce the ultimate infarct size (336). A multicentered, randomised, blind study from 1978 to 1988 did not find a beneficial effect of hyaluronidase on mortality or infarct size (352).

IV. HYALURONAN AS A SIGNALING MOLECULE

A. Hyaluronan Fragments

During the last 15 years, we and others have established the concept that HA plays different roles depending on its molecular weight (149,264,265,306,425,465). In its native state, such as in normal synovial fluid, HA generally exists as a high molecular weight polymer, usually in excess of 1000 kDa. However, under certain conditions such as tissue injury and inflammation, HA is more polydisperse, with a preponderance of lower-molecular-weight forms (357).

Fragmentation of HA occurs during many pathological conditions, a possible consequence of dysregulated expression of HA synthases and HA degradation enzymes during tissue injury and inflammation (55). Altered expression of HAS2 and HYAL2 is involved in the turnover of HA during the early phase of lung injury in irradiated rats (235). Hyaluronan fragmentation can also be a result of the release of reactive oxygen species during tissue injury (3,51,281,316,392). We recently demonstrated that extracellular superoxide dismutase inhibits inflammation in response to lung injury by preventing oxidative fragmentation of HA (106). Extracellular superoxide dismutase directly binds to HA and significantly inhibits oxidant-induced degradation of HA (106).

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 (144,148,149,151,152,264,265,306). HA inhibits human neutrophil elastase-induced airway responses and, within the range of 150–300 kDa, dose rather than molecular weight may be the most important determinant of pretreatment time resulting in a protective effect (375). HA fragments augment steady-state mRNA, protein, and inhibitory activity of PAI-1 as well as diminish the baseline levels of uPA mRNA and inhibit uPA activity in an alveolar macrophage cell line and in freshly isolated inflammatory alveolar macrophages from bleomycin-treated rats, suggesting that the regulation of PAI-1 and uPA by HA fragments may provide a mechanism for regulating fibrinolytic activity during lung inflammation (150). HA fragments induced macrophage metalloelastase mRNA, protein expression as well as enzyme activity in mouse macrophages and in inflammatory alveolar macrophages from bleomycin-injured rat lungs. HA fragments may be an important mechanism for the expression of macrophage metalloelastase by macrophages in inflammatory lung disorders (152).

West and colleagues showed that oligosaccharides derived from high molecular weight HA have distinct functions when compared with the larger molecular forms of HA (465). Whereas HA oligomers of 8 – 16 disaccharides stimulated angiogenesis in vivo and endothelial proliferation in vitro, native, high molecular weight HA had no effect (465). Furthermore, this group confirmed that the high molecular mass HA (about 1000 kDa) is anti-angiogenic, whereas HA fragments of 3–25 disaccharide units induce angiogenesis (74,354).

Fragmented HA containing 6 – 40-mers enhanced CD44 cleavage by tumor cells, whereas large polymer HA failed to enhance CD44 cleavage (405). A 6.9-kDa HA (36-mers) also promoted tumor cell motility in a CD44-dependent manner (404,405). Enhanced expression of matrix metalloprotease (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 (97). 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 (425). Others found that HA hexamers or smaller were not active.

Fragmented HA (80 – 800 kDa), but neither purified high molecular weight HA nor HA hexamers, markedly increased MCP-1 mRNA and protein expression (22), and ICAM-1 and VCAM-1 steady-state mRNA and cell-surface expression by murine kidney tubular epithelial cells (310).

B. HA-inducible genes

Many studies have shown that HA is biological active and is able to stimulate expression of a variety of genes. Below is the summary of these genes.

V. HYALURONAN BINDING PROTEINS

Much of HA exists in ECM in soluble form. HA also covalently binds to a variety of proteins to influence the functions of these proteins (432). HA binding proteins include receptors such as CD44, RHAMM, TNFIP6, Brevican, SHAP, LYVE1, and many other proteins. Some HA binding proteins are associated with cell membranes, whereas others are found in the extracellular matrix. Structurally, the link module (204) and the $B(X_7)B$ motif (where B is arginine or lysine and X is any nonacidic amino acid) (481) are thought to constitute the HA-binding region. 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 (113).

Day and colleague suggested that HA as a free polymer in solution exists in a stiffened highly dynamic ensemble of chaotically interchanging semiordered states (70). Under the organizing influence of proteins, these states may be coaxed towards a number of families of ordered and variously shaped structures of similar energy. When present as a simple polymer, HA is likely to be a perfect dynamic colonizer of the extracellular space (70). The specificity of protein–HA interactions, combined with the repeating nature of the polysaccharide, may drive the formation of periodic filamentous complexes that are likely to have significantly different quaternary organizations depending on the conformation of the bound sugar and the nature of the stabilizing protein–protein associations (70). These states may be interchangeable depending on the force. Furthermore, Day and associates proposed that that HA cross-linking is part of a protective mechanism, promoting adhesion of leukocytes to the HA complexes rather than enabling contact with inflammation-promoting receptors on the underlying tissues (69). Thus, leukocytes are maintained in a non-activated state by appropriate receptor clustering or receptor co-engagement. Hyaluronan networks serve as scaffolds to prevent the loss of extracellular matrix components during inflammation and to sequester proinflammatory mediators (69).

A. CD44

CD44 is the major cell-surface HA binding protein (10). CD44 is a widely researched molecule and it has been shown to have multiple roles in a variety of biomedical fields. 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 and by cell-type-specific glycosylation (232). It is widely expressed in almost every cell type in the human and in the mouse. It is also an abundant protein on many cell types. It is believed to be one of the most abundant cell surface proteins on macrophages. 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 (381). N-glycosylation regulates CD44 structure by altering both the affinity and avidity of CD44-HA binding (422). Most cells express the standard isoform, which is an 85-kDa protein that undergoes posttranslational modification (232). Most cells including stromal

cells such as fibroblasts and smooth muscle cells, epithelial cells, and immune cells all express CD44 (381).

HA-CD44 interactions play an important role in development, inflammation, T cell recruitment and activation, and tumor growth and metastasis (232). CD44 cytoplasmic domain can be phosphorylated when ligand binds to transduce signaling. CD44 is also required for BMP-7 signaling. The cytoplasmic domain of CD44 is required for BMP-7 induced Smad1 nuclear translocation (325). Smad1 was found to interact with the cytoplasmic domain of CD44 demonstrated with coimmunoprecipitation. The integrity of extracellular HA-cell interactions is required for BMP-7-mediated Smad1 phosphorylation, nuclear translocation of Smad1 or Smad4, and SBE4-luciferase reporter activation, suggesting a functional link between the BMP signaling cascade and CD44 (325).

When CD44 phosphorylation mutants were transfected into a murine fibroblast line expressing low levels of endogenous CD44, CD44 phosphorylation mutants were as efficient as wild type CD44 in mediating cell adhesion but were unable to support HA-dependent fibroblast migration, demonstrating a control mechanism specific for CD44-mediated cell motility (323). Work with transformed cells indicates that these cells use the cell surface matrix receptor CD44 for migration and invasion (139). Lung tissue from patients who died from acute alveolar fibrosis after lung injury reveals CD44-expressing mesenchymal cells throughout newly formed fibrotic tissue. CD44 was found uniformly over the cell surface and was found densely labeling filopodia and lamellipodia. By blocking the function of CD44 with monoclonal antibodies, fibroblast invasion into a fibrin matrix was inhibited (408). Fibroblast CD44 functions as an adhesion receptor for provisional matrix proteins and is capable of mediating fibroblast migration and invasion of the wound provisional matrix resulting in the formation of fibrotic tissue (408). Hyaluronan inhibits PDGF-BB-induced activation of PDGF receptor and cell motility, while blockage of the binding of HA to CD44 restored PDGF-receptor activation and motility, indicating that CD44 mediates the inhibiting effect on PDGF receptor (234). PDGF receptor and CD44 form a complex and the inhibitory effect of HA is neutralized by inhibition of tyrosine phosphatases, suggesting that HA-activated CD44 modulate PDGF receptor signaling by recruiting tyrosine phosphatase to the receptor (234).

The role of CD44 was investigated by genetic targeting in vivo. Transgenic mice expressing an antisense CD44 cDNA under the control of keratin-5 promoter had defective keratinocyte proliferation indicating the role of CD44 in the regulation of keratinocyte proliferation in response to extracellular stimuli (192). Given the importance of CD44 as demonstrated in many biological and pathological conditions, it was very surprising that the genetic disruption of CD44 in the mouse did not show much abnormality (371). Our laboratory has demonstrated that CD44 is required for the resolution of pulmonary inflammation (421). CD44-deficient mice succumb to unremitting inflammation following bleomycin-induced noninfectious lung injury, characterized by impaired clearance of apoptotic neutrophils, persistent accumulation of HA fragments at the site of tissue injury, and impaired activation of TGF-β1 (421). The phenotype was partially reversed by reconstitution with $CD44^{+}$ cells, thus demonstrating a critical role for this receptor on leukocytes in resolving lung inflammation. CD44 deficiency results in enhanced inflammation in *E. coli* but not *S. pneumoniae*-induced pneumonia, suggesting a role for CD44 in limiting the inflammatory response to E. coli (454). We and others demonstrated that inflammation is more aggravated in CD44-knockout mice than in wild type mice (237,294). We further found that the induction of the negative regulators of TLR signaling IL-1R-associated kinase-M (202), Toll-interacting protein, and A20 by intratracheal lipopolysaccharide in vivo and in macrophages in vitro was significantly reduced in $CD44^{-/-}$ mice, suggesting that CD44

plays a role in preventing exaggerated inflammatory responses to LPS by promoting the expression of negative regulators of TLR4 signaling (237).

In addition, recent studies suggested that CD44 expression was strongly induced in the infarcted myocardium and was localized on infiltrating leukocytes, wound myofibroblasts, and vascular cells. Although CD44 deficient mice showed enhanced inflammation following myocardial infarction, CD44 deficient infarcts showed decreased fibroblast infiltration, reduced collagen deposition, and diminished proliferative activity (156). Isolated primary CD44 deficient cardiac fibroblasts had reduced proliferation upon stimulation with serum and decreased collagen synthesis in response to TGF-β in comparison to wild type fibroblasts, suggesting that CD44-mediated interactions are critically involved in infarct healing (156). After tissue injury, fibroblast migration from the peri-wound collagenous stroma into the fibrin-laden wound is critical for granulation tissue formation and subsequent healing. Fibroblast transmigration or invasive migration from a collagen matrix into a fibrin matrix required the presence of fibronectin, several integrins, CD44 (59), and syndecan-4 (238). A recent study suggested that CD44 expression is not a general requirement for cell migration and gradient sensing; rather, it elicits a ligand-specific response (441). Expression of CD44 alone is not sufficient to drive chemotaxis towards HA, as NIH-3T3 fibroblasts were unable to respond to a HA gradient even when transfected with high levels of human CD44 (441). For NIH-3T3 cells to bind exogenous HA, it was necessary to both increase the level of receptor expression and remove a HA pericellular matrix (441). CD44 is required for myoblast migration and differentiation, since primary myoblasts from CD44-deficient mice displayed attenuated differentiation and subsequent myotube formation at early times in a differentiation-inducing in vitro environment (287). Chemotaxis of CD44 deficient myoblasts toward hepatocyte growth factor and basic fibroblast growth factor was totally abrogated (287). Very recently, it was demonstrated that CD44-deficient fibroblasts have fewer stress fibers, and focal adhesion complexes (1). Migration of CD44-deficient fibroblasts was increased in velocity, but was directionless (1).

B. RHAMM

RHAMM (for receptor for hyaluronan-mediated motility expressed protein, also called CD168) binds to biotinylated HA (129,482). RHAMM is a functional receptor in many cell types including endothelial cells (240,365). It is believed to be an HA receptor involved in tumor cell locomotion (129). Transfection experiments in fibroblasts suggest that RHAMM plays a role in Ras-dependent oncogenesis (122), but this role has been challenged by others (145), reflecting the complexity of the interactions between HA and HABPs in biological and pathological conditions. Nevertheless, RHAMM-HA interactions do play an important role in tissue injury and repair (491). Upregulation of RHAMM expression in bovine aortic smooth muscle cells was readily detected in an in vitro injury model (366). While HA stimulated the random locomotion of bovine aortic smooth muscle cells, an antibody to RHAMM that blocks HA binding with this receptor abolished smooth muscle cell migration following injury (366), suggesting that RHAMM is necessary for the migration of smooth muscle cells during wound repair.

Increased expression of RHAMM in macrophages after bleomycin injury in rats has been reported with a function blocking anti-RHAMM antibody (491). HA-stimulated macrophage chemotaxis was also inhibited by anti-RHAMM antibody (491). Daily administration of anti-RHAMM antibody to injured animals resulted in a 40% decrease in macrophage accumulation and lung architecture were improved with anti-RHAMM antibody treatment (491).

Inflammation is more aggravated in CD44-knockout mice than in wild type mice (294). Compensation for the loss of the CD44 gene occurs not because of enhanced expression of

the redundant RHAMM gene, but rather because the loss of CD44 allows increased accumulation of the HA substrate, with which both CD44 and RHAMM engage, thus enabling augmented signaling through RHAMM (294). RHAMM knockout mice are viable and show no significant defect, suggesting that RHAMM is not an absolute requirement for normal development (431). RHAMM is expressed at high levels in aggressive fibromatosis. When crossed with mice that harbor a targeted mutation in the tumor suppressor *APC* gene predisposing animals to tumors including aggressive fibromatosis tumor, RHAMM deficiency significantly decreased the number of aggressive fibromatosis tumors formed (431). RHAMM promotes fibroblast proliferation under conditions of low density (431). RHAMM-deficient fibroblasts fail to repair wounds in an *in vitro* wound healing assay (430). ERK1,2 activation and fibroblast migration/differentiation is also defective during the repair of RHAMM-deficient excisional skin wounds and results in aberrant granulation tissue in vivo. These results identify RHAMM as an essential regulator of CD44-ERK1,2 fibroblast motogenic signaling required for wound repair (430).

C. TSG-6/TNFIP6

Tumor necrosis factor-stimulated gene-6 (TSG-6), also called TNFα-induced protein 6 (TNFIP6) contains Link module and binds HA (204,228,322). The solution structure of the Link module from human TNFIP6 was determined and found to consist of two alpha helices and two antiparallel beta sheets arranged around a large hydrophobic core. This defines the consensus fold for the Link module family, which includes CD44, cartilage link protein, and aggrecan (204). The interaction of TNFIP6 with HA is pH-dependent (32). TNFIP6 expression is upregulated in many cell types in response to a variety of proinflammatory mediators and growth factors. TNFIP6 is induced by IL-1 and TNFα (228,474), and LPS (474). This protein is detected in several inflammatory disease states such as rheumatoid arthritis and in the context of inflammation-like processes is often associated with extracellular matrix remodeling. TNFIP6 is a potent inhibitor of neutrophil migration in an in vivo model of acute inflammation (473).

The recombinant Link module from human TNFIP6 has an inhibitory effect on neutrophil influx into zymosan A-stimulated murine air pouches, equivalent to that of full-length protein, resulting in a significant reduction in the concentrations of various inflammatory mediators in air pouch exudates (110). TNFIP6 is expressed by cultured vascular smooth muscle cells in response to serum and growth factor IL-1 but not to TNF or IL-6 (484). TNFIP6 is upregulated in proliferating vascular smooth muscle in the rat neointima after injury (484). TNFIP6-overexpressing cells grew faster than control vascular smooth muscle cells (484). TNFIP6 forms both covalent and non-covalent complexes with inter-α-inhibitor and potentiates its anti-plasmin activity (273).

Knockout of Tnfip6 in mice with a BALB/c background did not change the onset of proteoglycan-induced arthritis, but progression and severity were significantly greater in Tnfip6-deficient mice when compared to wild type BALB/c mice (409). An early and more extensive infiltration of the synovium with neutrophil leukocytes was the most prominent histopathologic feature of proteoglycan-induced arthritis in Tnfip6-deficient mice (409). Thus, TNFIP6 is a multifunctional anti-inflammatory protein that is produced at the site of inflammation and can be retained by the HA-rich extracellular matrix. A major effect of TNFIP6 is the inhibition of the extravasation of PMN cells, predominantly neutrophils, into the site of inflammation, most likely via a CD44/HA/TNFIP6-mediated blocking mechanism (409). TNFIP6 is a down-regulated gene during osteoblastic differentiation. TNFIP6 inhibits osteoblastic differentiation of human mesenchymal stem cells induced by osteogenic differentiation medium and BMP-2 (437). TNFIP6 is specifically expressed by expanding cumulus cell-oocyte complexes (103). Cumulus cell-oocyte complexes fail to expand in Tnfip6-deficient female mice because of the inability of the cumulus cells to assemble their

HA-rich extracellular matrix, demonstrating that TNFIP6 is a key catalyst in the formation of the cumulus extracellular matrix and indispensable for female fertility (104).

D. Brevican

Brevican (also call BEHAB, for brain enriched hyaluronan binding) is a brain-specific proteoglycan in perineuronal nets. Brevican occurs as secreted and cell-surface, glycosylphosphatidylinositol-anchored, isoforms (166). Brevican binds not only to HA (167,378) but also to chondroitin sulfate (480). Brevican increases the invasiveness of glioma cells in vivo and has been suggested to play a role in central nervous system fiber tract development (108,167). Brevican deficient mice are viable and fertile and have a normal life span (41). Extracellular matrix perineuronal nets formed but appeared to be less prominent in mutant than in wild type mice. Brevican-deficient mice showed significant deficits in the maintenance of hippocampal long-term potentiation, but no significant deficits in learning and memory (41). Expression of brevican was upregulated in response to a stab wound to the adult rat brain (166).

E. Neurocan

Neurocan is a nervous tissue-specific chondroitin sulfate proteoglycan of the aggrecan family which has been shown to interact with neural cell adhesion molecules (348) and tenascin (347). Neurocan binds to HA and chondroitin sulfate in the brain (75). Through its interactions with neural cell adhesion and extracellular matrix molecules, neurocan is a potent inhibitor of neuronal and glial adhesion and neurite outgrowth (249). Neurocan-deficient mice are viable and fertile and have no obvious deficits in reproduction and general performance (499). Brain anatomy, morphology, and ultrastructure are similar to those of wild type mice, indicating that neurocan has a redundant function in the development of the brain (499). The distribution and function of neurocan and HA are associated with the remodeling process of brain (346). Hyaluronan is organized into fiber-like structures along migratory pathways in the developing mouse cerebellum. Thus, HA-rich fibers are concentrated at sites where specific neural precursor cell types migrate, and the anisotropic orientation of these fibers suggests that they may support guided neural migration during brain development (18). Tissue injuries such as brain injury (12,266) and spinal cord injury (181) induced neurocan expression. Proteoglycan decorin suppresses neurocan and brevican expression and inflammation, and promotes axon growth across adult rat spinal cord injuries (68).

F. HABP1/C1QBP

HABP1 (for hyaluronan-binding protein 1, also called C1QBP, for complement component 1, q subcomponent binding protein) was first purified from the liver (67) and the brain (66). cDNA cloning confirmed that HABP1 is identical to complement component 1, q subcomponent binding protein (C1QBP) (111), and to pre-mRNA splicing factor SF2 (147,208). It is about 64 to 68 kDa and binds to HA (66,67) as well as mannosylated albumin (213). In addition to its mitochondrial location, HABP1 was also localized in the Golgi and completely dispersed throughout the cell during mitosis. This distinctive distribution pattern of HABP1 during mitosis resembles its ligand HA (377).

HABP1 exists as a highly acidic, noncovalently associated trimer in equilibrium with a small fraction of a covalently linked dimer of trimers (172). Structural studies demonstrated that HABP1 exhibits structural plasticity, which is influenced by the ionic environment under in vitro conditions near physiological pH (173). HA interacts only with compact HABP1, while C1q and mannosylated albumin can bind to loosely held oligomeric HABP1 as well. Thus, structural changes in HABP1 mediated by changes in the ionic environment are responsible for recognizing different ligands (173). The more stable conformation of

HABP1 was attained at higher ionic strength or at acidic pH showed maximum affinity toward HA (171). Therefore, cellular HA-HABP1 interaction can be regulated by pH and ionic strength. Endogenous HABP1 can be phosphorylated, and was coimmunoprecipitated with activated extracellular signal-regulated kinases (246). HABP1 is a substrate for extracellular signal-regulated kinases and an integral part of the mitogen-activated protein kinase cascade (246). In addition to HA, HABP1/C1QBP also binds to the globular heads of complement subcomponent C1q molecules and inhibit C1 activation (111).

HABP1 overexpressing cells showed extensive vacuolation and reduced growth rate, underwent apoptosis in normal rat skin fibroblasts (187,267). Overexpression of HABP1 was seen during *Leishmania donovani* infection. HABP1 binds with 2 proteins of promastigotes as well as amastigotes of *L. donovani*, suggesting a possible role for HABP in adhesion during the interaction of promastigotes and macrophages (344). It is unknown whether HA is required in this interaction. HABP1 has been demonstrated to play a role in spermatogenic differentiation. Stage-specific expression of HABP1 precursor during spermatogenesis in the rat (24). The sperm surface HABP1 level was correlated with the degree of sperm motility, an important criterion for fertilization (112). The appearance of HABP1 proprotein in the pachytene spermatocytes and the round spermatids during the initial stages of postnatal testis development suggests that this expression may be crucial for spermatogenesis (427).

G. HARE

HARE (for hyaluronan receptor for endocytosis, also called stabilin-2 and FEEL-2) was identified in the abundant expression of 175- and approximately 300-kDa HARE species from sinusoidal endothelial cells of the liver, lymph node, and spleen (497). The expression of HA receptors Stab2 and LYVE1 depended on the tissue and developmental stage of liver endothelial cells in mice (307). The human gene that encodes HARE is on chromosome 12 (495). The HARE protein features multiple $B(X_7)B$ HA-binding motifs, several fasciclin-like adhesion domains, and 18–20 epidermal-growth-factor domains (329). HARE protein binds to HA (130,262), dermatan sulfate, and chondroitin sulfates A, C, D, and E, but not chondroitin, heparin, heparan sulfate, or keratan sulfate (131,459).

While HARE/Stab-2 mRNA is confined to the liver and spleen, HARE/Stab-2 protein expression can be detected in the endothelial sinuses of the liver, lymph nodes, spleen, and bone marrow, and in specialized structures of the eye, heart, brain, and kidney in mice (93). Cloning and functional expression of the rat 175-kDa HARE demonstrated that the recombinant HARE is an authentic endocytic receptor for HA and was expressed on the cell surface (498). HARE was substantially colocalized with clathrin, but not with internalized HA that was delivered to lysosomes (498). The liver contains two distinct endothelial cell types: vascular and sinusoidal. The anti-HARE monoclonal antibodies showed diffuse strong staining of nonneoplastic liver sinusoidal endothelium. No staining of nonsinusoidal endothelium or the endothelial lining of the hemangiomas was seen with anti-HARE (82). Antibodies to HARE are not only markers for endothelial cells and the sinusoidal cells of rat liver, spleen, and lymph nodes, but also proved a useful tool in studying the functions of HARE and the physiological significance of HA clearance (458,497). HARE mediates systemic clearance of glycosaminoglycans from the circulatory and lymphatic systems via coated pit-mediated uptake. A majority of each HARE isoform was intracellular, within the endocytic system, suggesting transient surface residency typical of an active endocytic recycling receptor (130).

H. LYVE1

LYVE1 (for lymphatic vessel endothelial hyaluronan receptor 1, also called CRSBP-1 for cell surface retention sequence binding protein-1) was cloned as a lymph-specific HA receptor on the lymph vessel wall in human (19) and in mice (337). It is a type I integral membrane glycoprotein, homologue of CD44, contains a link module, and binds both soluble and immobilized HA (19). Expression of mouse LYVE1 remains restricted to the lymphatics in homozygous knockout mice lacking a functional gene for CD44 (337). However, LYVE1 is also present in normal hepatic blood sinusoidal endothelial cells in mice and humans (282). LYVE1 expression was also found on the endothelial cells of the lymphatic sinus and in reticular cells in the lymph nodes. (476). LYVE1 is expressed in DC-SIGN$^+$ macrophages within the chorionic villi in the rapidly differentiating placenta (33). TNFa promotes LYVE1 internalization from cell surface to lysosomes leading to its degradation. However, the internalization of LYVE1 is independent of HA (179). LYVE1 has being widely used as a lymphatic vessel specific marker (4,163,280,479). It also offers a prognostic parameter for head and neck squamous cell carcinomas, since intratumoral LYVE1$^+$ lymphatic vessels were clearly associated with a higher risk for local relapse as well as with poor disease-specific prognosis (259). LYVE1 is expressed in tissue macrophages. In murine tumor models and excisional wound healing, LYVE1 expression occurred in a subset of CD11b$^+$, F4/80$^+$ tissue macrophages (370). DC-SIGN$^+$CD163$^+$ macrophages have been shown to express LYVE-1 (33). LYVE1 is also implicated in the trafficking of cells within lymphatic vessels and lymph nodes (162).

LYVE1 plays a role in the transport of HA from tissue to lymph by uptaking HA via lymphatic endothelial cells (163,337). The occurrence of LYVE1-expressing lymphatic compartments and the alteration of chemokine CCL21 expression in the lymphatics may be involved in defective thymocyte differentiation and migration, and play a significant role in insulitic and diabetic processes (174). Despite the implied importance of this molecule, LYVE1-deficient mice are grossly normal (105,155). 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 LYVE1-deficient mice (105). HA homeostasis is unperturbed in LYVE-deficient mice (105). This suggests that LYVE1 is not obligatory for normal lymphatic development and function and that either compensatory receptors exist or LYVE1 has a more specific role than previously envisioned (105). However, a second study identified morphological and functional alterations of lymphatic capillary vessels in certain tissues, marked by constitutively increased interstitial-lymphatic flow and lack of typical irregularly shaped lumens in LYVE1-deficient mice (155). LYVE1 is not required for either entry or migration of dendritic cells through the afferent lymphatics, and leukocyte populations are unaltered in LYVE1-deficient mice (105), in line with the finding that lymphatic adhesion/transmigration is largely mediated by ICAM-1 and VCAM-1 rather than LYVE1 (178).

I. SHAP

SHAP (for serum-derived hyaluronan-associated protein) was originally identified as a serum-derived HA-associated protein since it covalently binds to HA (154,487). It was later found that SHAP is identical to inter-α (globulin) inhibitor heavy chain 2 (ITIH2), which belongs to the inter-α (globulin) inhibitor (ITI) family of structurally related plasma serine protease inhibitors involved in extracellular matrix stabilization. The ITI family consists of multiple proteins made up of a given combination of polypeptide chains after complex posttranslational maturation. There are 4 heavy chains and 1 light chain of ITI in the human, produced in the liver and circulating in peripheral blood. Mass spectrometric analyses showed that the C-terminal aspartic acid of each ITI heavy chain was esterified to the C6-hydroxyl group of an internal N-acetylglucosamine of HA chain (494). Biochemical analysis

demonstrated the multivalent feature of the SHAP-HA complex, where an HA chain with a molecular weight of 2000 kDa has as many as five covalently bound SHAP (486). Under both static and flowing conditions, CD44-positive cells adhered preferentially to the immobilized SHAP-HA complex than to HA. The enhanced adhesion is exclusively mediated by the CD44-HA, but not by ITI-HA interaction (501). The ITI complex also includes bikunin, a 30-kD subunit (35). The bikunin precursor, α-1-microglobulin, is a single chain plasma glycoprotein involved in regulation of the inflammatory process (268). Alpha-1-microglobulin is processed to mature α-microglobulin and bikunin (447). Bikunin circulates in peripheral blood as a free peptide and as a complex with IgA (268).

SHAP-HA complex was isolated from pathological synovial fluid from human arthritis patients, and the amount of complex correlates positively with the degree of inflammation (494). Furthermore, SHAP-HA complex levels in rheumatoid arthritis sera were extremely high compared to control levels, but in osteoarthritis sera no marked increase was observed compared to controls (196), suggesting that the SHAP-HA complex plays different roles in rheumatoid arthritis and in osteroarthritis. Both serum levels of the SHAP-HA complex and HA in the patients with of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma were significantly higher than those of normal individuals, and these levels correlate with of the stages of liver fibrosis. Thus SHAP-HA complex level is an indicator for the progression of the stages of liver fibrosis (379). Targeting the bikunin gene in mice abolished the formation of the SHAP-HA complex, leading to the absence of matrix in ovulated oocytes and severe female infertility (503). Restoration of the formation of the SHAP-HA complex by administration of ITI fully rescued the defects (503). The chondroitin sulfate moiety of bikunin is essential for presenting SHAP to HA, which is indispensable for ovulation and fertilization in mammals (502).

VI. ROLE OF CD44 IN HA SIGNALING

Although numerous studies suggest that HA fragments may signal through a CD44-dependent tyrosine kinase pathway (37,193,389) and that anti-CD44 antibodies partially inhibit HA fragment signaling in macrophages (144), other studies suggest that anti-CD44 antibodies have no effect on macrophage metalloelastase expression (152). With the aid of CD44-deficient mice, studies have demonstrated that HA is able to stimulate chemokine production in absence of CD44, suggesting that the presence of CD44 is not required to mediate HA signaling (97,175,193). The cumulative interpretation of these data is that the role of CD44 in regulating HA interactions depends on the cell type.

VII. HYALURONAN SIGNALING THROUGH TOLL-LIKE RECEPTORS

HA is a component of the cell coat of groups A and C of *Streptococcus* (241,243) and *P. multocida* (50). The repeating disaccharide structure of HA has features of pathogen-associated molecular patterns. Many pathogen-associated molecular patterns on pathogens utilize Toll-like receptors to initiate innate immune responses (2,413). The innate immune system uses TLRs to recognize microbes and initiate host defense (2,413). TLR4 has been unequivocally identified as the transmembrane signaling protein required for LPS signal transduction in macrophages (414). TLR2 is the mediator of macrophage recognition of mycobacteria (442) and gram-positive organisms (414). Stimulation of chemokine gene expression by HA fragments was abolished in the MyD88-deficient macrophages (175). Chemokine MIP-2 expression was reduced but remained present in both TLR2- and TLR4-deficient macrophages. HA fragment-induced chemokine and cytokine expression was completely abolished in TLR2 $^{-/-}$ TLR4 $^{-/-}$ peritoneal macrophages (175). The human HA degradation products purified from the serum of patients with acute lung injury were of similar molecular mass (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 (175).

HA oligosaccharides induce maturation of dendritic cells via TLR4 (423). HA oligosaccharide treatment results in distinct phosphorylation of MAP kinases, nuclear translocation of NF-κB, and TNFα production (423). Priming of alloimmunity by HA-activated dendritic cells is dependent on signaling via TIRAP, a TLR adaptor downstream of TLR2 and TLR4 (426). However, this effect is independent of alternate TLR adaptors, MyD88, or TRIF (426). 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 (426). Injury-induced, Toll-like receptor-triggered signaling pathways, such as HA fragments (426), high-mobility group box 1 protein (345), involved in establishing innate alloimmunity utilize adaptor proteins and transcription factors that play a crucial role in the host's defense against pathogens (218). Horton and colleagues reported similar findings, using a commercial source of HA fragments, although they suggested a primary role for TLR2 (368).

Hyaluronan upregulates IL-1R-associated kinase-M, a negative regulator of TLR signaling, to deactivate human monocytes, mediated through the engagement with CD44 and TLR4 (79). Small HA fragments stimulate expression of MMP2 and IL-8, leading to NF-κB activation, enhanced the motility of melanoma cells, in part in a TLR4-dependent manner (451). HA inhibits osteoclast differentiation through TLR4 by interfering with M-CSF signaling, suggesting a role of HA-TLR interaction in the regulation of bone metabolism (56).

VIII. HYALURONAN AS AN IMMUNE REGULATOR

A. T cells

CD44 is expressed on T cells (221). The interaction of cell-surface HA and CD44 on T cells 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 (8). 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 (231). Lesley and colleagues suggested that CD44 functions associated with HA binding involve a regulated process (230,231).

T cell activation is associated with increased surface levels of CD44. CD44 expression on T cells but not dendritic cells plays a critical role in antigen-specific T cell responsiveness (81). Activation of CD44 and ability to engage in rolling occurs directly through polyclonal as well as antigen-specific T cell receptor-initiated signaling, suggesting potential roles for the CD44-HA interaction (78). CD44 activation does not appear to be the result of overt changes in glycosylation (78).

Leukocytes extravasate from the blood in response to physiologic or pathologic demands by means of complementary ligand interactions between leukocytes and endothelial cells, via a multistep process. Binding of CD44 on activated T lymphocytes to endothelial HA mediates a primary adhesive interaction under shear stress, permitting extravasation at sites of inflammation (76,77). The integrin α4 (VLA-4), not integrin αL, is used in secondary adhesion after CD44-mediated primary adhesion of human and mouse T cells in vitro, and by mouse T cells in an in vivo model. Thus, extravasation of activated T cells initiated by CD44 binding to HA depends upon integrin α 4-mediated firm adhesion, which may explain the frequent association of these adhesion receptors with diverse chronic inflammatory

processes (385). CD44 and integrin α 4 are physically associated on the cell surface and that the cytoplasmic portion of CD44 is necessary for this association. Disruption of the association through deletion of the CD44 cytoplasmic tail concordantly prevents firm adhesion of integrin α 4 on its ligand VCAM-1 under shear stress, while leaving rolling interactions between CD44 and HA intact, demonstrating that co-anchoring within CD44 and VLA-4 bimolecular complex between a primary and secondary adhesion molecule regulates the ability of a cell to firmly adhere (292).

Skin γδT cells play specialized roles in keratinocyte proliferation during wound repair. γδT cells are required for HA deposition in the extracellular matrix and subsequent macrophage infiltration into wound sites (165). γδT cell-derived keratinocyte growth factors induce epithelial cell production of HA. In turn, HA recruits macrophages to the site of damage. These results demonstrate a novel function for skin γδT cells in inflammation and provide a new perspective on T cell regulation of ECM molecules (165).

While lymphocytes from CD44 $^{-/-}$ mice preferentially homed to lymph nodes, their entry into the inflamed synovial joints was delayed when compared to wild type cells (401). CD44-deficient lymphocytes from animals with chronic arthritis expressed markedly reduced levels of the lymph node homing receptor, L-selectin. Down-modulation of L-selectin from CD44 $^{-/-}$ cells in arthritic condition might be a counter-regulatory response, which, by extending lymphocyte transit time in the circulation at the expense of lymph node homing, allows CD44-deficient cells to gain entry to the site of chronic inflammation via secondary adhesion mechanisms (401).

$CD4^+CD25^+$ regulatory T cells (Treg) are fundamental to the maintenance of peripheral tolerance. Siegelman and colleague found that the level of functionally active, HA-binding form of $CD44^{act}$ is strikingly correlated with superior suppressor activity, suggesting that CD44 is more than a cell surface marker and plays a role in regulating Treg cell functions (98). The expression of other surface markers and Foxp3 are similar irrespective of HA binding and associated degree of suppressor potency (98). Furthermore, high molecule weight HA enhances human $CD4^+CD25^+$ regulatory T cell functional suppression of responder cell proliferation, whereas low molecule weight HA does not (34). In addition, high molecule weight HA also up-regulates the transcription factor FOXP3 on $CD4^+CD25^+$ regulatory T cells. These effects are only seen with activated $CD4^+CD25^+$ regulatory T cells and are associated with the expression of CD44 isomers that more highly bind high molecule weight HA (34).

B. PMN

Both polarization and directed migration of neutrophils are dependent on the expression of CD44 and its interaction with HA, which could modulate neutrophil migration into inflamed tissues (7). TNFIP6 inhibits polymorphonuclear cell efflux and neutrophil invasion into the inflammatory site (409). A recent study suggested that CD44 does not appear to affect subsequent migration within inflamed tissues, although CD44 mediates neutrophil adhesion and emigration (195). CD44 deficiency leads to enhanced neutrophil migration and lung injury in *Escherichia coli* pneumonia in mice CD44 deficiency results in enhanced inflammation in *E. coli* but not *S. pneumoniae*-induced pneumonia, suggesting a role for CD44 in limiting the inflammatory response to *E. coli* (454). This study is consistent with our recent observation that inflammation is more aggravated in CD44-knockout mice than in wild type mice (237). Endothelial CD44 rather than neutrophil CD44 mediates neutrophil migration (356). Ligation of CD44 on neutrophils with anti-CD44 and HA induced IL-6 gene transcription and IL-6 protein secretion. Interferon γ-induced IL-6 production is dependent on CD44 cross-linking (373). The cross-linking of specific epitopes of the CD44 molecule can rapidly induce neutrophil apoptosis in vitro and inhibit neutrophil-dependent

renal injury in vivo (412). Thus physiological ligands of the CD44 molecule may play an important role in eliminating neutrophils from sites of inflammation, including inflammatory kidney disease (412).

C. Macrophages

Ligation of human macrophage surface CD44 by bivalent monoclonal antibodies rapidly and profoundly augments the capacity of macrophages to phagocytose apoptotic neutrophils in vitro (450). CD44-deficient mice succumb to unremitting inflammation following noninfectious lung injury, characterized by impaired clearance of apoptotic neutrophils, persistent accumulation of HA fragments at the site of tissue injury, and impaired activation of transforming growth factor-β1 (421). This phenotype was partially reversed by reconstitution with $CD44^+$ cells, thus demonstrating a critical role for this receptor in resolving lung inflammation (421). CD44 regulates phagocytosis of apoptotic neutrophil granulocytes, but not apoptotic lymphocytes, by human macrophages (132).

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 (426). Activation of dendritic cells with HA enhances their ability to stimulate allogeneic and antigen-specific T cells markedly (81).

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 (283). Small HA fragments of tetra- and hexasaccharide size increase dendritic cell production of the cytokines IL-1β, TNFα, and IL-12 as well as their allostimulatory capacity (425). These small HA fragments induce dendritic cell maturation independently of CD44 or RHAMM and are dependent upon TLR4 (423).

D. Mast cells

Mast cell granules are a rich source of HA, and this may account for the striking concurrence of HA accumulation with a mastocytotic condition in many tissues undergoing pathologic changes (84). Cultured human mast cells adhere to HA-coated surfaces (102). Human mast cells expressing standard form of CD44, but not the v5, v6, v7, and v8 variants, appears to mediate the attachment of these cells to HA (102). However, CD44 was not found to be consistently elevated in serum obtained from patients with mastocytosis or individuals experiencing anaphylaxis (102).

The number of mast cells was higher in patients with sarcoidosis than in controls (86). Sarcoidosis patients had significantly increased bronchoalveolar lavage (BAL) fluid concentrations of HA, fibronectin, and type III procollagen peptide, as well as albumin and lymphocytes, compared to controls (86). Mast cell activation and stress play a role in the pathogenesis of interstitial cystitis (363). Intravesical sodium hyaluronate has been used to treat interstitial cystitis due to its possible replenishment of bladder glycosaminoglycans (186). HA inhibited mast cell activation and the secretion of proinflammatory mediators, providing a mechanism of HA as a therapeutic option for interstitial cystitis (36).

E. Eosinophils

The infiltration, accumulation and degranulation of eosinophils in the lung is a hallmark of active asthma (42). HA increased GM-CSF, TGF-β and ICAM-1 expression by eosinophils, leading to eosinophil survival. HA-eosinophil interaction may contribute to the regulation of airway inflammation and airway remodeling (313). HA stimulates the growth of $CD34^+$

progenitor cells into specifically differentiated, mature eosinophils (126). HA contributes to long-term eosinophil survival in vivo by enhancing GM-CSF production in asthma conditions (90). HA triggers granulocyte GM-CSF mRNA stabilization in eosinophils yet engages differential intracellular pathways and mRNA binding proteins (90). Recently, Pin1, a cis-trans isomerase, was identified as an essential component of the ribonucleoprotein complex responsible for GM-CSF mRNA stabilization, cytokine secretion and the survival of HA-activated eosinophils (380).

The expression of CD44 on alveolar eosinophils and the concentration of soluble CD44 were increased in BAL of patients with eosinophilic pneumonia patients, and the increase was correlated with Th2 cytokine IL-5 in BAL (190). In addition, a high concentration of HA was observed in BAL of eosinophilic pneumonia patients (190). Intraperitoneal administration of anti-CD44 monoclonal antibody prevented both lymphocyte and eosinophil accumulation in the lung, blocked antigen-induced elevation of Th2 cytokines, chemokines (CCL11, CCL17), and inhibited the increased levels of HA and leukotriene concentrations in BAL, demonstrating a critical role for HA-CD44 interaction in development of allergic respiratory inflammation (189).

IX. HA AND NON-LEUKOCYTES

A. Epithelial cells

Hyaluronan induces MCP-1 expression in renal tubular epithelial cells (22). HA is synthesized in high molecular weight form at the apical pole of human airway epithelial cells, covering the luminal surface (279). High molecular form HA is broken down by reactive oxygen species to form low molecular weight fragments that signal via RHAMM to stimulate ciliary beat frequency (248). Low molecular weight HA fragments stimulated ciliary beat frequency, an effect blocked by anti-RHAMM antibody and genistein (248), suggesting a role of HA-RHAMM interaction in airway mucosal host defense.

The transforming effects of hepatocyte growth factor and β-catenin are dependent on HA-cell interactions. Increased expression of HA is sufficient to induce epithelial-mesenchymal transition and acquisition of transformed properties in phenotypically normal epithelial cells in vitro (505). HA plays a central role in the transition of epithelia to mesenchyme in the embryo and in the acquisition of transformed properties in carcinoma cells (434). Overexpression of HAS2 on airway epithelial cells under control of the Clara cell specific CC10 promoter leading to an increased production of high molecular weight HA was protective against mortality, lung injury, and epithelial cell apoptosis (175).

B. Endothelial cells

Oligosaccharides of HA enhance the production of collagens by endothelial cells (354). Early-response gene signaling is induced by angiogenic oligosaccharides of HA in endothelial cells, while is inhibited by high molecular weight HA (74). HA fragments, but not high molecular weight HA, induce iNOS in liver, having the greatest effects on endothelial and Kupffer cells. Thus, HA fragments may be an important stimulus for NO production in various forms of liver disease, particularly as a cofactor with inflammatory cytokines (353).

Local cytokine production within inflamed vascular beds may enhance surface HA expression on endothelial cells, thereby creating local sites receptive to the CD44/HA interaction and thus extravasation of inflammatory cells (277). IL 15 induces endothelial HA expression in vitro and promotes activated T cell extravasation through a CD44-dependent pathway in vivo (91). Endogenous components of the extracellular matrix HA can stimulate endothelia to trigger recognition of injury in the initial stages of the wound defense and

repair response (417). HA-induced proliferation of endothelial cells is CD44 receptor mediated and accompanied by early-response-gene activation (389). HARE/stabilin-2 is found on sinusoidal endothelial cells of the liver, lymph node, and spleen (496). HARE mediates the systemic clearance of HA from the circulatory and lymphatic systems, through a clathrin-dependent, coated pit-mediated uptake (497).

Hyaluronan fragments stimulate endothelial cell differentiation. Hyaluronan dodecasaccharides induce capillary endothelial cell sprouting by the binding to CD44 and by the induction of CXCL1/GRO1 (411). HA regulates vascular endothelial cell barrier function through CD44v10 isoform interaction with S1P receptors, S1P receptor transactivation, and RhoA/Rac1 signaling to the EC cytoskeleton (386). CD44 is an important regulator of HGF/c-Met-mediated in vitro and in vivo barrier enhancement, a process with essential involvement of Tiam1, Rac1, dynamin 2, and cortactin (387).

C. Fibroblasts

Fibroblasts are the main cell type that release HA (164,242,397,429). Fibroblasts synthasize all three isoforms of HA synthases and release HA upon tissue injury (17,255) and the stimulation of inflammatory factors such as IL-1β and TNFα (472). On the other hand, HA fragments stimulate fibroblasts to release cytokines and to regulate inflammatory responses (242,255), and facilitate TGF-β-mediated fibroblast proliferation (270). Furthermore, upon myofibroblastic differentiation, more HA appeared in the conditioned medium and became associated with the cells (170). Moreover, HA modulates TGF-β dependent myofibroblast differentiation (456,457).

Hyaluronan-binding proteins regulate fibroblast functions through their interactions with HA. For example, acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44 (408). HA modulates contact inhibition of cell growth and migration (157). RHAMM deficient fibroblasts are defective in CD44-mediated ERK1/2 motogenic signaling, leading to defective skin wound repair, demonstrating a role of RHAMM in CD44 signaling and in fibroblast migration (430). A recent study suggested that HA-activated CD44 modulates PDGF receptor signaling by recruiting tyrosine phosphatases to the receptor and influences cell motility (234).

Consistent with the role of CD44 in mediating fibroblast invasion and subsequent tissue fibrosis, immunohistochemical analysis of lung tissue from patients who died from acute alveolar fibrosis after lung injury reveals CD44-expressing mesenchymal cells throughout newly formed fibrotic tissue (408). Anti-CD44 antibody blocked fibroblast migration on the provisional matrix proteins fibronectin, fibrinogen, and HA (408). Therefore, fibroblast CD44 functions as an adhesion receptor for provisional matrix proteins and is capable of mediating fibroblast migration and invasion of the wound provisional matrix resulting in the formation of fibrotic tissue (408). A recent study reported that high molecular weight HA increased Snail2 leading to fibroblast invasion (60).

D. Smooth muscle cells

HA is able to promote smooth muscle cell migration in various conditions (448,471). In addition, growth factors and cytokines regulate HA production in smooth muscle cells. For example, PDGF stimulates HA production in vascular smooth muscle cells (318,319), while IL-15 inhibits HA production and smooth muscle cell migration (161). Furthermore, HA binding proteins regulate smooth muscle cell proliferation and migration during tissue injury. For example, RHAMM is necessary for the migration of smooth muscle cells and the expression and distribution of this receptor is tightly regulated following wounding of

BASMC monolayers (366). HA-induced migration depends exclusively on RHAMM-mediated PI3K-dependent Rac activation (117).

X. HYALURONAN AND STEM CELLS

A. HA and embryonic stem cells

CD44 is expressed in discrete embryonic structures and the differentiation process of embryonic stem cells is accompanied by an induction of CD44 mRNA and protein (469). HA synthase 2 and RHAMM are differentially expressed during all stages of preimplantation human embryos and human embryonic stem cells (58). RHAMM expression is significantly downregulated during differentiation of human embryonic stem cells, in contrast to HAS2, which is significantly upregulated. RHAMM knockdown results in downregulation of several pluripotency markers in human embryonic stem cells, induction of early extraembryonic lineages, loss of cell viability, and changes in the human embryonic stem cell cycle, highlighting an important role for RHAMM in maintenance of human embryonic stem cell pluripotency, viability, and cell cycle control (58). HAS2 knockdown results in suppression of human embryonic stem cell differentiation without affecting human embryonic stem cells pluripotency, suggesting an intrinsic role for HAS2 in the human embryonic stem cells differentiation process (58). Addition of exogenous HA to the differentiation medium enhances human embryonic stem cell differentiation to mesodermal and cardiac lineages (58). During stem cell differentiation, HA (and other glycosaminoglycans) synthesis was enhanced by 13- and 24-fold, most likely due to increased expression of HA synthase-2 (289).

B. HA and hematopoietic stem cells

The fate of hematopoietic stem cells (HSC) is determined by microenvironmental niches. Hyaluronan is part of the extracellular environment in bone marrow. HA not only provides a physical scaffold or support within the marrow to facilitate localization and retention of HSCs to the stem cell niche but moreover, through ligation with its counter-receptors is able to directly affect the cellular functions of HSCs (133).

HA is required for *in vitro* hematopoiesis, since the removal of HA with hyaluronidase in long-term bone marrow cultures results in reduced production of both progenitor and mature cells (193). HA enhanced hematopoietic activity, committed progenitors, and the total number of mature bone marrow cells (256). HA synthesized by primitive hemopoietic cells participates in their lodgment at the endosteum following transplantation (301). Therefore, HA is required for the regulation of the hematopoiesis-supportive function of bone marrow accessory cells and participates in hematopoietic niche assembly (256). In vivo exposure to cigarette smoke and in vitro treatment of long-term bone marrow cultures with nicotine, a major constituent of cigarette smoke, result in inhibition of hematopoiesis (193,194,256).

CXCL12/SDF-1 is a major chemokine in promoting HSC homing, migration, and proliferation (219,269,372). HA oligosaccharides enhance CXCL12-dependent chemotactic effect on peripheral blood hematopoietic $CD34^+$ cells (367). HA-CD44 interaction is essential for homing into the bone marrow and spleen of non-obese diabetic/severe combined immunodeficient mice and engraftment by human HSCs (15). Hematopoietic progenitor cells migrating on HA toward a gradient of CXCL12 acquired spread and polarized morphology with CD44 concentrating at the pseudopodia at the leading edge. These morphologic alterations were not observed when the progenitors were first exposed to monoclonal anti-CD44 antibodies, demonstrating a crosstalk between CD44 and CXCR4 signaling (15).

Although RHAMM and CD44 are expressed by the mobilized blood hematopoietic progenitor cells, function blocking monoclonal antibodies identified RHAMM as a major HA binding receptor, with a less consistent participation by CD44. The G-CSF-associated alterations in RHAMM distribution and the RHAMM-dependent motility of hematopoietic progenitor cells suggest a potential role for HA and RHAMM in the trafficking of hematopoietic progenitor cells and the possible use of HA as a mobilizing agent in vivo (328).

C. HA and mesenchymal stem cells

CD44 has long been used as a marker for mesenchymal stem cells (MSC) (504). Later studies demonstrated that HA and HA-binding proteins play an active role in maintaining and differentiation of mesenchymal stem cells. HA improves human mesenchymal stem cell culture growth and promotes chondrogenic differentiation of human mesenchymal stem cells (135,191). CD44 and HA help mesenchymal stem cells move to injury area to promote tissue regeneration (331). HA promotes a CD44-dependent migration of the mesenchymal stem cells (142). $CD44^{+}$, not $CD44^{-/-}$, stem cells injected into mice with acute renal failure migrated to the injured kidney where HA expression was increased, to accelerate morphological or functional recovery (142). Thus, CD44-HA interactions recruit exogenous MSC to injured renal tissue and enhance renal regeneration (142). MSCs actively migrated to cardiac allografts and contributed to graft fibrosis and, to a lesser extent, to myocardial regeneration (477). MSC, in response to PDGF stimulation, express high levels of CD44 standard isoform, which facilitates cell migration through interaction with extracellular HA. Such a migratory mechanism could be critical for the recruitment of MSCs into wound sites for the proposition of tissue regeneration, as well as for the migration of fibroblast progenitors to allografts in the development of graft fibrosis (500).

An HA-based scaffold has been developed for tissue regeneration. Cartilage regeneration using mesenchymal stem cells and a hybrid poly-(lactic-co-glycolic acid)-gelatin/chondroitin/hyaluronate hybrid scaffold had better chondrocyte morphology, integration, continuous subchondral bone, and much thicker newly formed cartilage (94). HA mixed esters of butyric and retinoic Acid drive cardiac and endothelial fate in term placenta human mesenchymal stem cells and enhance cardiac repair in infarcted rat hearts (445). MSCs survive well in the HA-based prototype ligament scaffold, as assessed after 2 days from seeding, and express CD44, a receptor important for scaffold interaction, and proteins responsible for the functional characteristics of the ligaments (61).

XI. HA-TLR INTERACTION IN NONINFECTIOUS TISSUE INJURY

It is becoming clear that Toll-like receptors 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. Recent studies from Medzhitov and colleagues suggest that TLRs may have homeostatic functions in the gut epithelium (342). These investigators demonstrated that the stimulation of TLR by commensal intestinal flora is critical for protecting against intestinal epithelial injury (342). This elegant work emphasizes the importance of cross talk between factors involved in innate immunity and cytoprotection in the maintenance of intestinal epithelial homeostasis. We demonstrated that $TLR2^{-/-}TLR4^{-/-}$ mice developed a reduced inflammatory response to lung injury, with a decrease in transepithelial neutrophil migration and reduced expression of MIP-2. However, these mice had a higher mortality rate when compared with wild type mice (175). Inhibition of HA binding with the peptide in vivo recapitulated the phenotype observed in the $TLR2^{-/-}TLR4^{-/-}$ mice after lung injury, enhanced lung injury and increased epithelial cell apoptosis, and decreased transmigration of neutrophils (175). 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 (175). 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 bleomycin-induced apoptosis (175). We found that bleomycin induces both NF-κB activation and apoptosis in primary lung epithelial cells (175). Thus, HA regulates basal NF-κB activation in epithelial cells. NF-κB regulates apoptosis (224) and HA fragments can activate NF-κB in macrophages (306). Primary epithelial cells from $TLR2^{-/-}TLR4^{-/-}$ mice have a significant increase in spontaneous apoptosis relative to wild type (175). Furthermore, we made the remarkable observation that cell-surface HA is severely abrogated in $TLR2^{-/-}TLR4^{-/-}$ epithelial cells (175), although the cause of the loss of HA in these mice is unclear. Epithelial cell-surface HA promotes basal NF-κB activation in a TLR-dependent manner and this activation has a protective effect against injury (309).

TLR4 plays a protective role in oxidant-mediated lung injury by maintaining appropriate levels of antiapoptotic responses in the face of oxidant stress (493). TLR4 also maintains constitutive lung integrity by modulating oxidant generation, preventing the development of emphysema (492). On the other hand, TLR4 (and TLR2) have a deteriorative role during non-infectious tissue injury. Hemorrhage-induced lung TNFα production, neutrophil accumulation, and protein permeability, but not NF-κB activation, is dependent on a functional TLR4 (20). 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 (95). Recently, it was shown that TLR4 signaling through the MyD88-dependent pathway was required for the full development of kidney ischemia/reperfusion injury, as both TLR4- and MyD88-deficient mice were protected against kidney dysfunction, tubular damage, neutrophil and macrophage accumulation, and expression of proinflammatory cytokines and chemokines (478). Upregulation of the endogenous ligands HA, high-mobility group box 1, and biglycan, providing circumstantial evidence that one or more of these ligands may be the source of TLR4 activation (478). The discrepancy on the roles of TLR in tissue injury may be due to the injury models utilized, the degree of the injury, and the timing of endpoints examined.

Although both HA and LPS activate TLR, they provoke different sets of gene expression (418). For example, cultured cells exposed to HA showed a pattern of gene induction that mimics the response seen in mouse skin after sterile injury with an increase in molecules such as TGF-β2 and matrix metalloproteinase-13. These factors were not induced by LPS despite the mutual dependence of both HA and LPS on TLR4, suggesting that TLRs may play different roles in infectious inflammation and in sterile inflammation (418). Gallo and associates made an important observation recently that a unique complex of TLR4, MD-2, and CD44 recognizes HA in noninfectious inflammation (418), different from the TLR4, MD-2 and CD14 complex that recognizes LPS during infection. However, MD-2 is usually associated lipid recognition and binding (199,314,321). As HA is a pure glycosaminoglycan and contains no lipid, it is against the involvement of MD-2 in HA-TLR interaction. How MD-2 fits into HA-TLR interaction is to be determined. Furthermore, Gallo and colleagues reported that LPS-TLR4-CD44 signaling is mediated (in part) by A20 (286).

A TLR4 polymorphism (Asp299Gly) attenuates receptor signaling and diminishes the inflammatory response to gram-negative pathogens (315). A recent study found that the Asp299Gly TLR4 polymorphism is associated with a decreased risk of atherosclerosis. This finding pointed to the notion that the innate immunity plays a role in atherogenesis (197).

Similarly, inactivation of the MyD88 pathway led to a reduction in atherosclerosis through a decrease in macrophage recruitment to the artery wall that was associated with reduced chemokine levels (29). How HA plays a role in this process, it is to be seen.

XII. LUNG DISEASES

A. Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a fatal disease of unknown origin with an average survival of 2–3 years from the time of diagnosis for which no effective medical therapies currently exist (304). Lung transplantation is available for IPF patients under the age of 60, but most patients are older than this when the diagnosis is made (253). Although there is a familial form of pulmonary fibrosis, it is rare and the vast majority of cases are sporadic (25). Patients suffocate from unremitting deposition of collagen in the gas exchanging portions of the lung. The molecular mechanisms of lung injury, inflammation and fibrosis are largely unknown. HA levels were higher in BAL fluid in patients with IPF than in healthy controls (27). The HA levels were also correlated with the severity of the disease. Patients with deteriorated conditions had higher lavage fluid concentrations of HA than the patients those whose disease was stable (27). Fibroblast clones derived from primary fibroblast cultures from the lung tissue of patients with pulmonary fibrosis secreted much greater amounts of HA and proteoglycan decorin than the clones from normal individuals (468). HA levels in BAL fluid in sarcoidosis patients were significantly higher than those of controls (26). The increases were significantly higher in clinically active than in inactive sarcoidosis (26,31). The concentrations of HA and fibronectin were higher in patients with sarcoidosis compared to the healthy nonsmoking controls (86). We recently reported that serum inter-alpha-trypsin inhibitor and matrix hyaluronan promote angiogenesis in fibrotic lung injury (107).

Elevated HA levels have been reported in experimental pulmonary fibrosis. There was a transient histological accumulation of HA in the alveolar interstitium, corresponding to increases in HA levels in BAL fluid and lung tissue extracts in bleomycin-induced alveolitis in rats (295,296). Increased extracellular matrix components, fibronectin and HA, as well as polymorphonuclear cells, were detected in BAL fluid in quartz exposed rats (85). While HA in lung tissue and BAL fluid peaked on days 3–7 and then gradually declined towards normal values on days 21–30, lung tissue collagen contents increased between days 7 and 30 (141). The mechanism involved in bleomycin-induced increased HA production in rat lung was associated with growth factors such as PDGF-BB (420), and a decreased HA binding capacity of alveolar macrophages may account for the impairment of internalization and thereby degradation of excessive HA during the early phase of fibrotic lung injury (419).

Although many studies have suggested that HA plays a causative role in the pathogenesis of pulmonary fibrosis, it has been difficult to demonstrate this directly in vivo. One of the challenges is that the disruption of HAS2 allele leads to embryonic leathality and leaves no surviving mice to be subject to injury models. Conditional knockout HAS2 in specific cell types will provide excellent models to dissect the role of HA in biology and pathology.

B. Asthma

HA appears in low concentrations in BAL fluid from healthy individuals, while increased amounts have been reported in lavage fluid from patients with allergic asthma (358,391). Furthermore, HA levels in BAL fluids were significantly increased in patients with persistent asthma, in comparison to patients with intermittent asthma (449), were correlated with the pulmonary function of the patients (39).

One of the reasons for increased HA levels in asthma is an increase in TGF-β levels which promote airway smooth muscle cells to secret glycosaminoglycans such as HA (30). IL-1β and TNFα were the most potent stimulators of HA synthesis and when combined, caused synergistic increases in HA accumulation in human lung fibroblasts. Fluticasone inhibited IL-1β and TNFα induced HA synthesis, and attenuated IL-1β and TNFα stimulated HA synthase-2 mRNA (472). On the other hand, low molecular weight HA increased GM-CSF, TGF-β and intercellular adhesion molecule-1 expression by eosinophils, leading to eosinophil survival. HA-eosinophil interaction may contribute to the regulation of airway inflammation and airway remodeling (313).

Fibroblasts from subjects with the most hyperresponsive airways in asthma produced much more total proteoglycans such as HA, perlecan, and versican than cells from subjects with less hyperresponsive or normal responsive airways (467). Hyaluronan, secreted from submucosal gland cells, plays a role in mucosal host defense by retaining lactoperoxidase and possibly other substances important for first line host defense at the apical surface ‘ready for use’ and protected from ciliary clearance (359).

Aerosol HA administration significantly reduces the bronchial hyper-reactivity to muscular exercise in asthmatics (326). Such an effect could be attributed to the correction of the pathological remodeling, one of the main features of asthma: a correction which could be attributed to the unique physicochemical properties of this major component of the loose connective amorphous matrix of the airways, which is undoubtedly involved in the remodeling process (326). Others found that inhaled HA does not significantly protect against exercise-induced bronchoconstriction in a randomized double-blinded placebo-controlled crossover study, suggesting that HA at the testing dose is not effective as a prophylaxis for exercise-induced bronchoconstriction in patients with asthma (214).

C. COPD/Pulmonary Emphysema

Young COPD patients with higher concentrations of HA and fibronectin in BAL had higher inflammatory cells in BAL, and lower in pulmonary function measurements, than the COPD patients with lower HA levels (393). HA levels were significantly higher in the sputum from patients with COPD than in those from controls. The COPD population appeared to consist of two subpopulations with either high or moderate HA levels. The subgroup of patients with high HA levels had lower FEV_1 than the moderate HA group (393). In addition, neutrophil influx and levels of IL-8, and the soluble TNF receptors were significantly higher in patients with high HA levels than in those with moderate HA levels and controls, indicating a relationship between HA levels, local inflammation and severity of disease, and suggesting enhanced degradation of HA in the lungs of patients with COPD (80). Hyaluronan, chondroitin 4-sulfate, and chondroitin 6-sulfate levels decreased significantly in animals exposed to ozone for 20 months when compared with those in control animals (338). Cigarette smoke exposure leads to enhanced deposition of mostly low molecular weight HA in alveolar and bronchial walls by altering the expression of HA modulating enzymes (40). However it is uncertain if the changes of these glycosaminoglycans contribute the pathogenesis of emphysema.

Aerosolized low molecular weight HA following endotoxin administration significantly increased lung inflammation, whereas pretreatment with HA had the opposite effect (288). Aerosolized HA may be an effective means of preventing pulmonary emphysema and perhaps other lung diseases that involve elastic fiber injury (48). Although clinical trials involving nebulized HA are not expected to yield a measurable treatment effect for at least several years, it is proposed that the special ability of this polysaccharide to retain water may increase the elasticity of lung elastic fibers, producing a relatively rapid improvement in pulmonary mechanics (49). Compared to untreated/smoked controls, aerosolized HA-treated

animals showed statistically significant reductions in mean linear intercept and elastic fiber breakdown products in BAL fluid. The aerosolized HA showed preferential binding to elastic fibers, suggesting that it may protect them from injury (47). The effect of HA on inflammation appears to be related to its molecular size, with larger polysaccharide chains having anti-inflammatory activity and smaller ones having proinflammatory properties. The breakdown of inhaled HA into smaller fragments could possibly induce an inflammatory reaction in the lung that counteracts any beneficial effect. Consequently, the proposed therapeutic use of HA will require development of treatment strategies aimed at minimizing its proinflammatory activity (46).

D. Respiratory distress syndrome

The HA concentrations in BAL fluid and in serum from patients with adult respiratory distress syndrome was much higher than that seen in control patients (125,209). HA staining can be seen in lung sections of patients with severe established adult respiratory distress syndrome (252). The observed accumulation of HA in the small airways in adult respiratory distress syndrome may be expected to immobilize water and thereby contribute to the interstitial and alveolar edema. The inverse correlation was seen between BAL fluid HA and pulmonary oxygenation index (125). In addition, HA concentrations in lung extracts increased with progressively severe respiratory distress syndrome in premature monkeys (184). Bleomycin and hyperoxia cause an increase in lung HA (185,421). We demonstrated that CD44 plays a role in removing accumulated HA fragments at the site of tissue injury to resolve lung inflammation (421).

XIII. Kidney diseases

A. Nephritis

Crescent formation is a major feature of rapidly progressive glomerulonephritis and is generally associated with a poor prognosis. Crescents are formed by accumulation of monocyte/macrophages and plasma proteins in Bowman’s space, by proliferation of parietal epithelial cells and fibroblasts, and by deposition of the extracellular matrix. Marked accumulation of hyaluronate was demonstrated in developing and sclerosing crescents, in association with local infiltration of T lymphocytes and monocyte/macrophages, cells known to express CD44 (302). CD44 is constitutively expressed in the normal kidney and is dramatically up-regulated in rat crescentic anti-glomerular basement membrane disease, suggesting possible roles for the CD44-HA interaction in leukocyte recruitment, renal fibrosis and tubular cell-matrix and cell-cell interactions during the induction and progression of crescentic glomerulonephritis (183). The expression of CD44, HA, and osteopontin was up-regulated at the early stage of the crescent formation in patients with crescentic glomerulonephritis, suggesting that cell-matrix interactions mediated by the CD44-osteopontin and CD44-HA may play important roles in the formation and progression of the crescents (290). Furthermore, the expression of CD44 in the interstitium correlated with the severity of chronic glomerular lesions (362). The glomerular and interstitial CD44 and HA expression correlated with proteinuria, and the interstitial CD44 and HA expression correlated with creatinine clearance rate. It is believed that CD44 participates in the progression of IgA nephropathy by binding HA and osteopontin (362). CD44 is expressed de novo by tubular epithelial cells in areas of tubular injury in kidneys of *kd/kd* mice which develop a spontaneous and chronic tubulointerstitial renal disease, but not in normal control kidneys (384). CD44 positive lymphocytes and macrophages also infiltrate the kidney to *kd/kd* mice. HA also accumulates in *kd/kd* kidneys in the interstitial space, particularly in cortical areas of tubular injury. The expression of osteopontin is enhanced in *kd/kd* kidneys, predominantly in areas of tubular injury (384).

HA plays a significant role in thromboxane-mediated immune events in the kidney, where HA stimulates cyclooxygenase-2 expression and subsequent thromboxane A2 production (406). In experimental anti-Thy-1 nephritis, there was an early glomerular influx of $CD44^+$ macrophages and de novo CD44 expression by mesangial cells, suggesting that cell-matrix interactions mediated by the CD44-HA receptor is involved in mesangial cell proliferation in rat anti-Thy-1 nephritis (300). Leukocyte infiltration into tissues in inflammation is a multistep process involving the sequential engagement of adhesion molecules such as selectins. Synthesized sulphated HA showed strong inhibitory effects on the binding of P- and L-selectin in vitro and is minimally antigenic. Thus, sulphated HA is considered to be a candidate selectin-blocking agent for clinical use. Sulfated HA inhibits intraglomerular infiltration of macrophages and prevents progression of experimental crescentic glomerulonephritis (257,312). Sulfated HA reduced proteinuria, macrophage infiltration, and crescent formation in a dose-dependent manner and reduced urinary protein excretion. Sulfated polysaccharides might be beneficial for the treatment of crescentic glomerulonephritis (257,312).

B. Lupus Nephritis

Increased serum concentration of HA was noted in dermatomyositis patients (211). HA expression was increased in the mesangium, and in the periglomerular and tubular distribution in kidney biopsies of patients with lupus nephritis (490). Lupus nephritis patients showed increased levels of circulating HA, especially during active disease, which correlated with anti-DNA antibody titers (490). Fibroblasts derived from active lesions of nephrogenic fibrosing dermopathy synthesize elevated levels of HA when compared with normal controls (83). Given that HA plays a pivotal role during inflammatory responses, influences cellular behavior and assists in the recruitment of lymphocytes to sites of injury, it is likely that HA contributes to the pathogenesis of lupus nephritis (490).

C. Renal failure

Serum HA levels were significantly increased in patients with renal insufficiency and with end-stage renal failure when compared with the levels measured in healthy controls. Significant correlations were found between serum HA and degree of impaired renal function (123,146). Hyaluronan levels were significantly greater in the subgroup with lower glomerular filtration rates, were associated with an inflammatory state, suggesting impaired renal elimination of proinflammatory cytokines, increased generation of cytokines in uremia, or an adverse effect of inflammation on renal function (324). Similarly, Terney and associates found that patients with deteriorated clinical condition often had greater HA levels, suggesting that HA may be a biochemical marker of patients whose condition deteriorates despite renal replacement therapy (440). Serum HA is mainly in a high molecular weight form (440). In addition, serum HA is raised in active vasculitis (466).

Serum HA concentrations predict survival in patients with chronic renal failure on maintenance haemodialysis (475). Serum HA is an accurate predictor of mortality and morbidity over an 18-month period in patients treated by continuous ambulatory peritoneal dialysis. Large quantities of HA are excreted in peritoneal dialysate, which in part represents local HA production (239). Markedly elevated serum HA levels are found in predialysis patients with malnutrition, inflammation, and atherosclerotic cardiovascular disease and that serum HA is a risk predictor of poor survival in dialysis (399).

HA turnover is cleared rapidly in the circulation by both the liver and the kidney. Evidence suggests that high molecular size HA chains, which are anti-inflammatory, antiangiogenic, and immuno-suppressive are cleared by the liver (298). By contrast, intermediate-sized fragments, which are highly angiogenic, inflammatory, and a stimulus for fibrous

deposition, are cleared by the kidney. The accumulation of HA fragments in renal failure can account for HA deposition in the dermis and may be a mechanism for the nephrogenic fibrosing dermopathy that can accompany these lesions (298).

XIV. ARTHRITIS

Hyaluronan was first isolated from synovial fluid from patients with rheumatoid arthritis more than 50 years ago (101,340). The serum HA concentration in patients with rheumatoid arthritis was significantly greater than that in healthy controls (89,247). The intrinsic viscosity of HA in synovial fluid decreases significantly in mild and severe arthritis compared to that in normal individuals (203). However, while some studies found that the serum levels of HA may not correlate with the severity of the disease (233), others found that serum levels of HA correlate with the severity of the disease and with an objective functional capacity score and with an articular index based on the total amount of cartilage in involved joints (114). Amount of serum HA may be a useful measure of disease activity in patients with rheumatoid arthritis and is a better correlate of clinical disease activity in patients with rheumatoid arthritis than erythrocyte sedimentation rate or C-reactive protein (88). In an experimental condition, serum HA increased as the arthritic lesions developed, correlating with the severity of the disease (28,115).

HA is susceptible to degradation by excessive reactive oxygen species in rheumatoid arthritis patients and HA markedly decreased the O_2^-, H_2O_2, and $OH^\bullet$ in protecting articular tissues from oxidative damage (364). Degradation of hyaluronate in arthritic synovial tissue may be inhibited by radical scavengers (369). The hyaluronate-derived low-molecular-mass oligosaccharide species and formate ($HCOO^-$) are suggested as novel markers of reactive oxygen radical activity in the inflamed rheumatoid joint during exercise-induced hypoxic/reperfusion injury (118).

Sodium HA has been used in an intra-articular treatment of arthritis in race-horses (11) as well as in patients with arthritis (205). Sodium hyaluronate and glucocorticoid treatments had a significant positive effect according to the patients' subjective evaluation (205). In a clinical trial in Japan, significant improvement in pain symptoms and inflammation was observed after the 5 injections of sodium hyaluronate intra-articularly into the knees of 25 patients with rheumatoid arthritis (116). Local therapeutic effects of HA in antigen-induced arthritis in rats are clearly biphasic, with inhibition of inflammation and cartilage damage in the early chronic phase but with promotion of joint swelling, inflammation and cartilage damage in the late chronic phase (355).

It has been suggested that HA-binding proteins play a role in arthritis. CD44 is up-regulated on many synovial cell types in patients with rheumatoid arthritis, and the level of CD44 present in synovial tissue is correlated with the degree of synovial inflammation (134). An anti-CD44 antibody abrogates tissue swelling and leukocyte infiltration (272). On the other hand, treatment with recombinant TNFIP6 protein had a potent ameliorative effect, manifested by decreases in the disease incidence, arthritis index, and footpad swelling, significantly reduced levels of IgG1, IgG2a, and IgG2b antibodies against bovine and murine type II collagen (274). In comparison with wild type mice, the progression and severity of proteoglycan-induced arthritis were significantly greater in Tnfip6-deficient mice with more extensive infiltration of the synovium with neutrophil leukocytes, and elevated serum levels of IL-6 and amyloid A (409).

XV. BRAIN INJURY

Significantly elevated HA levels were found in cerebrospinal fluid patients with spinal stenosis, head injury and cerebral infarction (223). In addition, HA-binding proteins were

also upregulated during brain injury. CD44 expression was strongly activated in the area surrounding the injury within 2 days and then persisted for over 2 months (403). BEHAB/brevican is upregulated in response to central nerve system injury (166). Versican was upregulated in central nerve system injury (13). The concomitant induction of CD44 and HAS-2 mRNA expression was detected in the microglia, macrophages, and microvessels of the ischemic brain tissue (452). HA accumulates after injury and activates microglia and macrophages. Although HA-mediated cytokine release and MAPK signaling in microglia was lower than from peritoneal macrophages, resident microglia does respond to extracellular mediators after brain ischemia (453).

In a rat middle cerebral artery occlusion model HA accumulation was seen in stroke-affected areas and hyaluronidase-1 and 2, and CD44 were upregulated after stroke (5). Increased oligosaccharide HA production soon after stroke may be detrimental through enhancement of the inflammatory response, while activation of HA-induced cellular signaling pathways in neurons and microvessels may impact on the remodeling process by stimulating angiogenesis and revascularization, as well as the survival of susceptible neurons (6). HA binding to the cultured astrocytes stimulated Rac1 signaling and cytoskeleton-mediated migration. HA binding to astrocytes stimulated Rac1-dependent protein kinase N-γ kinase activity which, in turn, up-regulated the phosphorylation of the cytoskeletal protein, cortactin, and attenuated the ability of cortactin to cross-link F-actin. HA-CD44-induced astrocyte function may provide important insights into novel therapeutic treatments for tissue repair following central nervous system injury (38). HA accumulates in demyelinated lesions from individuals with multiple sclerosis and in mice with experimental autoimmune encephalomyelitis (16). The addition of high molecular weight HA to oligodendrocyte progenitor cultures reversibly inhibits progenitor-cell maturation, whereas degrading HA in astrocyte-oligodendrocyte progenitor cocultures promotes oligodendrocyte maturation. High molecular weight hyaluronan may therefore contribute substantially to remyelination failure by preventing the maturation of oligodendrocyte progenitors that are recruited to demyelinating lesions (16).

An HA-based scaffold has been developed for tissue regeneration (94). When a HA-poly-D-lysine copolymer hydrogel with an open porous structure was implanted in brain tissue, macrophages and multinucleated foreign body giant cells were found at the site of implantation of the hydrogel, and astrocytes between the hydrogel and the surrounding tissue, demonstrating the promise of the HA-poly-D-lysine hydrogel as a scaffold material for the repair of defects in the brain (428). Similarly, HA hydrogels modified with laminin created a scaffold supported cell infiltration and angiogenesis, and simultaneously inhibit the formation of glial scar (153).

XVI. HEART DISEASES

HA has been implied in the development and the progression of atherogenesis. HA is expressed in all aortic layers. The highest concentration of the human aorta HA was found in the tunica media, exhibiting a negative concentration gradient from the tunica media to the atheromatic plaque (320). HA acts as a negative regulator on the PDGF-induced vascular smooth muscle cell proliferation and as a positive regulator on the PDGF-induced vascular smooth muscle cell migration (320). HA deposits and cyclooxygenase-2 expression are colocalized in the human internal carotid artery (407). In atherosclerosis, HA associates with leukocytes and vascular smooth muscle cells, and is involved in vascular remodeling. HAS1 and HAS2 are upregulated in response to prostaglandins via Gs-coupled prostaglandin receptors in human vascular smooth muscle cells (99).

Accumulation of HA in myocardial interstitial tissue parallels development of transplantation edema in heart allografts in rats (124). Low molecular weight HA increases the uptake of oxidized low-density lipoprotein into monocytes (410). HA retains low density lipoprotein by forming a HA-low-density lipoprotein complex through the macrophage scavenger receptor CD204 (376). CD44 is upregulated in atherosclerotic lesions of apoE-deficient mice. Low molecular weight forms of HA stimulate VCAM-1 expression and proliferation of cultured primary aortic smooth muscle cells, whereas high molecular weight forms of HA inhibit smooth muscle cell proliferation. CD44 plays a critical role in the progression of atherosclerosis through multiple mechanisms (65). Organization of HA- and versican-rich pericellular matrices may facilitate migration and mitosis by diminishing cell surface adhesivity and affecting cell shape through steric exclusion and the viscous properties of HA proteoglycan gels (92). Versican interacts with HA to create expanded viscoelastic pericellular matrices that are required for arterial smooth muscle cell proliferation and migration (470). Versican is prominent in advanced lesions of atherosclerosis, at the borders of lipid-filled necrotic cores as well as at the plaque-thrombus interface, suggesting roles in lipid accumulation, inflammation, and thrombosis (470).

Overproduction of HA in the aorta resulted in thinning of the elastic lamellae in HAS2 transgenic mice, leading to increased mechanical stiffness and strength (53). Overproduction of HA in the genetic background of the ApoE-deficient mouse strain promoted atherosclerosis development in the aorta, suggesting that accumulation of HA accelerates the progression of atherosclerosis (53).

Infarct healing is dependent on an inflammatory reaction that results in leukocyte infiltration and clearance of the wound of dead cells and matrix debris. CD44 expression was markedly induced in the infarcted myocardium and was localized on infiltrating leukocytes, wound myofibroblasts, and vascular cells (156). $CD44^{-/-}$ mice showed enhanced inflammation, decreased fibroblast infiltration, reduced collagen deposition, and diminished proliferative activity (156). Isolated $CD44^{-/-}$ cardiac fibroblasts had reduced proliferation upon stimulation with serum and decreased collagen synthesis in response to TGF-β in comparison to wild type fibroblasts. Thus, CD44-mediated interactions are critically involved in infarct healing in resolution of the postinfarction inflammatory reaction and regulates fibroblast function (156).

CD44 is expressed abundantly in the embryonic myocardium. The differentiation process is accompanied by an induction of CD44 mRNA and protein (469). Synthesized mixed esters of HA with butyric and retinoic acid primed the expression of cardiogenic genes and elicited a remarkable increase in cardiomyocyte yield in mouse embryonic stem cells (444), demonstrating the potential for chemically modifying the gene program of cardiac differentiation without the aid of gene transfer (444). The mixed esters of HA with butyric and retinoic acid enhanced the expression of VEGF, VEGF receptor KDR, and HGF, primed stem cell differentiation into endothelial cells, and increased the transcription of the cardiac lineage-promoting genes GATA-4 and Nkx-2.5 (445,446).

The Asp299Gly TLR4 polymorphism is associated with a decreased risk of atherosclerosis (197). Similarly, inactivation of the MyD88 pathway led to a reduction in atherosclerosis through a decrease in macrophage recruitment to the artery wall that was associated with reduced chemokine levels (29). These findings pointed to the notion that the innate immunity plays a role in atherogenesis. Whether (or how) HA plays a role in this process, it is to be seen.

XVII. DIABETES

Inflammatory destruction of insulin-producing β cells in the pancreatic islets is the hallmark of insulin-dependent diabetes mellitus. Increased HA levels were seen in diabetic patients (299) and in the glomeruli from diabetic rats (245). Insulin treatment promoted the proliferative response of aorta to injury and this was associated mainly with increased HA production (54). Increased HA, hyaluronidase production and HA degradation were observed in injured aorta of insulin-resistant rats (55). However, others found a decrease of HA in skin in diabetic patients (23) or rats with chronic diabetes mellitus (52). This discrepancy may be due to the measurement of HA during different stages of the disease, inflammatory status and treatment.

Cardiovascular disease contributes to mortality in type 1 diabetes mellitus. Accumulation of HA around smooth muscle cells in lesions of atherosclerosis in diabetic patients. Serum HA levels correlate with poor blood glucose control and diabetic angiopathy and that it could be used as a marker of diabetic angiopathy (275). HA and hyaluronidase were significantly increased in type 1 diabetes when compared to controls. Plasma HA and hyaluronidase correlated in type 1 diabetes. Type 1 diabetes patients show structural changes of the arterial wall associated with increased HA metabolism. These data may lend further support to altered glycosaminoglycan metabolism in type 1 diabetes as a potential mechanism involved in accelerated atherogenesis (299). In a porcine model of atheroscerosis, diabetes was associated with multiple extracellular matrix changes including an increase in HA staining that have been associated with increased lesion instability, greater atherogenic lipoprotein retention and accelerated atherogenesis (261).

In addition, HA binding proteins were also increased in patients with diabetes or in experimental diabetic animal models. The occurrence of LYVE1-expressing lymphatic compartments and the alteration of CCL21 expression in the lymphatics may be involved in defective thymocyte differentiation and migration, and play a significant role in insulitic and diabetic processes (174). Injection of anti-CD44 monoclonal antibody 1 hour before cell transfer of diabetogenic splenocytes and subsequently on alternate days for 4 weeks induced considerable resistance to diabetes in recipient mice, reflected by reduced insulitis (462). A similar antidiabetic effect was observed even when the anti-CD44 monoclonal antibody administration was initiated at the time of disease onset (462). Administration of the enzyme hyaluronidase also induced appreciable resistance to insulin-dependent diabetes mellitus, suggesting that the CD44-HA interaction is involved in the development of the disease.

HA plays an active role in the development of diabetes. HA recruits monocytes to the injury area (128). HA production in response to a raised glucose environment in diabetes can contribute to mesangial hypercellularity (244). HA increases vascular smooth muscle cell expression of PAI-1, a phenomenon that may alter the balance between proteolysis and its inhibition in vessels of patients with type 2 diabetes, thereby contributing to the acceleration of macroangiopathy (254). HA facilitates corneal epithelial wound healing in diabetic rats, which suggests that one possible mechanism of its stimulatory effect lies in its binding to a provisional fibronectin matrix, in both diabetic and non-diabetic rats (291). A regimen consisting of moist wound healing using HA-containing dressings may be a useful adjunct to appropriate diabetic foot ulcer care (443).

Recently, HA-insulin complex was developed to test whether it could be used to treat diabetic patients by oral administration. Glucose-lowering activity was demonstrated after oral administration of the HA-insulin complex to diabetic rats (168). The HA-insulin complex was active after oral administration and the complexed insulin significantly decreased blood glucose concentrations within 1 hour after oral administration in rats (169).

XVIII. LIVER DISEASES

Serum HA levels have long been used as a marker for liver fibrosis. Serum concentrations of HA were higher in patients with active hepatitis compared to that of normal individuals (119,120,343). HA is believed to be a better marker than other markers such as amino terminal propeptide of type III procollagen with regard to prediction of development of cirrhosis as well as prediction of symptoms (284,308). Furthermore, HA levels have a negative correlation with time of survival and HA is a sensitive marker for liver damage in primary biliary cirrhosis (308) and in patients with compensated hepatitis C virus cirrhosis (121). Serum HA levels in children with chronic hepatitis B is a better fibrosis marker than laminin for diagnosing children with advanced liver fibrosis (225). A modification of the Child-Pugh classification of liver cirrhosis by inclusion of HA significantly improves the predictive power of the Child-Pugh classification in alcoholic cirrhosis (207), and in patients with compensated hepatitis C virus cirrhosis (121). Serum HA can also be used as a marker for liver fibrosis in patients with asymptomatic chronic viral hepatitis B with portal inflammation, in assessing and monitoring time trends in liver disease, substituting for repeated biopsies (330). Moreover, HA levels can be used to monitor the antifibrotic effect of lamivudine in children with chronic hepatitis B long-term lamivudine treatment (225). The serum HA level is regarded as a useful predictor for hepatic regeneration after hepatectomy (311). In addition, hyaluronidase levels are elevated after liver injury and measures of circulating hyaluronidase activity may be used to assess liver damage (109).

Increased ascitic levels of HA in liver cirrhosis is found in patients with cirrhosis, suggesting that simultaneous increased synthesis of HA by the peritoneal cells and a reduction of degradation by liver endothelial cells occur in patients with cirrhosis with ascites. This event of increased HA synthesis may be contributory to remodeling and regeneration of the peritoneal lining (217). Hyaluronan analysis indicated that a certain glycosaminoglycan level is required in ascites before its appearance in plasma. The simultaneous increased HA levels in ascitic fluid do not seem to be derived from the systemic circulation. Correlation analyses for TGF-β and IL-6 indicated a strong dependence of the production of HA on cytokine levels and, to a lesser extent, on IL-1β levels, in ascitic fluid of cirrhotic patients (361).

Hyaluronan is excreted in human from the liver and urine (222). The increased level of circulating endogenous HA found in patients with alcoholic cirrhosis is caused by a combination of increased supply to and decreased extraction from plasma (140). HA turnover occurs systemically from the lymph and serum as well as locally by the same cells responsible for its synthesis. Local turnover involves receptor-mediated uptake and delivery to lysosomes. Some fraction of the HA bound to CD44 becomes internalized and delivered to lysosomes regulated possibly by alternatively spliced isoforms of CD44, changes in CD44 phosphorylation, changes in cytoskeletal binding proteins or the activity of extracellular proteolytic activity (201).

In an IL-2-induced vascular leak syndrome model, $CD44^{-/-}$ mice showed a markedly reduced vascular leak syndrome in the lungs and liver, suggesting that CD44 plays a key role in endothelial cell injury by cytotoxic lymphocytes (339). Treatment with HA enhanced the IL-2-induced edema and lymphocytic infiltration in these organs and caused marked increase in IL-2-induced lymphokine-activated killer cell activity, whereas administration of anti-CD44 monoclonal antibodies caused a significant decrease in edema and lymphokine-activated killer cell activity but similar levels of lymphocytic infiltration (285). On the other hand, in a Con A induced liver injury model, $CD44^{-/-}$ mice showed a markedly increased hepatitis, increased production of cytokines such as TNFα, IL-2 and IFN-γ, T cells from $CD44^{-/-}$ mice were more resistant to activation-induced cell death when compared with the

wild type mice. Activated T cells use CD44 to undergo apoptosis, and dysregulation in this pathway could lead to increased pathogenesis in a number of diseases, including hepatitis (57). Administration of staphylococcal enterotoxin B to $CD44^{-/-}$ mice caused significantly enhanced liver damage which correlated with elevated numbers of T cells, NK cells, NKT cells, and macrophages in the liver and increased production of TNFα and interferon-γ compared to wild type mice (263). These studies demonstrated a protective role for CD44 during hepatic injury.

XIX. SUMMARY

Hyaluronan as an important part of extracellular matrix accumulates during inflammation and tissue injury. Hyaluronan is degradated to smaller species by reactive oxygen species and possible by hyaluronidases at the injury site. Hyaluronan regulates cytokines and other inflammatory substances and influence inflammatory cell recruitment and chemotaxis. The actions of hyaluronan are dependent on its interacting proteins and cells. Hyaluronan binds to an array of proteins to elicit its biological roles. Hyaluronan-CD44 interactions have long been suggested important in leukocyte homing and recruitment. The recent demonstration of hyaluronan-Toll like receptor interactions provides the molecular insight into the mechanisms of hyaluronan signaling in inflammatory cells as well as in epithelial cells. Hyaluronan and its binding proteins play a role in the pathogenesis of many human diseases and in numerous experimental conditions. Therapeutic developments targeting hyaluronan and its binding proteins are developing. Understanding the role of hyaluronan and its binding proteins in the pathobiology of disease will facilitate the development of novel therapeutics for many critical diseases.

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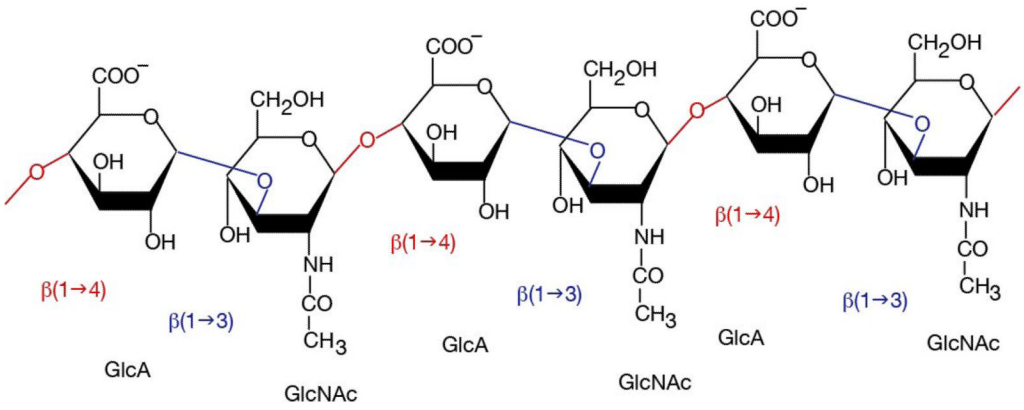

Figure 1. Hyaluronan structure

Hyaluronan is composed of repeating polymeric disaccharides D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) linked by a glucuronidic β(1→3) bond. Three disaccharide GlcA-GlcNAc are shown.

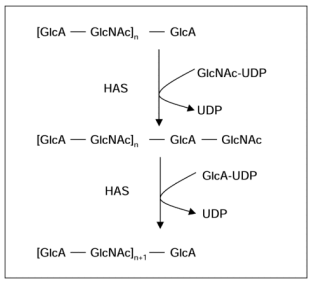

Figure 2. HA synthase reaction

Hyaluronan synthase catalyzes the reaction by adding *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) alternatively to expand HA chain.

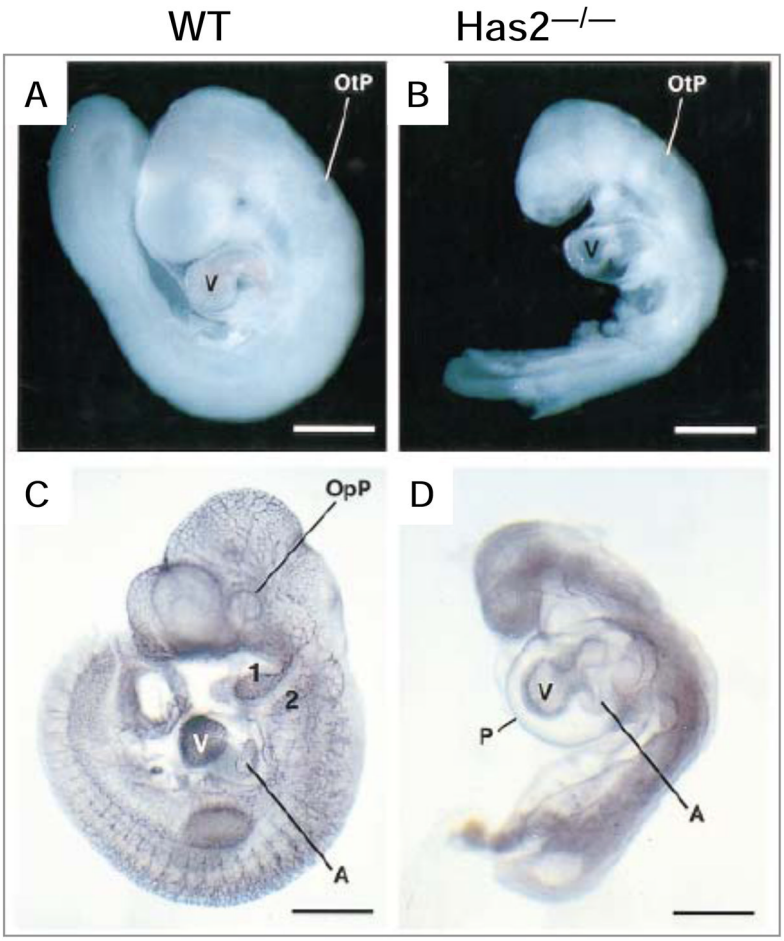

Figure 3. HAS2-deficient mice

Disruption of hyaluronan synthase 2 resulted in the defect in heart formation. Representative wild-type (A) and *Has2*$^{-/-}$ (B) embryos at E9.5. Note the diminished size, the bloodless heart, and distorted somites of the *Has2*$^{-/-}$ embryo. E9.5 wild-type (C) and *Has2*$^{-/-}$ (D) embryos stained for the endothelial marker PECAM. Note the absence of an organized vascular network expressing PECAM in the *Has2*$^{-/-}$ embryo. P, pericardium; E, endoderm; M, mesoderm; OpP, optic placode; OtP, otic placode; first and secondpharyngeal pouches are numbered. From Camenisch et al., *J. Clin. Invest.* **106**:349–360 (2000), with permission.

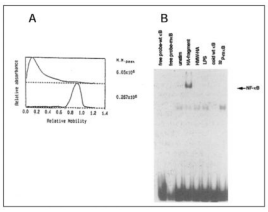

FIGURE 4. HA FRAGMENT

Purified HA fragments but not HMW-HAinduced NF-KB DNA binding activity in MH-S cells. (A) Densitometric scanning demonstrating the molecular weights of HMW-HA *(top)*, and purified HA fragments *(bottom*). (B) Electrophoretic mobility shift assay of nuclear extracts prepared from MH-S cell stimulated for 2 h with either serum-free media (unstim), HMW-HA, HA-fragment, or LPS. HA fragments induced NF-KB DNA binding activity. From Noble et al., *J. Exp. Med.* 183:2373–2378 (1996), with permission.

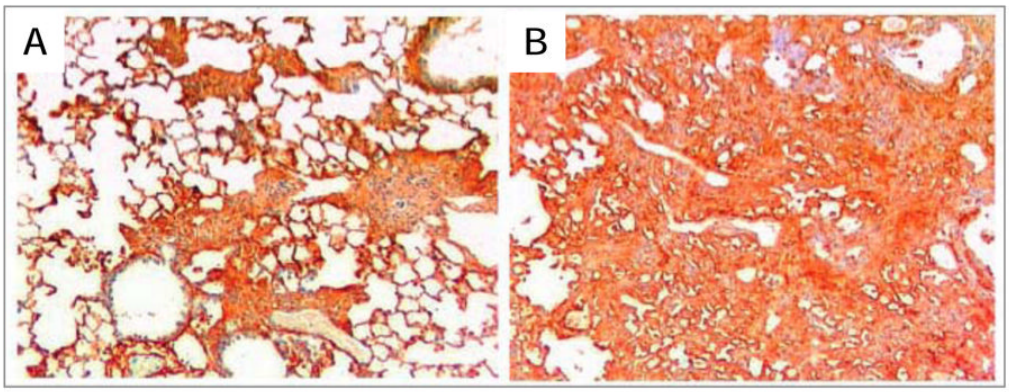

FIGURE 5. CD44 NULL HA STAINING

Accumulation of HA after bleomycin treatment. Lung tissue stained for HA at day10 through 14. Wild-type (A) and CD44-deficient mice (B). From Teder et al., *Science* **296**, 155 (2002), with permission.

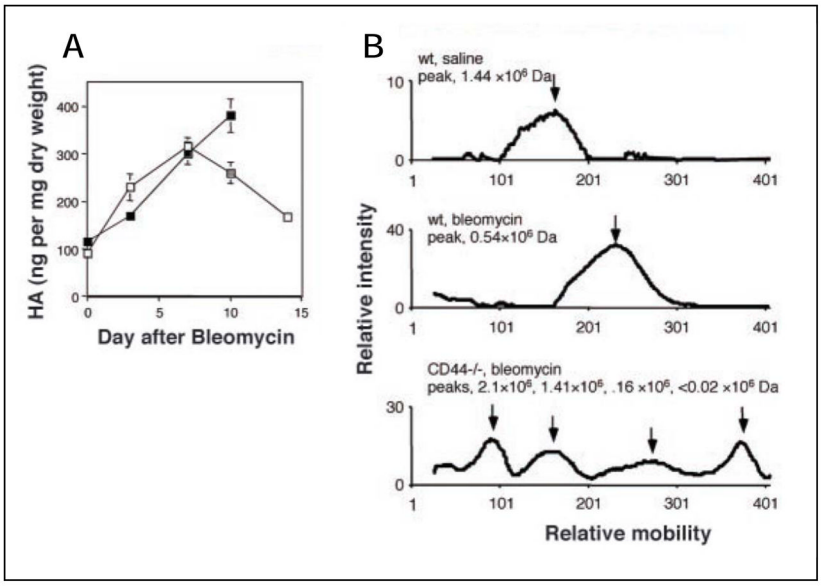

FIGURE 6. HA FRAGMENT ACCUMULATION IN CD44 MICE

Accumulation of HA after bleomycin treatment. (**A**) HA content was measured by an HA-specific enzyme-linked immunosorbent assay in lungs of wild-type (open square) and CD44-deficient mice (closed square). (**B**) HA MW at day 7 in lungs of saline-treatedwild-type mouse (top panel, MWaverage 1.443106), bleomycin-treated wild-type mouse (middle panel, MW average 0.54 3 106), and bleomycin-treatedCD44-de?cient mouse (bottom panel, MW averagesfrom left to right 2.10 3 106, 1.41 3 106, 0.16 3 106, and,0.02 3 106). From Teder et al., *Science* **296**, 155 (2002), with permission.

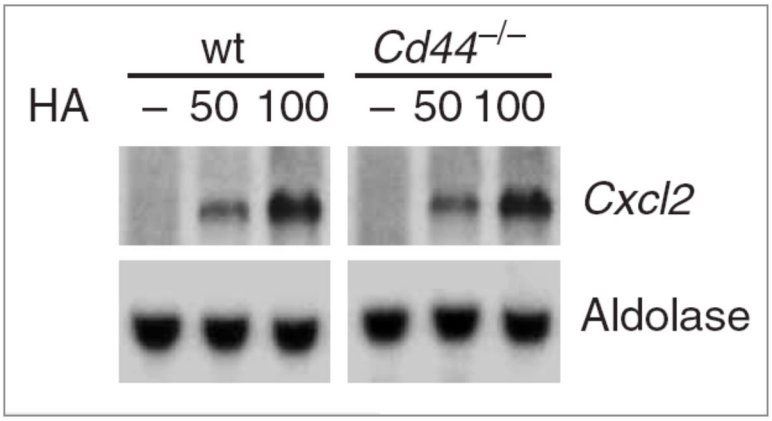

FIGURE 7. CD44 AND HA SIGNALING

HA signaling is independent on the presence of CD44. Chemokine CXCL2 expression by peritoneal macrophages was not affected by the deficiency of CD44. From Jiang et al., ***Nat Med***, 11:1173 (2005), with permission.

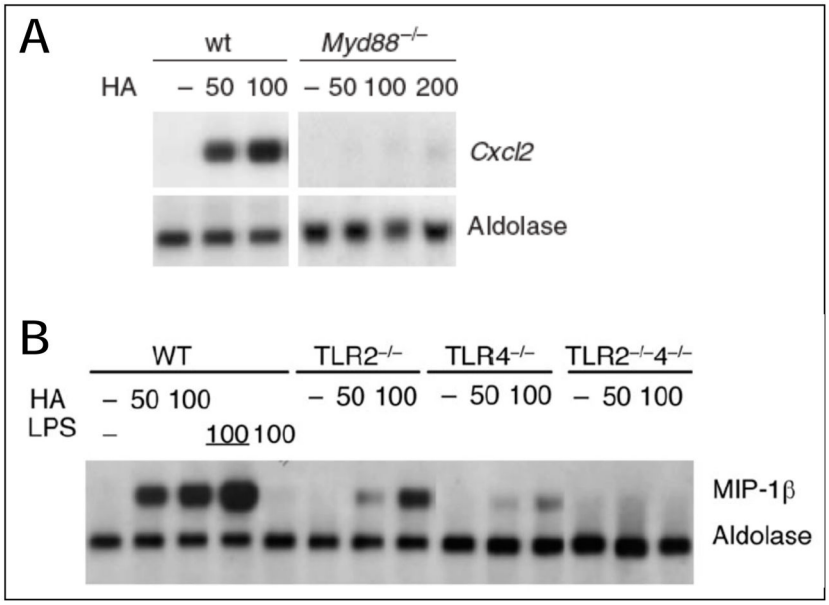

FIGURE 8. TLR AND HA SIGNALING

HA signaling is independent on the presence of CD44. Chemokine CXCL2 expression by peritoneal macrophages was not affected by the deficiency of CD44. From Jiang et al., ***Nat Med***, 11:1173 (2005), with permission.

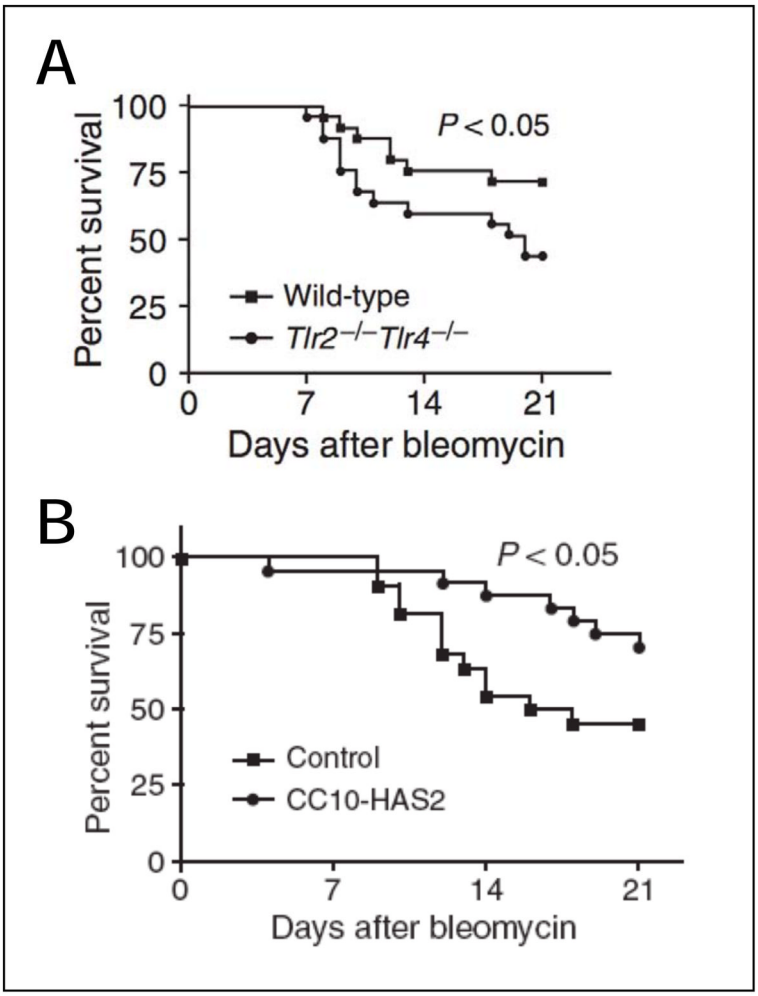

Figure 9. TLR and lung injury

TLR2 and TLR4 double deficient mice were more susceptible (A), while mice overexpressing HAS2 on epithelial cells were more resistant, to bleomycin induced lung injury (B).

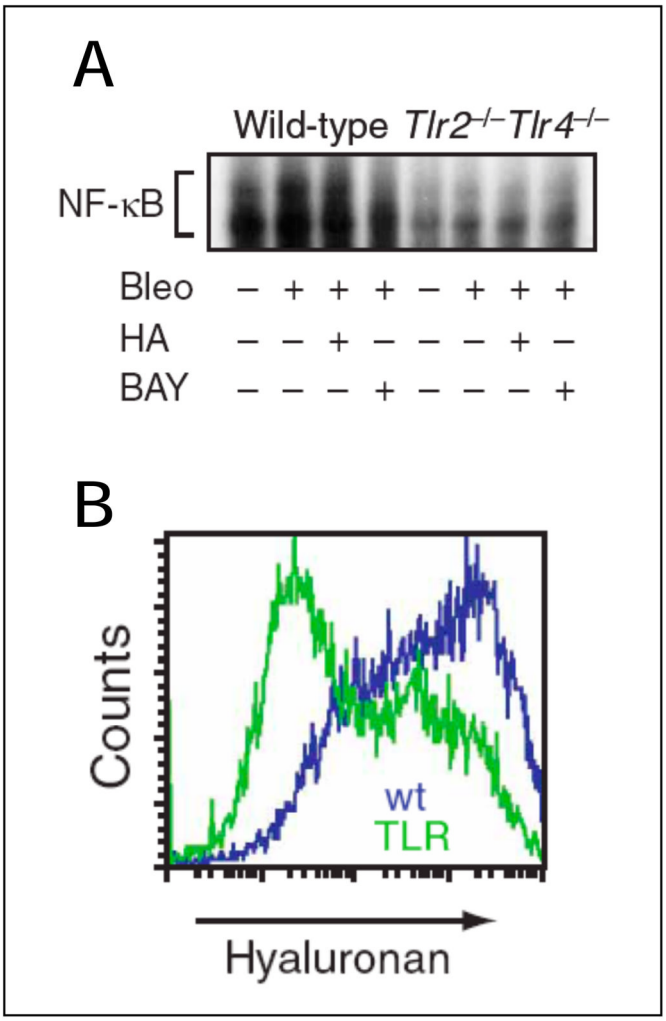

Figure 10. TLR, HA, and NF-kB

TLR2 and TLR4 double deficient mice displayed lower basal NK-kB activity (A). The epithelial cells from TLR2−/− TLR4−/− mice expressed reduced cell surface HA (B).

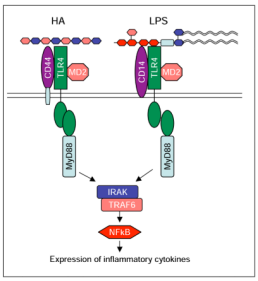

Figure 11. Analogous signaling of HA and LPS

Analogous signaling of HA and LPS. LPS uses TLR4, CD14 and MD2 to transduce its signal, while HA interacts with TLR4, CD44 and MD2 to elicit its signaling.

TABLE 1

Selected genes induced by HA fragments.

Category	Gene/protein	Cell type	References
Chemokines	CCL3	Macrophages	(265)
	CCL4	Macrophages	(265)
	CXCL2	Macrophages	(17,175)
	CCL5	Macrophages	(265)
	CCL2	Renal tubular epithelial cells	(22,265)
	CXCL10	Macrophage	(149,265)
	CXCL9	Macrophage	(149)
	CXCL1	Endothelial cells	(411)
	CCL5	Macrophages	(265)
	IL-8	Endothelial cells, epithelial cells	(255,417,451)
	CXCL1	Macrophages	(148,175)
Cytokines	IL-12	Macropahges, dentritic cells	(144,425)
	TNFα	Dentritic cells	(425)
	IL-1β	Dentritic cells	(425)
Growth factors	TGF-β2	Monocytes	(418)
	IGF-1	Macropahges	(305)
Transcription factors	IκBα	Macrophages	(306)
	AP-1	Endothelial cells	(74)
	Rest	Monocytes	(418)
ECM	MMP-10	Endothelial cells	(417)
	MMP-13	Monocytes, dentritic cells	(97,418)
	PAI-1	Macrophages	(150)
	uPA	Macrophages	(150)
	MME	Macrophages	(151)
	MMP-9	Dentritic cells	(97)
	Collagen VIII	Endothelial cells	(354)
HSPG	Syndecan-4	Endothelial cells	(417)
Others	iNOS	Hepatocytes, endothelial, Kupffer, and stellate cells	(353)
	Cox2	Renal tubular epithelial cells	(406)
	MDR-1	Lymphocytes	(436)
	Trdn	Monocytes	(418)
	Frk	Monocytes	(418)