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REVIEW ARTICLE

Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer

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Hyaluronan (HA) is a linear nonsulfated glycosaminoglycan of the extracellular matrix that plays a pivotal role in a variety of biological processes. High-molecular weight HA exhibits different biological properties than oligomers and low-molecular weight HA. Depending on their molecular size, HA fragments can influence cellular behavior in a different mode of action. This phenomenon is attributed to the different manner of interaction with the HA receptors, especially CD44 and RHAMM. Both receptors can trigger signaling cascades that regulate cell functional properties, such as proliferation migration, angiogenesis, and wound healing. HA fragments are able to enhance or attenuate the HA receptor-mediated signaling pathways, as they compete with the endogenous HA for binding to the receptors. The modulation of these pathways could be crucial for the development of pathological conditions, such as inflammation and cancer. The primary goal of this review is to critically present the importance of HA molecular size on cellular signaling, functional cell properties, and morphology in normal and pathological conditions, including inflammation and cancer. A deeper understanding of these mechanisms could contribute to the development of novel therapeutic strategies.

Abbreviations
ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; CAF, cancer-associated fibroblasts; CD44s, CD44 standard isoform; ECM, extracellular matrix; ECs, endothelial cells; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; FAK, focal adhesion kinase; GAG, glycosaminoglycan; HA, hyaluronan; HASes, HA synthases; HER2, human epidermal receptor 2; HMW HA, high-molecular weight HA; HYALs, hyaluronidases; ICAM-1, intercellular adhesion molecule 1; IGF-IR, insulin-like growth factor receptor; IL, interleukin; LMW HA, low-molecular weight HA; MAPK, mitogen-activated protein kinase; MDR1, multidrug resistance protein 1; MMP, matrix metalloproteinase; MMW HA, medium molecular weight HA; MSCs, mesenchymal stem cells; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; o-HA, HA oligomers; PAI-1, plasminogen activator inhibitor-1; PDGFR, platelet-derived growth factor receptor; PGs, proteoglycans; PI3K, phosphoinositide 3-kinase; RHAMM, receptor for HA-mediated motility; SEM, scanning electron microscopy; stat-3, signal transducer and activator of transcription protein 3; TEM, transmission electron microscopy; TGFβRI, transforming growth factor beta receptor I; TLRs, toll-like receptors; TNF-α, tumor necrosis factor α; uPA, urokinase plasminogen activator; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; vHMW HA, very high-molecular weight HA.

Introduction

Hyaluronan (HA) is a ubiquitous glycosaminoglycan (GAG) of extracellular matrix (ECM) composed of disaccharide repeats of *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA). The structure of the repeating units is $(\rightarrow 4GlcA1\beta\text{-}3GlcNAc1\rightarrow)_n$. It is a relatively simple molecule without the typical modifications present in other GAGs such as GlcA-epimerization or sulfation [1,2].The resulting anionic polymer has a semiflexible structure adopting an expanded worm-like random coil, with a variable molecular mass. Under normal conditions, HA is generally synthesized as a high-molecular weight polymer (HMW HA) that ranges from 1000 to 6000 kDa. When ECM homeostasis is disrupted by pathological conditions, such as cancer, inflammation, oxidative stress and tissue remodeling, endogenous HMW HA can be degraded faster by hyaluronidases (HYALs) and ROS, thus unbalancing the equilibrium toward a higher concentration of low-molecular weight HA (LMW) species, with a molecular mass $\leq$ 250 kDa. LMW HA can be further fragmented into shorter oligomers (o-HA) [3].

Many of HA functions depend on specific HA-binding proteins and proteoglycans (PGs) such as versican, aggrecan, and neurocan that bind HA through noncovalent interactions and contribute to create a highly hydrated and charged domain on the cell surface as well as in the extracellular space [4].The structure-based physical properties of HA determine different functions of the polysaccharide: the anionic nature of HA along with its hydrodynamic volume create a protective coat for the interaction of specific targets with their receptors embedded in the cell membrane [5]. HA can also act like a size-selective barrier that regulates the diffusion of small molecules, while large molecules are partially or completely excluded. Thanks to its hygroscopic properties, HA provides viscoelasticity and lubrication of liquid connective tissues, being one of the major components of articular joints synovial fluids [6] and vitreous body. In these fluids, HA is mainly present as vHMW HA [7], while other fluids such as milk, blood, saliva, and urine contain HA as a LMW molecule [8–12]. Solid and healthy tissues are usually associated with HMW HA, like cartilage [13–15] and skin [16], where HA provides a three-dimensional scaffold for cells by the assembly of pericellular ECM. As an ECM component, HA mediates several physiological processes. Its presence is fundamental during embryogenesis and epithelial-to-mesenchymal transition (EMT), promoting neural crest cell migration, the formation of heart valves, and brain development [17–19]. HA is also one of the major mediators of wound healing; it is constantly produced as HMW HA, especially during the first stages of the process and it is constantly cleaved by its degrading enzymes to sustain fibroblast proliferation [20], collagen deposition, and angiogenesis [21,22]. Furthermore, a recent study reported that wound repair and fibroblast migration are differently stimulated by specific sizes of HA oligosaccharides. According to this study, only the 6-mer oligosaccharides were able to induce fibroblast motility, wound closure, and inflammatory response, whereas 4-mer failed to induce cell migration and 10-mer even inhibited early wound closure [23].

Several reports demonstrated that alterations in HA amount and size are characteristics of pathological conditions, such as cancer and inflammation [24–26]. In particular, an abnormal accumulation of HA in cancer stroma is considered as a marker of malignancy for several types of solid tumors [27–34]. Moreover, the inhibition of HA production by 4-methyl-umbelliferone (4-MU) and the use of siRNA and miRNA against HA synthases (HASes) suppresses tumor growth [35–41]. Remarkably, the presence of LMW HA is predominant during tissue injury and stimulates the production of proinflammatory cytokines [31]. For example, HA fragments < 500 kDa induce the expression of inflammatory genes in renal tubular epithelial cells and bladder cancer cells [42,43]. Furthermore, fragments among 1.9–3.8 kDa can stimulate nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) signaling leading to proinflammatory chemokine production and breast tumor invasion [44]. This evidence suggests that the increased synthesis and the further fragmentation of HA by tumor cells stimulate inflammation, which in turn sustains tumor malignancy and progression. In this review, we focus on the HA metabolism and the molecular size-dependent effects of HA on cellular signaling, cell functional properties, cell morphology and receptor binding in normal and pathological conditions, including inflammation and cancer, providing perspectives for future pharmacological targeting.

HA synthesis and degradation: metabolic and epigenetic regulation

Hyaluronan is synthesized by three transmembrane isoenzymes called HASes (HAS1, HAS2, and HAS3). These enzymes are similar in the amino acid sequence and structure yet differ in enzymatic activity and regulation. HAS1 and HAS2 synthesize HMW HA chains, while HAS3 produces shorter polymers ($\sim 2 \times 10^3$ kDa *vs* $\sim 2 \times 10^2$ kDa, respectively) [1,45,46]. Furthermore, the three isoforms exhibit a different subcellular localization [47] and undergo different

regulative processes [48–50]. Among HASes, HAS2 is considered the most important enzyme because of its fine regulation and its essential role for animal survival, hence HMW HA is critical during embryogenesis. High levels of HMW HA during development induce growth of several tissues, such as blood vessels, brain, heart, and limbs. HMW HA is also responsible for tissue hydration due to its ability to bind high amounts of water. Because of this property, HA is able to lubricate and space-fill tissues [51–53]. HAS2 produces HMW HA and its deficiency results in embryonic lethality and failure of the endocardial cushion formation, along with defects in yolk sac and vasculogenesis [54,55]. HA synthesis can be controlled by multiple mechanisms. The cytoplasmic content of UDP-sugars (UDP-GlcA and UDP-GlcNAc) is critical to regulate HA production, as the compound 4-MU inhibits the synthesis of HA depleting GlcA cytoplasmic levels [56]. Although 4-MU could limit UDP-GlcA availability to multiple glycosyltransferases, its inhibitory action is specific for the substrate of HAS, as it is localized in the inner plasma membrane; in fact, the majority of glycosyltransferases are located in the Golgi, that is not permeable to 4-MU. The cytoplasmic concentration of GlcNAc is another critical issue in the synthesis of HA. Firstly because the treatment with GlcN to the growth medium increases UDP-GlcNAc levels and thus HA production [57–59]. Secondly, GlcNAc can be the substrate for the enzyme OGT (O-GlcNAc transferase), that is able to transfer GlcNAc moieties on HAS2 protein, with an enzymatic reaction called O-GlcNAcylation. This post-translational modification increases the half-life of the enzyme, stabilizing the protein in the plasma membrane and increasing HA synthesis [60]. Interestingly, the UDP-sugar precursor availability can also influence HA size in prokaryotic cells, like in the Gram-negative *Pasteurella multocida* [61]. Besides the precursor availability in the cytoplasm, the synthesis of HA strongly depends on HAS2 mRNA expression and it can be controlled at post-transcriptional levels. The phosphorylation mediated by energy sensor AMPK on Thr-110 inhibits the synthesis of HAS2 and in turn the production of HA [62], suggesting an important metabolic control of HA synthesis that depends on the energy status of the cells and nutrient availability. A recent study reported that also ubiquitination of Lys-110 and the action of deubiquitinating enzymes (USP4 and USP17) differently affect the dimerization of the protein, acting on its stability and function [63,64]. Besides post-transcriptional modifications, HAS2 expression is finely regulated by epigenetics through the action of the long noncoding RNA, HAS2-AS1. HAS2-AS1 is a natural antisense transcript able to induce HAS2 expression after O-GlcNAcylation [65]. This modification would act *in cis* modifying the architecture of the chromatin, making HAS2 promoter accessible to transcription factors. However, the activity and the influence of HAS2-AS1 on HAS2 expression can be considered tissue and cell specific: the first research group to describe HAS2-AS1 demonstrated that the overexpression of HAS2-AS1 reduced HAS2 mRNA levels and HA production, inhibiting the proliferation of human osteosarcoma cells [66], whereas other studies reported a coordinated expression between HAS2-AS1 and HAS2 [67–69]. Recent studies reported that HAS2 can undergo the control of microRNAs (miRNAs). Röck *et al.* [70] demonstrated that miR-23a-3p targeted and inhibited HAS2 expression inducing cellular senescence. Similarly, HAS2 can be also targeted by the miRNA let7 [71] and miR-26b [72], while miR-7 can indirectly decrease the expression of HAS2 targeting the epidermal growth factor receptor (EGFR), negatively affecting the HA-mediated CD44-EGFR signaling pathway [73]. We recently discovered that ERβ suppression in triple-negative breast carcinoma cells markedly reduced their functional properties and affected the mesenchymal phenotype. This was associated with alterations in the expression of matrix molecules including proteolytic enzymes, proteoglycans, and growth factor receptors. Notably, ERβ ablation altered the expression of miRNAs directly involved in the aggressiveness of breast cancer cells as well as in the biosynthesis of matrix molecules, including miR-10b, miR-200b, and miR-145, which in turn are crucial regulators of functional properties, EMT program and matrix composition of ERβ-suppressed cancer cells [74]. We recently discovered that ablation of ERβ caused a significant reduction in the expression of HAS2 as well as a reduction in HYALs and CD44 isoforms (v3, v6, and v9) (not shown).

The presence of different sizes of HA suggests that the cells have a very efficient mechanism for the HA turnover and metabolism. HA fragmentation is conducted by HYALs or through oxidative/nitrosative damage. HYALs are hydrolases that cleave the β-(1,4) linkage and they are active in a large pH range. There are six HYALs so far recognized in humans: HYALs 1-4, HYALP, and PH-20 [4,52]. The degrading activity of HYALs is not strictly related to HA, but can be also extended to other GAGs; HYAL4, for example, is able to cleave chondroitin sulfate [75], with no evidence of HA catabolic activity. Among all HYALs, HYAL1 and HYAL2 are the predominant isoforms to cleave HA. HYAL1 degrades HA into small fragments

of one to six disaccharides [52,76], whereas HYAL2 mainly produces larger fragments (20 kDa). Degradation of HA by HYAL1 primarily takes place in the lysosome after binding and internalization of HA performed by HA receptors. Interestingly, experiments of colocalization demonstrated that HYAL2 is present on the cell membrane together with CD44, generating HA fragments that could be released into the medium or internalized to be further degraded by HYAL1 [77]. HYAL1 and HYAL2 show a catalytic activity at low pH and are associated with processed HA into endocytic vesicles. A recent study described the new HYAL, TMEM2, as a cell surface HYAL; the enzyme acts at pH 6–7 and degrades HMW HA into an intermediate size (~ 5 kDa) before the internalization and degradation in the lysosomes [78]. Aside from the specific enzymatic activity of HYALs, HMW HA can be fragmented by reactive oxygen or nitrogen species produced during tissue inflammation, sepsis, ischemia–reperfusion injury and cancer [79]. In damaged tissues, HA fragmentation can be considered one of the first danger signals to trigger signaling pathways leading to inflammation and repair. Although the mechanisms by which LMW HA sustains inflammation and malignant progression are still poorly understood, it has been proposed that LMW fragments may alter clustering and thus the signaling of cell surface receptors activated by native HMW HA, such as CD44 and receptor for HA-mediated motility (RHAMM) [80].

HA surface receptors: signaling and functions

Hyaluronan is a versatile macromolecule, able to interact with different receptors on cell surface and activate distinct downstream signaling. Interestingly, its versatility lies in its size, as HA chains of different lengths trigger diverse responses upon the binding with the same receptor (Fig. 1). Although several HA receptors have been identified, the most relevant for inflammation and cancer are CD44 and RHAMM [27,81]. CD44 is a ubiquitous single-span transmembrane glycoprotein that regulates a variety of cell–cell and cell–matrix interactions, such as cell traffic, lymphocyte activation, cell aggregation, and the presentation of signaling molecules for migrating cells [31]. In humans, CD44 is encoded by a single gene located on chromosome 11 and contains 20 exons, generating *ca* 20 variants/isoforms (CD44v). The standard isoform (CD44s) is encoded by 10 exons constant in all isoforms. CD44 variant isoforms derive from alternative splicing and all encode for segments of the extracellular domain, representing additional binding motives for the interaction with other molecules in the microenvironment. CD44s is ubiquitously expressed, whereas CD44 variants are expressed primarily during inflammation and cancer [31]. For example, CD44v6 is highly expressed in lung, breast, ovarian, and colon cancer [82–85], while increased CD44v expression and decreased CD44s expression were found in three different cell lines and human specimens of metastatic pancreatic carcinoma [86]. Nevertheless, it has been reported that CD44s is a marker of poor prognosis in the early stages of ampullary adenocarcinoma, while CD44v are representative of advanced cancer cases with recurrence [87]. Interestingly, the switch between CD44s and CD44v is important in the regulation of EMT and in the plasticity of cancer cells [88]. The interaction between HA and CD44 occurs in the N-terminal region thanks to the HA-binding motif BX7B, which is also expressed in other HA-binding proteins including RHAMM. The amino terminal "link" domain leads to ligand-induced clustering and the activation of signaling axes, such as ERK1/2, Akt, Wnt/β-catenin and focal adhesion kinase (FAK). The mechanisms by which CD44 affects cancer progression are under investigation and mainly focus on the ability of the receptor to bind HA, thus to trigger different cellular responses like the stimulation of oncogenic pathways, miRNA functions, and chemo/radiation resistance [89].

The size of HA can differently influence the activation of the receptor, thus the stimulation of specific pathways. As mentioned above, HMW HA stimulates CD44 clustering, however, the addition of oligomers seems to reduce the clustering strength induced by HMW HA [80]. Indeed, HMW HA possess multivalent sites to bind CD44, while oligomers possess just one or two sites [90], suggesting that oligomers can act as antagonists reducing the affinity between HMW HA and the receptor.

As the interaction between CD44 and HA is mediated by hydrogen bonds, the expression of CD44 on cell membrane is important to determine the ligand–receptor interaction. An artificial membrane system consisting of a bilayer densely coated with CD44 showed that the interaction between HMW HA and CD44 creates irreversible bounds, which are independent from HA concentration. However, according to the same study, LMW HA (< 10 kDa) could not create irreversible interactions, as the binding strength became weaker and dependent on HA concentration [90]. HMW HA inhibits the mitogen-dependent induction of cyclin D1, reducing the proliferation of vascular smooth muscle cells and fibroblasts via CD44 [91]. HMW HA and LMW HA bind CD44 with similar

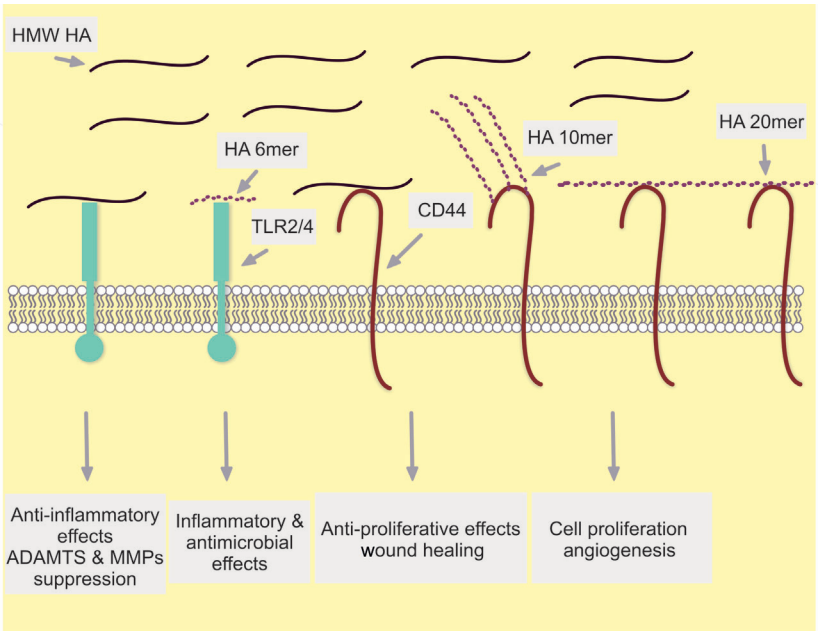

Fig. 1. Hyaluronan interactions with HA receptors depend on HA molecular size. HA interacts with HA receptors, regulating inflammation and cancer depending on its molecular size. HMW HA binds to TLRs 2 and 4 exhibiting anti-inflammatory effects and suppressing MMPs and ADAMTs activation. HA hexamers bind to TLRs 2 and 4 promoting wound healing and inflammation. HMW HA binds to CD44 leading to wound healing and exhibiting antiproliferative properties. On the other hand, o-HA form clusters on CD44 initiating angiogenesis and promoting cell proliferation (multifragment binding). o-HA size needs to be up to six saccharides in order to be able to bind to CD44. As the number of saccharides is increasing the binding to the receptor becomes more stable. HA fragments of more than 20 saccharides are able to bind more than one CD44 receptor (multivalent binding).

affinity. However, they regulate the cyclin D1 expression via different pathways. HMW HA binding to CD44 inhibits the Rac-dependent signaling that triggers the expression of cyclin D1. On the other hand, CD44 activation by LMW HA leads to ERK1/2 activation and ERK1/2-dependent cyclin D1 expression [92].

Signaling and cell–matrix interactions mediated by CD44 may be regulated by the proteolytic cleavage of the receptor. o-HA stimulates CD44 cleavage and promotes cell migration in pancreatic carcinoma cell line. This effect is specific for o-HA ranging from 6-mers to 36-mers, but not for HMW HA and HA disaccharides obtained after the digestion with *S. dysgalactiae* [93], highlighting the importance of HA size in the receptor binding. This mechanism is largely used by tumor cells, where the production of HA fragments enhances their own CD44 cleavage, stimulating cell motility and tumor progression [94]. LMW HA can also stimulate the formation of a unique receptor complex made of CD44 and toll-like receptors (TLRs), stimulating the release of proinflammatory chemokines in breast cancer cells through MyD88-NF-kB signaling [44]. Similarly, oligosaccharides made of 6-mer induce inflammation by engaging both TLR4 and CD44 in human chondrocytes [95] and in neuron-like SH-SY5Y cells [96]. On the contrary, 4-mer interacts with TLR2 and TLR4 but not with CD44 [97], underlying the specificity of the number of disaccharides in receptor binding and activation.

Receptor for HA-mediated motility is a coiled coil-type protein expressed on the cell surface and in the cytoplasm, as well as in the cytoskeleton and nucleus. Unlike CD44, RHAMM expression is tightly regulated under physiological conditions. Thus, RHAMM is a cytoplasmic protein, whose translocation on the cell surface occurs via unconventional transport, as the receptor lacks a signal peptide. Like CD44, RHAMM undergoes alternative splicing [98,99], indeed truncated isoforms were detected in cells following injury and tumor [100,101]. Several studies reported an increased expression of RHAMM during breast, colon brain, prostate, and endometrial cancer [102–105], as well as in inflammation like skin wounds, osteoarthritis, and bleomycin-induced lung injury [106–108]. The binding with exogenous HA and the signaling cascade are mediated by the interaction with other receptors such as platelet-derived growth factor receptor (PDGFR),

transforming growth factor beta receptor I (TGFβRI), and CD44 [31]. These extracellular interactions trigger the activation of inflammatory pathways, like ERK1/2 leading to cell migration, wound healing, tumorigenesis, and EMT. Intracellular RHAMM binds to the cytoskeleton interacting with actin filaments and microtubules contributing to microtubule-mediated cell polarity and motility. Nuclear RHAMM also binds mitogen-activated protein kinase (MAPK), which mediates the activation of matrix metalloproteinase 9 (MMP-9), inducing inflammation and cell migration [109].

Although the mechanisms that mediate the interaction between CD44 and HA of different molecular weights are well described, little is known about the interaction between RHAMM and HA of different sizes. Kouvidi *et al.* [110] reported that LMW HA (15–40 kDa) specifically binds RHAMM in fibrosarcoma cells and stimulates cell adhesion onto fibronectin, while HMW HA inhibited cell adhesion and failed to induce RHAMM expression. Moreover, o-HA sized 2–10 disaccharides units stimulate angiogenesis via RHAMM-mediated signaling pathways in epithelial cells during wound healing, where CD44 failed to activate signal transduction [111]. The action of RHAMM can occur in synergy with CD44. In fact, it has been reported that the presence of both receptors is essential during wound closure stimulated by HA 6-mer, that in turn stimulate the accumulation of wound M1 and M2 macrophages and TGFβ1 [23]. These observations are consistent with the fact that RHAMM is expressed during stressful conditions such as inflammation and tumorigenesis, in which HA is mainly found as a low-molecular size molecule.

HA molecular size-dependent biological functions

The most intriguing fact about HA is its variety of roles, which depends on its molecular size. HA fragments of different molecular sizes exhibit different biological actions and biological responses. Depending on their molecular size, HA fractions can be classified into: o-HA (< 10 kDa), LMW HA (10–250 kDa), medium molecular weight HA (MMW HA, 250–1000 kDa), HMW HA, (> 1000 kDa), and very high-molecular weight HA (vHMW HA, > 6000 kDa). vHMW HA can only be found in naked mole rats and it is considered to be responsible for their increased life span and cancer resistance [52]. HMW HA is found in most of the human tissues and it is a major ECM component. It is involved in wound healing, tissue homeostasis, protects the integrity of the epithelial tissue, and promotes anti-inflammatory, antiproliferative, and antiangiogenic effects [1,52,80,112–116]. It also seems to exert chondroprotective effects and it is able to reduce pain by attenuating nerve impulses and sensitivity. This fact contributes to explain its long-term efficacy on articular cartilage [117,118]. Regarding the antiangiogenic effects of HMW HA, it has been demonstrated that it inhibits vascular smooth muscle cell proliferation by locking the cells in the G1 phase, whereas LMW HA triggers the cell cycle progression through the G1 phase promoting cellular proliferation [119]. MMW HA has also been proved to have different effects than HMW HA, since it leads to proinflammatory responses in mice kidney epithelial cells in contrast to HMW HA. In particular, MMW HA stimulates the upregulation of the adhesion molecules Intercellular Adhesion Molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) mRNA levels via activating the transcription factors NF-κB and AP-1 [120]. Regarding LMW HA, Zhao *et al.* [121] found that a 117-kDa HA inhibits migration and invasion of mice and human breast cancer cells via CD44/Twist signaling pathway. It also increases the expression levels of E-cadherin, whereas it suppresses the expression of vimentin and MMP-9. On the other hand, a 35-kDa HA had the opposite effects since it promoted cellular migration and invasion, increased vimentin expression, and reduced the expression of E-cadherin [121]. Consequently, the exact molecular size of the HA is crucial for its mode of action since HA fragments that are classified in the same HA category based on their molecular size exhibit different effects.

Smaller HA fragments, o-HA, are able to stimulate various signaling pathways leading to a range of cellular responses, such as angiogenesis, cell proliferation, invasion, and inflammation [55,80,112,122,123]. These smaller HA fragments usually created during pathologic conditions, such as fibrosis, inflammation, and cancer, act as cellular alarm signals [51,52,124,125]. The opposing effects of HA fragments of different sizes occur due to the different modes of action by which the HA fragments interact with the HA receptors as described above [80,122,126]. There is a variety of studies that demonstrate the different size-dependent effects of HA fractions. HA fragments of 3–10 disaccharides have been proven to stimulate the proliferation and migration of the endothelial cells initiating angiogenesis in contrast to native HMW HA that exhibits antiangiogenic properties [127,128]. Montesano *et al.* [129] demonstrated that o-HA (3–10 disaccharides) are able to stimulate bovine microvascular endothelial cell (EC) invasion in a 3D matrix promoting angiogenesis in collaboration with vascular

endothelial growth factor (VEGF). o-HA also stimulates the gene expression and activation of urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1). HMW HA had no effect on the bovine microvascular ECs [129]. A study on rat aortic ECs indicated that o-HA and HA hexamers could stimulate proliferation and angiogenesis of these cells. They could also reduce platelet adhesion and activation of EC layers in contrast to HMW HA (1500 kDa) that inhibited it. Moreover, o-HA elevated the expression levels of EC activation markers ICAM-1 and VCAM-1 and triggered the secretion of cytokines [130]. o-HA stimulates ECs proliferation, promoting angiogenesis [131–134]. The mechanism underlying these o-HA effects on ECs according to Wang *et al.* [135] was the phosphorylation of Src, FAK, and ERK1/2 via CD44 that led to the expression of c-jun and c-fos, which caused EC proliferation and tube formation. On the other hand, HA oligomers could reduce proliferation and migration of vascular smooth muscle cells in response to the platelet-derived growth factor. Moreover, the o-HA changed the cellular morphology, from fusiform to a more spread and flattened form. Larger HA fragments seemed to have no effect at these cells [136]. HA fragments have also been shown to play pivotal role during wound healing. o-HA, 3–10 disaccharides in particular, are able to induce wound repair *in vivo* by promoting neovascularization through G protein phosphorylation and ERK1/2 activation, increasing the granulation production, the proliferation of fibroblasts, and the collagen deposition. These effects probably occur due to the o-HA ability to induce EC collagen production and proliferation via CD44 or RHAMM [111,137,138]. In addition, o-HA fragments stimulate the production of several cytokines such us tumor necrosis factor alpha (TNFα), interleukin 8 (IL-8), and IL-1β by macrophages and fibroblasts promoting cellular migration and wound healing [134,139].

HA fractions in pathological conditions

HA molecular size-dependent effects in inflammation

Increased deposition of HA into the ECM is a hallmark of inflammatory disease [24,140–143]. Interestingly, cable-like structures made of HA polymers of indeterminate size and HA-binding proteins were found in several inflammatory conditions [141,144–146]. These structures originate from the cell surface and are capable of spanning multiple cells reaching several millimeters in length. Jokela *et al.* [146] demonstrated that the stimulation of keratinocytes with inflammatory molecules, such as TNFα, IL-1, or high glucose concentration induced the formation of HA cables and the adhesion of leukocytes, without alteration in the secretion of HA. Such a rearrangement of HA structure may have a protective function during inflammation; monocytes can bind HA cables regardless of their activation state avoiding the contact with inflammation-promoting receptors. Moreover, HA networks might serve as a barrier to prevent the loss of ECM components and might sequester proinflammatory mediators [147,148]. As mentioned above, during inflammation HA is usually found like a low-molecular size molecule, presenting a variety of polymers with overlapping lengths and functions. On the contrary, HMW HA polymers elicit protective effects to suppress the inflammatory response. It has been demonstrated that HMW HA is protective against acute lung injury preventing apoptosis in a TLR-dependent basal activation of NF-κB [149]. Moreover, high-molecular size polymers ranging from 780 to 1200 kDa were able to prevent T cell-mediated liver injury, reducing the production of proinflammatory cytokines like TNFα, interferon gamma (IFNγ), and IL-4 [150]. HWM-HA is also able to counteract the inflammatory stimulus of LPS in corneal fibroblast, reducing the expression of IL-6, IL-8, and CXCR1 through TLR4 upregulation [151]. Interestingly, the intra-articular administration of HMW HA is a widely used treatment for inflammatory-based pathologies such as osteoarthritis. In this pathology, the anti-inflammatory effect of HMW HA is not only mediated by its mechanical and viscoelastic properties but also by the suppression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and MMP action and the modulation of plasmin/plasminogen system, that prevents cartilage degradation and fibrinolysis [152].

The HA fragments with a molecular size ≤ 500 kDa have been shown to exhibit several proinflammatory effects in some tissues or conditions. In particular, they can trigger the activation of macrophages and dendritic cells, as well as stimulate the expression of proinflammatory genes including TNFα, IL-1β, IL-1, and MMPs [153–155]. The induction of such inflammatory mediators by HA enhances the pre-existing inflammatory response, creating a positive feedback loop where inflammation promotes further inflammation. Experiments conducted *in vivo* demonstrated that LMW HA promotes inflammation by downregulating the anti-inflammatory A2a receptor in a CD44-dependent manner [156] and that small HA fragments can play an

important role as mediators of inflammation in allergic pulmonary disease [157]. Furthermore, HA molecular size plays a pivotal role in the macrophage activation and phenotype. LMW HA triggers macrophage changes that lead to proinflammatory responses while HMW HA stimulates changes that are correlated with proresolving responses [158]. In addition, HA tetramers triggered a marked inflammatory response in mouse synovial fibroblast, characterized by the upregulation of TLR-4, TLR-2, p38-MAPK as well as NFκB activation [159]. However, it is important to note that some of the proinflammatory effects attributed to HA fragments may be the results of the presence of contaminants in the preparation used, as reported in the study by Dong *et al*. [160].

Although it is widely recognized that LMW HA is an important mediator of the inflammatory response, a number of studies demonstrated that small HA molecules can have a protective function. For example, HA fragments < 750 kDa participate in colonic epithelial repair in a murine model of colitis via TLRs [161]. In addition, TLRs mediate the expression of human β-defensin 2 upon the stimulation of keratinocytes with HA < 200 kDa [162]. Moreover, the production of β-defensin 2 upon LMW HA treatment and TLR activation, has been described also in vaginal epithelium, where it favors epithelial repair after injury and mediates self-defense from pathogens [163]. Interestingly, LMW HA is able to attenuate the inflammatory process and protect from hepatocellular apoptosis during liver injury in murine models [164].

A more detailed discussion must be dedicated to the role of HA during wound healing, an intricate series of complex and overlapping reactions where HMW HA species are prominent in the earliest stages, whereas more fragmented forms are generated progressively until the end of the process. The disruption of tissue architecture and bleeding are the triggering events to initiate the inflammatory stage of wound healing. Platelets in the clot produce large amounts of HMW HA, that is essential for the recruitment of fluids leading to edema [165]. The presence of HMW HA within the edema is crucial to create a porous net to facilitate the infiltration of neutrophils and the subsequent release of TNFα, IL-1β, IL-8 [139] along with the activation of myeloperoxidase. The secretion of inflammatory chemokines and the activity of the myeloperoxidase contribute to HMW HA fragmentation that stimulates the recruitment and the interaction with leukocytes and monocytes through the binding to CD44. In this scenario, LMW HA is also able to bind TLR2/4 receptors expressed by macrophages, stimulating the transcription of TNF-α and the insulin-like growth factor-1 [20], to sustain with a positive loop the inflammatory phase. LMW HA present at the injured site is able to stimulate cell proliferation and recruit fibroblasts, originating the proliferative phase of the process. It has been demonstrated that HA fragments made up of 6–20 disaccharides can stimulate dermal fibroblast migration and proliferation [112] with the subsequent deposition of type III collagen that contributes to the formation of a new ECM. Fibroblasts also produce a high amount of new HA after TGFβ and Smad signaling which is continuously cleaved by HYALs and ROS in fragments of ~ 70–100 kDa [166]. The final stage of the proliferative phase is epithelialization. In wound healing, HA-CD44 complexes face the wound margin and regulate keratinocytes proliferation until the formation of a delicate cover of epithelial cells and wound closure.

The effects of HA of different molecular size on inflammation are summarized in Table 1.

Biological importance of HA fractions in cancer

Solid tumors can be defined as cellular masses which are created through dynamic interactions between the tumor cells and a mixed population of stromal cells. Crosstalk between oncogenic and adjacent stromal cells leads to the formation of a peritumoral microenvironment, capable of influencing tumor cell behavior [167–170]. HA is in fact a modulator of the tumor microenvironment by interacting with specific receptors and intracellular signal transduction which can promote the malignant phenotype and secondly by modulating the hydration and osmotic balance in the tumor microenvironment. Consequently, HA synthesis is increased in various cancer types, such as breast, prostate, lung, and ovarian cancer [2,171–174]. Interactions between HA and cancer cells are important biological events which may be of key importance in order to understand how cancer cells invade the ECM, penetrate lymphatic and blood vessels, and colonize in distant tissues. Elevated levels of HA have been correlated with aggressiveness, poor prognosis, and resistance to chemotherapy [12,25,171,172,175–181]. Moreover, deposition of HA and type I collagen leads to a tumor-associated fibrosis which contributes to cancer initiation and progression [181]. HA is not only produced by the tumor cells but also by the tumor stromal fibroblasts or cancer-associated fibroblasts (CAFs) which are derived from mesenchymal stem cells (MSCs). Notably, CD44 knockdown in MSCs blocks both their ability to be recruited to the tumor site and their tumor-promoting functions [182]. HA promotes CAF motility toward tumor cells and tumor

Table 1. Hyaluronan molecular size-dependent effects on inflammation.

Size	HA function	HA receptor	References
Indeterminate	HA cables: bind monocytes and prevent their contact with inflammation-promoting receptors; barrier to prevent the loss of ECM components and sequestration of proinflammatory mediators	–	[139,141,143,144]
HMW HA	Protects against acute lung injury, inducing apoptosis	TLR	[146]
780–1200 kDa	Prevents T cell-mediated liver injury and decrease the production of inflammatory cytokines	–	[148]
HMW HA	Contrasts the inflammatory stimulus of LPS in corneal fibroblast	TLR4	[149]
HMW HA	Viscoelastic properties; Suppression of ADAMTs and MMPs function; Modulation of plasmin/plasminogen system	–	[150]
< 500 kDa	Activation of macrophages and dendritic cells; Stimulation of proinflammatory genes expression	–	[151–153]
LMW HA	Down regulation of the anti-inflammatory receptor A2A in murine models	CD44	[154]
Small HA fragments	Mediators of inflammation in allergic pulmonary disease	–	[155]
4mers	Upregulation of proinflammatory mediators (p38 MAPK and NF-kB) in mouse synovial fibroblast	TLR2-TLR4	[156]
< 750 kDa	Colonic epithelial repair in a murine model of colitis	TLRs	[159]
< 200 kDa	Stimulates the expression of human β-defensine 2 in keratynocytes	TLRs	[160]
LMW HA	Stimulates the expression of human β-defensine 2 in vaginal epithelium leading to vaginal repair after injury	–	[255]
LMW HA	Protects from apoptosis during liver injury in murine models	–	[162]
1600 kDa	Downregulates the inflammation responses in nasal epithelial cells	–	[234]

cell motility. CAFs not only utilize HA in order to migrate close to the tumor cells but their autocrine production of HA also stimulates the migration of the HA-binding tumor subpopulation [181]. The reduction in HA synthesis and the downregulation of the major HA synthase, HAS2, have been proved useful for the inhibition of cancer progression [39,41,183–189].

It is well established that the metastatic potential of cancer cells is related to their morphology. This fact is particularly evident in EMT process, which represents a change in shape of primary tumor cells when they invade the surrounding stroma [190,191]. Even though cancer cell ability to migrate or invade the ECM is strongly correlated with the cellular shape and size, it is also associated with the formation of cytoplasmic structures such as filopodia and lamellipodia, invadosomes and podosomes, extracellular vesicles (microvesicles and exosomes) which are better detectable by ultrastructural analysis [i.e., scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM)] [192–198]. Very few studies have been performed in order to investigate the relationship between cancer cell morphology and HA. Dickinson *et al.* using SEM observed that colon (LS174T) and breast (MDA-MB-231) cancer cells cultured for 24 h on HA substrates revealed evident adhesive protrusions or edge of cancer cells spreading onto the HA substrate. However, when cells were seeded onto the substrate in the presence of anti-CD44 they were unable to attach, confirming that the adhesion occurs through CD44 [199]. In basal-like breast cancer, EGFR is overexpressed [200]. Louderbough *et al.*, testing different substrates in MDA-MB-231 cell cultures, found that HMW HA embedded in type I collagen gel matrix could inhibit EGFR activation and consequently alter invasive cellular phenotypes by reducing the formation of filopodia, as observed under SEM. Interestingly, cells delayed adhering on the matrix and migrated mainly using lamellipodia. In contrast, when cells were cultured in media containing soluble HMW HA, they adhered easily to the collagen matrix and migrated using filopodia [201]. Invasive mammary carcinoma observed by means of TEM showed that many intercellular junctions or epithelial desmosomes were severely altered, as some of them were internalized. Furthermore, the most invasive cells developed invadopodia which penetrated deeply inside the tumor stroma. Tumor cells generated and released membrane vesicles both from the cellular body and invadosomes; ranging from 30 to 2000 nm. These invadosomes included both exosomes and microvesicles that were dispersed into the extracellular microenvironment [202]. Rilla *et al.* using SEM and TEM demonstrated that HA synthesis is one of the major molecular mechanisms that stimulate the production and shedding of plasma

membrane-derived microvesicles. In particular they found that the tips of long cytoplasmic microvilli that were created in cells by overexpression of HASs were released in the culture medium as microvesicles. Also, the inhibition of HA synthesis reduced their formation. HA-producing tumor cells appeared as immersed in a glycosaminoglycan coat. HA synthesis was taking place on the surface of microvilli and microvesicles, permitting the secretion and transfer of hyaluronan to distal sites from the cell. Thus, microvesicles, which were also surrounded by a HA coat, had the potential to deliver the HA synthase machinery, membrane and cytoplasmic materials to other cells, influencing tissue regeneration, inflammation and tumor progression [203]. In fact, HA synthesis is associated with the formation of various actin-based plasma membrane protrusions, like filopodia, lamellipodia, invadopodia, retraction fibers, surface blebs, and membrane ruffles. Recent reports demonstrated that overexpression of enzymatically active GFP-HAS induces the growth of long, slender protrusions that share many features of both filopodia and microvilli in MCF-7 epithelial breast cancer cells [204].

Filopodia are finger-like cytoplasmic protrusions that exist to be typically adhered to a substrate or another cell, in contrast with microvilli that do not adhere. The HAS3-induced protrusions that may be located between these two types of protrusions are dynamic structures independent on adherence. These protrusions show a rapid growth and retraction, depending on ongoing HAS activity. However, they are degraded after enzymatic HA digestion. Even though the induced protrusions share most cytoskeletal features with filopodia, they do not require adherence to the substrate like traditional filopodia [205]. These thin and long cytoplasmic protrusions act as platforms for shedding of extracellular vesicles and play important biological roles in regulating invasion, adhesion, microenvironmental sensing, and modulation of cancer cells. In fact they are able to produce most of the total HA secreted by cells, and they may be responsible for the increased HA levels of both tissues and body fluids, in inflammation and cancer [204]. These microvesicles contain numerous proteins involved in organizing and remodeling ECM. Moreover, HA-rich pericellular matrices promote the adhesion of tumor cells to microvessel endothelium, facilitate intravasation and protect immigrant cells during circulatory transit. The pericellular HA coat, surrounding the circulating tumor cells, aids in extravasation at ectopic sites as well as in growth of migrant colonies in distant target tissue locations. Thus, it represents a "portable cancerized microenvironment" [206]. HA is extruded as HMW HA, which is rapidly depolymerized into fragments ranging from 10 to 40 kDa in invasive cell lines. These fragments are involved in neovascularization [172] and are produced by both host and tumor cells by enzymatic attack from one or more of several HYALs. They are associated with more aggressive types of disease [206].

The functional properties of cancer cells are regulated by various signaling pathways that are triggered by the HA–CD44 interactions [207]. In colon cancer, increased HA synthesis enhances the aggressiveness of cancer cells and induces EMT. Furthermore, HA–CD44 interactions initiate the HER2 (human epidermal receptor 2, ErbB2,) phosphoinositide 3-kinase (PI3K)/Akt, β-catenin, and cyclooxygenase-2/prostaglandin E2 pathway as well as the HER2, PI3K/Akt, β-catenin pathway [188]. Both of these pathways lead to colon cancer cell growth and survival [188]. In ovarian tumor cells, HA–CD44 interactions promote cancer cell proliferation and migration via activation of Rac1 and Ras signaling [208]. In breast cancer, CD44–HA interactions are responsible for chemoresistance [209–211]. HA binding to CD44 activates stem cell marker Nanog that leads to the activation of signal transducer as well as activator of transcription protein 3 (Stat-3) and gene expression of the multidrug transporter, MDR1. Also, HA–CD44 interactions trigger the binding of the cytoskeletal protein ankyrin to MDR1. As a result, the cancer cells exhibit resistance to chemotherapy [212]. The activation of nanog also leads to production of microRNA-21 (miR-21), upregulation of antiapoptotic proteins, and reduction in proteins that induce cell death, promoting breast cancer cell survival [213]. Furthermore, HA–CD44 interactions promote breast cancer cell invasion, motility, and EMT, via upregulation of serine protease and collagen-degrading enzymes expression and by triggering various signaling pathways like c-Src–mediated twist signaling pathway [2,214–220]. Similarly, HA–CD44 interactions stimulate HER2 activation and cancer progression in mammary and colon carcinomas [221,222]. Moreover, HA–CD44 interactions lead to the activation of various RTKs, such as EGFR, insulin-like growth factor receptor 1β (IGF-IRβ), PDGFR-β, and c-MET, in colon, breast, and prostate cancer cells [222]. These interactions promote breast and prostate cancer cells' metastasis as well [223]. In head and neck squamous cell carcinoma, HA promotes CD44 interactions with EGFR, initiating the EGFR signaling pathway that leads to chemotherapy resistance [224]. On the other hand, in esophageal cancer, the HA interactions with its receptor, RHAMM, promote cancer

cells' proliferation, migration, and filopodia formation [225].

Notably, several studies suggest that HA binding to its receptors can promote or inhibit cancer cells' proliferation, migration, and invasion depending on the molecular size of HA [1,112,114,218,226–228]. HMW HA is able to act as chemoattractant, promoting the migration of breast cancer cells [229]. Furthermore, HMW HA triggers the signaling through CXCR4, receptor of the cytokine CXCL12. This cytokine acts on ECs promoting angiogenesis. Cells that express the CXCR4 are able to metastasize to organs that express the CXCL12. On the other hand, o-HA (6–10 disaccharides) inhibit CXCR4-induced ERK1/2 phosphorylation in human liver carcinoma cell line, HepG2 and HUVEC [230]. A 7.46-kDa HA promoted the proliferation and invasion of malignant pleural mesothelioma cells via CD44 [231]. Furthermore, o-HA stimulates the adhesion of BT-549 breast cancer cells and the activation of ERK1/2 via HA-CD44 pathway [80]. Last but not least, 3–5-kDa o-HA promotes breast cancer cell invasiveness and production of IL-1β and IL-8 by stimulating the associations between CD44 and TLRs [232]. Regarding LMW HA, it has been proven that a 35-kDa HA promotes migration and invasion of human and mice breast cancer cells via CD44 while a 117-kDa HA exhibits the opposite effects [121]. Moreover, LMW HA (15–40 kDa) has been proven to increase the adhesion of fibrosarcoma cells on fibronectin via ERK1/2 and FAK phosphorylation in a RHAMM-dependent manner [110]. Regarding breast cancer cell adhesion, LMW HA has been proven to enhance the basal-like breast cancer cell adhesion on fibronectin matrices and bone marrow ECs [233]. Thus, HA fragments can trigger different cancer cell responses depending on their molecular size and the cell type they interact with.

The effects of HA of different molecular size on cancer are summarized in Table 2.

HA fractions as potential therapeutics: mechanistic aspects

Hyaluronan fractions, depending on their molecular size, exhibit different properties and stimulate several biological responses. This HA ability can be used in the development of therapeutic strategies. Studies prove that HA fragments can be effective therapeutics in several pathological cases [112,234–236]. For example, HA fragments can be used as therapeutics in cases of knee injury. It has been proven that intra-articular HA injections improve the healing and act protectively for articular cartilage in animal models. Moreover, when HA is provided immediately after injury it exhibits chondroprotective abilities and improves the metabolism of the chondrocytes in the injured area [237]. In cases of diabetic wounds, o-HA (2–10 disaccharides) treatment stimulates the proliferation and the migration of ECs leading to angiogenesis that facilitates wound closure. o-HA increases the collagen deposition in the wounded area as well. This phenomenon occurs due to the induction of TGF-β expression and Src and ERK1/2 phosphorylation. Thus, HA-based treatments can be used for wound repair [22,23,238].

Regarding inflammation, Albano *et al.* [239] proved that addition of HMW HA (1600 kDa) downregulates the inflammatory responses in nasal epithelial cells. IL-17A activates ERK1/2 and NF-κB pathways leading to inflammation. HMW HA attenuates this effect. Moreover, exogenous HMW HA exhibits protective effects against colitis via TLRs in mice [161]. LMW HA can be used for therapeutic purposes after skin injury, since LMW HA induces the production of β-defensin by keratinocytes. β-defensin is a protein that exerts a strong antimicrobial activity enhancing the skin protection against pathogens. LMW HA stimulates the β-defensin production by activating TLR-2 and -4, while CD44 does not seem to participate in this process. Through the c-fos-mediated, protein kinase C-dependent signaling pathway, the keratinocytes produce β-defensin protecting the skin tissue [162]. LMW HA exhibits also protective properties against inflammatory liver disease in mice [164] and induces wound healing via PI3K/Akt pathway [163].

In oncology, o-HA and LMW HA are suggested to be beneficial in many types of cancer as they can reduce cancer cell growth, migration, and invasion [112,226,240,241]. Treatment of colon cancer cells with o-HA in cultures leads to a reduction in expression and activity of cyclooxygenase-2, followed by a decrease in HA synthesis [188]. Moreover, o-HA inhibits tumor growth *in vivo*, and induces mammary and colon cancer cell apoptosis, as it triggers caspase-3 activity and suppresses the PI3K/Akt pathway which is responsible for cell survival [226]. LMW HA induces apoptosis of colorectal cancer cells and stimulates the immune response which is involved in tumor inhibition as well [241]. Regarding breast cancer, it has been proven that HMW HA (600–1200 kDa) and HA decasaccharides are able to reduce the MDA-MB-231 cell growth, migration, and invasion. Moreover, they reduce the HA production by the cancer cells. HA decasaccharides are capable of suppressing osteolytic activity, preventing bone metastasis expansion [240]. Our research group recently demonstrated that LMW

Table 2. Hyaluronan molecular size-dependent effects in cancer.

Size	HA function	HA receptor	References
35 kDa	Promotes migration and invasion of mice and human breast cancer cells, downregulates the expression of e-cadherin, and upregulates the expression of vimentin	CD44	[120]
117 kDa	Inhibits migration and invasion of mice and human breast cancer cells, downregulates the expression of vimentin, and upregulates the expression of e-cadherin		
HMW HA	Promotes CXCL12-induced CXCR4 activation in human liver carcinoma cell line, HepG2, and HUVEC	CD44	[225]
6–10 disaccharides	Inhibit CXCR4-induced ERK1/2 phosphorylation in human liver carcinoma cell line, HepG2, and HUVEC		
7.46 kDa	Promotes proliferation and invasion of human pleural mesothelioma cells	CD44	[226]
15–40 kDa	Promotes adhesion of fibrosarcoma cells on fibronectin via ERK1/2 and RAK phosphorylation	RHAMM	[109]
HMW HA	Inhibits the proliferation of vascular muscle cells and fibroblasts	CD44	[90,91,118]
LMW HA	Promotes the proliferation of vascular muscle cells and fibroblasts		
3–10 disaccharides	Stimulate ECs proliferation and migration, promoting angiogenesis	CD44	[110,130–134,136,256]
o-HA	Reduces proliferation and migration of vascular smooth muscle cells in response to platelet-derived growth factor and modify cellular morphology		[257]
3–10 disaccharides	Increase the adhesion of HK-2 and BT-549 cells and the phosphorylation of ERK1/2	CD44	[79]
3–5 kDa	Promotes breast cancer cells invasion and the production of IL-1β/IL-8	CD44-TLRs	[258]
220 kDa	Promotes adhesion of basal-like breast cancer cells to bone morrow ECs and fibronectin	CD44	[259]
200 kDa, 760 kDa	Acts as chemoattractant promoting the migration of MDA-MB-468 and MDA-MB-231 breast cancer cells	CD44	[224]
< 10 kDa	Promotes EMT in MCF-7 cells and enhances the aggressive phenotype of MDA-MB-231 Reduces cell migration Changes the gene expression of ECM modulators, growth factor receptors, and EMT markers	–	[237]
200 kDa	Favors a less aggressive phenotype in MCF-7 and MDA-MB-231 Reduces the proliferation, migration, and invasion of the cells Changes the gene expression of ECM modulators, growth factor receptors, and EMT markers		
o-HA	Decreases the expression and activity of cyclooxygenase-2 and the HA production	CD44	[183]
600–1200 kDa	Reduces the MDA-MB-231 breast cancer cell proliferation, migration, and invasion Decreases the HA synthesis	CD44	[235]
10 saccharides	Reduce the MDA-MB-231 breast cancer cell proliferation, migration, and invasion Decrease the HA synthesis Suppress the progression of osteolytic lesion		
100–300 kDa	Reduces colon carcinoma growth by inducing cancer cell apoptosis and immune response	CD44	[236]
Octasaccharides	Reduce the osteosarcoma cell lines MG-63 and LM-8 proliferation, motility, and invasiveness Reduce endogenous HA Suppress the formation of distant lung metastasis	CD44	[238]
3–10 disaccharides	Inhibit the growth of mammary and colon cancer cells inducing apoptosis	CD44	[216,221]

Table 2. (Continued).

Size	HA function	HA receptor	References
	Inhibit the tumor growth *in vivo* Reduce HER2 activation		
100–300 kDa	Modulates multidrug resistance by sensitizing lymphoma cells and chronic myeloid leukemia cell lines to chemotherapy	CD44	[242,243]
3–10 disaccharides	Inhibit growth and invasiveness of malignant glioma cells	CD44	[239]
3–12 disaccharides	Inhibit the B16F10 melanoma cells' growth	CD44	[240]
2–10 disaccharides	Inhibit HA–CD44 interactions reducing the aggressiveness and promoting drug resistance in ovarian carcinoma cells and malignant peripheral nerve sheath tumors	CD44	[241,244]
s-HA fragment	Induces apoptosis in prostate cancer cell lines and bladder cancer cells Reduces their motility and invasiveness Downregulates the expression levels of snail and twist while increases the expression levels of e-cadherin in bladder cancer cells Inhibits the Akt signaling Downregulates RHAMM and VEGF expression levels in prostate cancer cells Inhibits prostate and bladder cancer tumor growth *in vivo*	CD44, RHAMM	[245,246]

HA reduced breast cancer cell proliferation, migration, and invasion, by changing the expression of several genes including ECM modulators, EMT markers. Furthermore, the morphology of the cells was modified depending on the MW of the HA fragment used. LMW HA promoted a less aggressive cellular phenotype in contrast with o-HA that promoted phenotypic changes enhancing cellular aggressiveness (A. G. Tavianatou, Z. Piperigkou, C. Barbera, R. Beninatto, V. Masola, I. Caon, M. Onisto, M. Franchi, D. Galesso & N. K. Karamanos, unpublished work). o-HA octasaccharides, decrease the aggressiveness of osteosarcoma cell lines MG-63 and LM-8, by reducing their proliferation, motility, invasiveness, and endogenous HA production. Moreover, o-HA is able to reduce HA accumulation in local tumors, preventing distant lung metastasis [242,243], the growth and invasiveness of malignant glioma cells [244], suppress the growth of B16F10 melanoma cells [245], and reduce the aggressiveness of ovarian carcinoma cells [246]. HA fragments have been proven to sensitize cancer cells to chemotherapy. LMW HA regulates multidrug resistance of lymphoma cells and chronic myeloid leukemia cell lines to chemotherapy by reducing the activity of p-Akt and P-glycoprotein, leading to apoptosis [247,248]. o-HA sensitizes malignant peripheral nerve sheath tumors to chemotherapy [249]. Notably, sulfated HA (s-HA) fragments have been used in some studies with promising results, as they induce apoptosis in bladder and prostate cancer cells and inhibit the motility and the invasiveness of both cancer cell types via PI3K/Akt signaling pathway attenuation. They also downregulate the expression levels of RHAMM and VEGF in prostate cancer cells and CD44 and RHAMM expression levels in bladder cancer cells. The mRNA levels of transcription factors snail and twist are decreased after s-HA treatment in bladder cancer cells, whereas the expression levels of E-cadherin are increased. Furthermore, s-HA suppresses tumor growth in xenograft models of both cancer types without toxic side-effects [250,251]. An intriguing application of small HA fragments is their use in the construction of nanocarrier for targeted cancer therapy [252]. Edelman *et al.* took advantage of CD44 overexpression in human ovarian adenocarcinoma cell lines to build nanoparticles made of 6.4 kDa HA and BSA by the Maillard reaction. The covalent conjugates of BSA-HA self-assembled into nanoparticles, encapsulating hydrophobic cytotoxic drugs as paclitaxel and imidazoacridinones, that were selectively internalized by ovarian cancer cells overexpressing CD44, but not by cognate cells lacking the receptor [253]. A similar application was made by Maiolino *et al.* [254] using < 10 kDa HA to decorate nanoparticles targeting CD44 for the combined photo/chemotherapy of cancer, thus making these strategies very useful for the specificity and efficacy of cancer treatment. Using the same approach, the research group of Renier *et al.* developed a technological platform for the chemical conjugation of 200 kDa HA with well-known anticancer drugs such as paclitaxel and SN-38 (active metabolite of irinotecan). These coupled molecules,

named Oncofid P (formerly HYTAD1-P20) and Oncofid S, respectively, were shown to be active *in vitro* toward CD44-overexpressing human bladder and ovarian cancer cell lines, thanks to the receptor-mediated internalization of the anticancer drug, favored by HA conjugation [255,256]. The safety and effectiveness of the HA–anticancer drug conjugates were then confirmed *in vivo* in different tumor animal models [257,258] and lastly the Oncofid P therapy against bladder cancer entered the clinical evaluation phase [259].

As described above, HA fragments could be promising therapeutic agents in several pathological cases. Thus, the elucidation of the mechanisms underlying their actions is of great importance. Several studies have been conducted in order to identify the exact

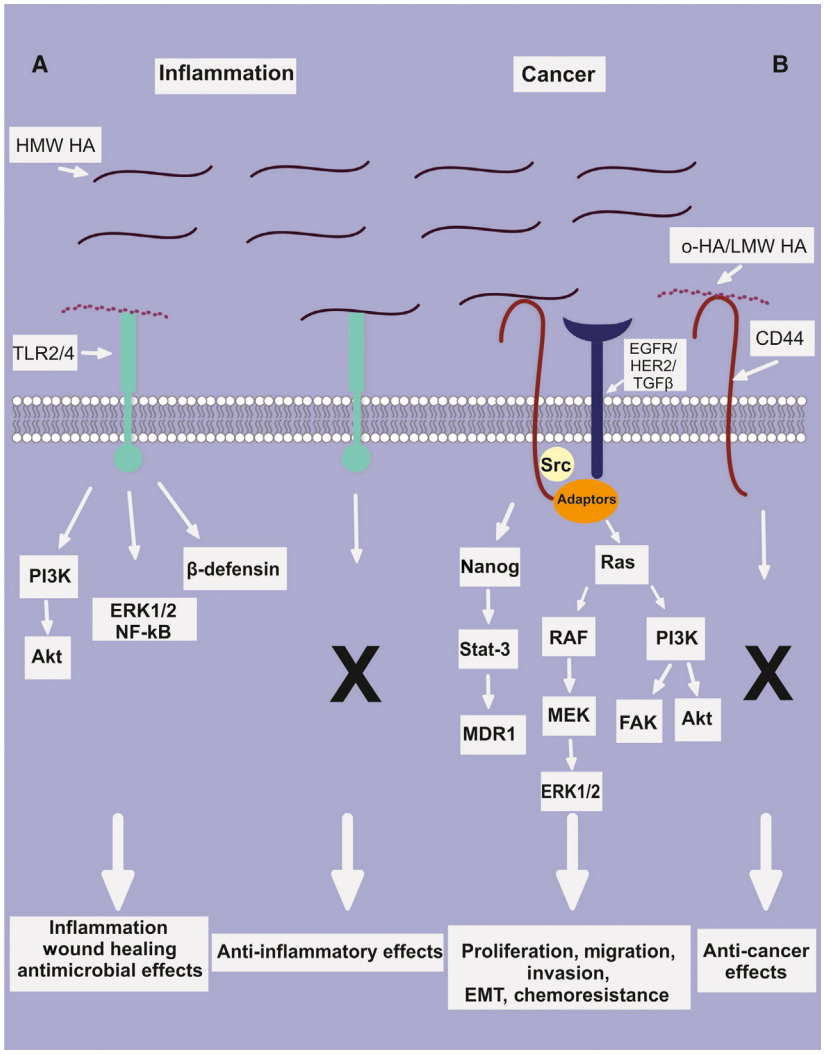

Fig. 2. High-molecular weight HA and LMW HA/o-HA competition for binding to HA receptors in inflammation and cancer. o-HA and LMW HA compete with endogenous HMW HA for binding to HA receptors, initiating or attenuating various signaling pathways. (A) LMW HA bind to TLRs 2 and 4 activating β-defensin that exhibits antimicrobial properties. Moreover, LMW HA binding to these receptors leads to PI3K/Akt, NF-κB, and ERK1/2 activation that stimulates wound healing and inflammatory responses. On the other hand, HMW HA binding to TLRs 2 and 4 exhibits anti-inflammatory effects, attenuating these processes. (B) Endogenous HA binding to CD44 triggers various signaling pathways by activating Src, EGFR, HER2, and TGFβ. The activation of these molecules triggers signaling cascades with several responses. RAS activation leads to PI3K activation and phosphorylation of Akt and FAK. Moreover, RAS activates RAF that phosphorylates MEK leading to ERK1/2 activation. The stimulation of these molecules leads to cancer cell proliferation, migration, invasion, and EMT. In addition HA binding to CD44 triggers the activation of nanog and stat-3. The activation of these factors leads to MDR1 activation and cancer cells resistance to therapy. In the contrary, HA fragments binding to CD44 attenuate these signaling cascades indicating the HA fractions utility as anticancer agents.

MW of the HA fragments that is needed in order to attenuate a signaling pathway or trigger a cellular response. According to several studies, hexasaccharides have been proved to be the smallest HA fragments that are able to bind effectively to the HA receptors [260–262]. Use of HA octasaccharides or decasaccharides instead of hexasaccharides was considered to be more efficient demonstrating the importance of the HA exact size in cellular responses. These observations are in agreement with Tammi *et al.* [263] study which indicated that HA decasaccharides are able to displace endogenous HA from CD44 in rat keratinocytes in contrast with HA hexasaccharides. In addition, HA fragments from 20 to 24 saccharides could bind to CD44 more effectively. This could be an indication of divalent binding to the receptor [261]. Yang *et al.* [80] also demonstrated that native HA induces CD44 clustering in contrast to o-HA, which antagonizes native HA and replaces multivalent HA–CD44 interactions with monovalent ones.

Consequently, HA fragments interact with the HA receptors via different modes, depending on their MW. Moreover, HA fragments antagonize endogenous HA for binding to HA receptors. Several studies have proven that the HA fragments can bind to CD44, disrupting HA–CD44 interactions and attenuating a variety of signaling pathways leading to different biologic responses [80,121,221,226,264] (Fig. 2). Misra *et al.* [222] demonstrated that addition of o-HA fragments could interrupt the HA-CD44 interactions and lead to a reduction in activation of several RTKs such as PDGFR-β and IGF-IR in breast and colon carcinoma cells. Moreover, HA-CD44 binding interruption leads to HER2 inactivation and reduction in cancer cell aggressiveness [221] HA–CD44 binding disruption by HA fragments results in PI3K/Akt cell survival pathway suppression and cell death [226,240,245,247,248]. The ability of HA fragments to disrupt HA-CD44 interactions and the subsequent reduced aggressiveness of several cancer types, is a very promising tool for targeted cancer therapeutic approaches [241,243,244,246,249].

Concluding remarks

Hyaluronan is a main, well-studied ECM component. The diversity of its modes of actions is strongly correlated with its molecular sizes. HA fragments of different MW regulate important biological multistep processes such as development, inflammation, and cancer. Depending on their MW, the HA fragments can be beneficial or harmful, attenuating or promoting disease progression. HA interactions with its receptors initiate various signaling pathways regulating cellular responses to a variety of signals. The disruption of these interactions could lead to major changes in cellular behavior. In case of inflammation the HA fragments initiate wound healing and angiogenesis facilitating the healing process or exhibit anti-inflammatory properties depending on their molecular size. In cancer cases the accumulation of native HA is strongly correlated with cancer cell aggressiveness and the HA fragmentation usually promotes the development of the disease. On the other hand, o-HA fragments attenuate cancer progression by HA–CD44 binding interruption, leading to tumor suppression. Consequently, the elucidation of the mechanisms that regulate the HA fragments' effects and the identification of the signaling pathways and molecules that are involved in each response could be useful in the development of the future pharmacological targeting strategies.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

AGT, IC, MF, ZP, DG and NKK wrote, reviewed and edited the manuscript. NKK organized, reviewed and submitted the manuscript.

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