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Review article

Hyaluronan fragments produced during tissue injury: A signal amplifying the inflammatory response

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ABSTRACT

Inflammation is a complex mechanism that plays a key role during diseases. Dynamic features of the extracellular matrix (ECM), in particular, during phases of tissue inflammation, have long been appreciated, and a great deal of several investigations has focused on the effects of ECM derivatives on cell function. It has been well defined that during inflammatory and tissue injury, ECM components were degraded. ECM degradation direct consequence is the loss of cell homeostasis, while a further consequence is the generation of fragments from larger precursor molecules. These bio-functional ECM shred defined matrikines as capable of playing different actions, especially when they function as powerful initiators, able to prime the inflammatory mechanism. Non-sulphated glycosaminoglycan hyaluronan (HA) is the major component of the ECM that undergoes specific modulation during tissue damage and inflammation.

HA fragments at very low molecular weight are produced as a result of HA depolymerization. Several evidence has considered the plausibility that HA breakdown products play a modulatory action in the sequential stages of inflammation, although the effective mechanism of these HA derivative compounds act is not completely defined.

This review will focus on the pro-inflammatory effects of HA fragments in recent years obtained by in vitro investigations.

1. Introduction

The ECM is a combination of extracellular molecules secreted by basement cells that provides structural and biochemical support to the adjoining cells. The ECM plays a fundamental role in giving mechanical strength and elasticity to tissues. ECM is also capable of modulating the interaction, the cells behavior, and the binding of several growth factors and cytokines [1]). The glycosaminoglycans (GAGS), fundamental components of the ECM are polysaccharides consisting of repeating disaccharide units, either in free forms such as HA or being part of proteoglycans PGs). The damage of these macromolecules occurring during inflammatory events, for instance by oxidation or enzymatic degradation, deeply modifies the structure of these components and their properties. Such alterations have been described to play a key role in a large number of human morbid affections [2]. In fact, the degradation of these bio-molecules has been showed to occur in several well defined pathologies such as carcinogenesis, atherosclerosis, kidney disease, rheumatoid arthritis, osteoarthritis, hepatitis, lung disease, and several other inflammatory diseases [3–12].

The altered assembly of connective tissue, resulting either from the synthesis or degradation of the ECM components, can markedly modify cell-matrix interactions by activating various signalling pathways that regulate cell behavior [1,2].

A large number of investigations have demonstrated that HA influences inflammatory responses [14–16]. The HA molecules consist of long unbranched chains of alternating units of D-glucuronic acid and *N*-acetyl-D-glucosamine. This linear polysaccharide can reach a size of 6–8 MDa. Hyaluronan interacts with several immunological and non-

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immunological cells through the cluster determinant 44 (CD44), It is a more specific cell surface receptor, and this receptor, by clearing the HA molecule is able to avoid HA accumulation and to maintain tissue homeostasis [17].

During inflammation and tissue damage, HA which is mainly found, in the native form, in a high molecular weight, is depolymerised in small pieces at low molecular weight. Many of these pieces are then further degraded to small oligosaccharides which have the ability to promote inflammation by inducing the production of several inflammation mediators and effectors such as reactive oxygen species, cytokines, chemokines, and destructive enzymes that prime and amplify the inflammatory response and consequently cell damage [14–16].

HA degradation occurs either enzymatically by hyaluronidases (HYALs) or non-enzymatically by mechanisms such as free radical-related depolymerization [18]. Free radical depolymerization of HA occurs in the presence of reactive oxygen species and Maillard products, resulting in HA oligomers of different length. In degradation of the HA chains by HYALs, the HA fragments are generated as the glycosidic bonds, between, the disaccharides are hydrolyzed. The HA pieces will then either be further depolymerised locally or drained from the tissue via the lymphatic system. One of the HA turnover pathways is its local degradation in the skin but the majority of the HA fragments leave the tissue with the lymph and is cleared in the lymph nodes. All that remains after passage through the nodes is degraded by the liver [18,19].

In particular, the functional effects of HA oligomers and their clearance from the jeopardized tissue, undergoing the inflammatory response are produced through their interaction to specific receptors such as CD44, RHAMM, and TLR-2,4 [20,21]. Internalized low molecular mass HA is removed by the lysosome and further degraded into tetra and hexasaccharides through the intracellular HYAL1 and HYAL2 [22]. The HA fragments generated by HYAL1 and HYAL2 are further depolymerised by two lysosomal exoglycosidases, a β-glucuronidase and a β-*N*-acetyl hexosaminidase [22]. Therefore the generation of these small HA pieces, thus obtained, can act as an endogenous danger signal, leading to the activation of both innate and acquired immunity.

The separate stimulation of TLR4 and CD44 receptors may prime/amplify the inflammatory response through NF-kB activation [23,24].

In fact, after the binding with the target, the TLRs dimerized and assumed the conformational change suitable for the recruitment of downstream signalling mediators. These molecules comprise the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), the Il-1R-associated kinases (IRAKs), the transforming growth factor-beta (TGF-β) activated kinase (TAK-1), the TAK-1 binding protein (TAB1 and TAB 2), and the tumor necrosis factor (TNF)-receptor-associated-factor-6 (TRAF-6) [25,26]. Tumor necrosis factor receptor-associated factors (TRAFs) are intracellular adaptor proteins that are proximal signal transducers for the TNFR superfamily [25,26]. The physiological action of TRAF-6 is mainly mediated by the activation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IkB) kinase complex and mitogen-activated protein kinase (MAPK) members which modulate the transcription of genes through NF-kB and AP1. It has been shown that the action of TRAF-6 in TLR signalling is particularly specific in the signalling pathways activated through TLR-4 stimulation [25,26].

Post-translational modifications of CD44 proteins regulate their capacity to bind with several ECM components with increased importance in tumor progression, including HA, collagens, fibronectin, laminin [27], fibrin, osteopontin (OPN) [28], and serglycin [29].

The activation of the CD44 receptor is often dependent on protein kinase C (PKC) modulation that phosphorylates the CD44 and other correlated proteins [30]. Of note, PKC activation leads to the expansion of CD44 receptors in membrane folds of cancer cells, thus playing a key role in tumor invasion [31,32]. In fact, PKC seems to possess the potential to regulate both the affinity of CD44 for HA [33]. In addition, it has been shown that the interaction of the CD44 receptor with HA leads to the PKC activation, thus showing an interdependent mechanism involving the PKC and the CD44 receptor [34]. Moreover, all these pathways are to a large part exacerbated by the increased production of inflammatory cytokines and other harmful mediators, which are modulated by the activation of the NF-kB [35].

This review briefly resumes the novelty related to the recent findings on the biochemical effects of HA fragments/oligosaccharides published in the last decade both in vitro and in vivo experimental models.

2. Hyaluronan depolymerization by hyaluronidases

Hyaluronan degradation occurs, in part, by HYALs. In mammals HYALs, are a specific family of enzymes consisting of six HYALs (1–6) [36,37]. HYALs are also typical of lower organisms such as bacteria, where they degrade HA to produce mainly disaccharides [38], and in various claims, where they mainly generated tetra- and hexasaccharide scraps [39]. HYALs belong to the endoglycosidases category and they are able to hydrolyze the b-1,4 bond of the HA polymer [40]. HYALs degrading HA may be distinct in various groups; HYAL1 and HYAL3 are active at acid pH, while HYA-5 also called (PH20 or SPAM1) possesses optimum activity at a neutral pH [41]. Among the 6 HYALs, HYAL1 and HYAL2 are the more present forms acting to degrade HA in somatic components. HYAL3 is more limited in its expression pattern and is present at low concentrations in the brain, liver, testis, and bone marrow [37]. Instead, HYAL4, is a chondroitinase with no clear evidence of degrading activity on HA [37]. It is mainly expressed in the placenta and skeletal muscle [37]. HYAL5 is specific to sperm and plays a function in the fertilization mechanism [41] as well as HYAL6, also know as HYALP1, is an expressed pseudogene with mutations in the genomic DNA and cDNA and its role is fundamental in spermiogenesis [42].

HA depolymerization into small oligosaccharides is operated by HYAL1 and HYAL2 acting together to degrade HA into tetrasaccharides. The acidic environment necessary for HYAL2 activity contributes to the HA fragments production of approximately 20 kDa, corresponding to about 50 disaccharide units [43]. In this way, these 20 kDa fragments are internalized and transported first to endosome and then to lysosomes, where HYAL1 further catabolizes the small HA pieces into tetrasaccharide units [44].

Recent findings have demonstrated that HYBID (hyaluronan-binding protein involved in hyaluronan depolymerization/KIAA1199/CEMIP) is a HA-binding protein that seems to have an important role in HA-specific degradation [45]. In this contest HYBID knockdown cells completely abrogated HA-degrading activity, thus suggesting that HYBID may represent an essential factor for HA depolymerization in some cell types [45]. However, it is still unclear whether HYBID enzymatically cleaves HA, as further studies are needed to clarify this mechanism. In addition, transmembrane protein 2 (TMEM2) has been reported as a novel cell surface hyaluronidase, as verified in over-expressed HEK293 cells or in an in vitro reaction using recombinant TMEM2 proteins [46]. More recent investigations demonstrated that TMEM2 can act as a hyaluronidase in HYBID-mediated HA depolymerization [47].

3. Hyaluronan degradation by nonspecific pathways

Native HA may also be degraded through nonspecific pathways. Free radicals, derived from oxygen, such as superoxide anions, hydrogen peroxide, nitric oxide, peroxynitrite, and hypochlorous acid, are produced under inflammatory conditions during the sepsis, generalized tissue inflammation, and ischemia-reperfusion [48]. The natural antioxidant defenses are not able to neutralize the high amount of ROS generated by these events and therefore they react directly with all cellular constituents, including native HA [48]. The principal evidence of this detrimental action is that it is possible to highlight it, for instance, in the synovial fluid, where the inflammation pathway leads to

depolymerization of native high molecular weight HA with a consequent reduction in synovial fluid viscosity and cartilage degradation [13]. Therefore, it seems very obvious that excessive production of ROS feeds the inflammation mechanism through the oxidative fragmentation of the HA. The plausible conclusion that it is possible to extrapolate from this evidence is that the blocking activity of ROS, for example, using superoxide dismutase, as well as the inhibition of HA degradation, limit the formation of HA fragments and the inflammation damage [49].

4. Characteristics of the small HA fragments

Although the specific mechanisms involved in the diverse signalling of HA are still poorly understood, it is known that HA can modulate many biological effects including cell adhesion, cell migration, morphogenesis, tumorigenesis, cell survival, apoptosis, and inflammation and that these biological effects can differ on the basis of HA size.

HA-derived oligosaccharides are belonging to the family of damage-associated molecular pattern molecules (DAMP) that are able to interact and activate the innate immunity system [50]. Their modulatory activity on the inflammation mechanism and the consequent functional responses are dependent on both to the exact oligosaccharide size and by the cellular type engaging the small polymers [51]. In fact, as HA oligosaccharides may stimulate different receptors, they may produce different downstream effects depending on the damaging condition [49,51]. Experimental evidence has shown that the inflammation and fibrotic period of remodeling tissues occur in the presence of variable mixtures of high and low molecular mass of HA, suggesting the conclusion that different HA polymer sizes are indispensable for the inflammatory response. However, the evidence is that the treatment with hyaluronidase (PH20) did not induce any inflammation response in tissues, while on the contrary, it was able to be pro-inflammatory in cultured cells. This is really of interest and it is the current subject of various investigations, although a plausible explanation for the lack of the inflammatory response could be the rapid removal of the small polymers from the treated tissue [52]. Starting by these evidence, it is possible to consider the overall complexity of HA biology during and after its degradation. Especially, It is also not surprising that the effects of specific sizes of HA oligosaccharides on cellular modulation is yet unclear and controversial. Future research will be focused on to clarify the size-dependent cell response taking into account these complex variations [53,54].

5. In vitro studies

Several in vitro studies involving HA fragments indicate that these degradation products may prime an inflammatory response. The fragmentation of GAGs with the production of small fragments has been linked to several human pathologies. An experimental study demonstrated the direct generation of these detrimental compounds. In fact, this investigation shows how HA and chondroitin sulfate (CS) was markedly degraded through different ROS radicals. It was reported that GAG fragmentation was dependent on the radical flux, independent of O_2 concentration, and it occurs in a site-specific way as suggested by the detection of oligomers. Electron paramagnetic resonance (EPR) spin trapping evaluation of all fragments including disaccharides and monosaccharides, using ^{13}C-labeled molecules, evidenced the production of selective carbon-centered sugar-derived radicals. In this contest, authors concluded that the time course of the generation of these ROS is very consistent with those responsible for GAG degradation [55]. Therefore this experiment represents a strong confirm of small HA fragment production by ROS.

5.1. Immunological cells

Gao et al. demonstrated that extracellular superoxide dismutase (EC-SOD) was able to prevent HA degradation by direct binding. In fact, an in vitro study using human polymorphic neutrophils suggested that the polymorphic neutrophil chemotaxis induced from the oxidative fragmentation of HA was fully abolished by the EC-SOD. Authors concluded that these data suggested that the inhibition of HA depolymerization could be one mechanism able to reduce the inflammation due to HA fragments [56]. In this context antioxidant therapies could support the treatment with anti-inflammatory drugs.

An other investigation aimed to study the involvement of HA fragments in inflammatory bowel disease. On the basis that inflammatory cells interact with HA, authors investigated the cell response and the molecular mechanism of the HA interaction with human mononuclear cells. Peripheral blood mononuclear cells (PBMC) were treated with very low and high molecular weight HA and then the production of the pro-inflammatory cytokines IL-6 and the monocyte chemo-attractant protein (MCP-1) was quantified. In order to verify the involvement of the receptors CD44 and TLR4, cells were co-incubated with specific blocking antibodies against these receptors. In addition, mononuclear cells from CD44-null and TLR4 mutant mice were also treated for HA at very low molecular weight. The small pieces of HA were able to increase IL-6 and MCP-1 production, while HA at high molecular weight had no effect on the production of these cytokines. The treatment of cells with small polymers of HA plus the antibodies against the CD44 limited the increment of both IL-6 and MCP-1. The addition of the HA fragments to both TLR4 mutant and CD44-null mice has not had a significant effect on IL-6 production. The further addition of a MAPK inhibitor fully abolished IL-6 production both in TLR4 mutant and CD44-null PBCM, instead, the addition of a specific inhibitor of MEK abolished the IL-6 production in CD44-null PBCM only and not in TLR4 mutant. In light of these results, authors suggested that the CD44 and TLR4 receptors play separate roles in the cytokine production during HA-induced inflammatory response in PBMC [57]. By these data it is possible to understand that the treatment with specific antagonists of the CD44 and TRL-4 receptors can modulate the inflammatory response produced by the fragments of HA. More targeted research could lead to the development of new anti-inflammatory drugs.

A further investigation confirming that HA fragments may act as an endogenous signal of inflammation and can prime cytokine increment in damaged tissue was studied by Yamasaki et al. Authors investigated whether the inflammasome component NOD-like receptor protein-3 (NLRP3)/cryopyrin was involved in the inflammation response induced by small HA pieces. Mice with a mutation in cryopyrin presented a regular increment in C-X-C chemokine ligand-2 (Cxcl2) after sterile damage induction while at the same time showed a reduced inflammatory response and increment in the IL-1β production. In the same way, the treatment of macrophages obtained from cryopyrin-deficient mice augmented the release of Cxcl2 but it did not have any effect on the IL-1β production. In order to elucidate this process, peritoneal macrophages obtained from CD44-deficient mice were added with antibodies against the CD44, or with specific inhibitors of lysosome activity, in this way, the IL-1β production was inhibited. Then the macrophages were treated with LPS and subsequently with HA oligosaccharides. Results demonstrated that the action of CD44 and the consequent HA catabolism prime the intracellular cryopyrin activating the IL-1β production. Hence authors suggested that these data support the hypothesis that HA is able to activate the IL-1β pathway and the cryopyrin mechanism during sterile inflammatory processes [58]. Also in this case the opportune inhibition of the CD44 may produce beneficial effect during inflammation.

As well known, the HA depolymerization during inflammation generates HA pieces of variable sizes that are able to stimulate multiple angiogenic and inflammatory responses in a size-specific way. An investigation reports that platelets contain only HYAL-2 but not HYAL-1. Platelet HYAL-2 is able to degrade HA into pieces that are capable of stimulating an inflammatory and angiogenic response; this mechanism happens without the presence of HYAL-1. On the contrary, HYAL-1, in

other cells, is fundamental to complete HA degradation. Therefore, platelet-derived HYAL-2 fragmented the HA into small pieces that activate leukocytes to release pro-inflammatory cytokines, including interleukin-6 and interleukin-8. On this basis, authors suggest that platelets seem deeply involved in the mediation of inflammation and angiogenic mechanism beyond that in hemostasis and wound healing [59]. Thus it is plausible to hypothesize that the changes in these specific steps may produce chronic inflammation, and the platelet may act as an interface between acute and chronic inflammation, as well as wound healing and the fibrotic events.

A further study investigated the effect of HA at a different molecular weight on human blood phagocytes. Phagocytes were treated with HA of weight 52, 250, and 970 kDa). Results have shown that each treatment stimulated the phagocytes to release free radicals and TNF-α. The higher effect was induced by the HA at 52 kDa while the HA at 970 kDa had a very slow action. By these data, authors concluded that HA may modulate the activation of human blood phagocytes and the potency is dependent on the molecular weight, including HA at high molecular weight [60]. This experiment further supports the detrimental action of HA at low molecular weight.

Several findings have reported that adenosine plays a role in the protection of tissues damaged during inflammation, acting on the adenosine A2a receptor (A2aR), on the other side, the products of HA depolymerization, generated during inflammation, perpetuate the mechanism. Thus, these two endogenous compounds play opposite functions in the regulation of inflammation. A study aimed to investigate the action of HA at a low molecular weight on A2aR activity in mice peritoneal macrophage. Results have shown that HA fragments produced a marked down-regulation of the A2a receptor. CD44 was deeply involved in this mechanism as the HA small pieces modulated the A2aR through the mediation of the protein kinase C (PKC). It has been also observed that HA fragments exert A2aR down-regulation in vivo model of inflammation and this HA effect may be abolished by the pre-treatment with a specific HA-blocking peptide [61]. These data strongly support the evidence that adenosine possesses a crucial role in reducing inflammation and in addition they confirm the hypothesis that HA at low molecular mass can further maintain and strengthen inflammatory response.

It has been reported that eicosanoids, produced from the activated cytosolic phospholipase A2 group IVA (cPLA2α) are potent pro-inflammatory mediators. By these evidence an investigation was conducted with the scope to study the involvement of HA at low molecular weight (LMW HA) on cPLA2α activity, arachidonic acid (AA) production, and the eicosanoid release in human monocytes and murine macrophages. Results showed that LMW HA significantly promoted AA release, stimulated cPLA2α, ERK1/2, p38, and JNK activation, enhanced COX2 expression and PGE2 levels both in monocytes and macrophages. Incubation of cells with a selective cPLA2α blocking compound, inhibited AA production and PGE2 augment in all cells. In addition, by treating cells with CD44, TLR4, TLR2, MYD88, RHAMM or STAB2 siRNA-transfected macrophages and monocytes, was revealed that AA produces cPLA2α, ERK1/2, p38, and JNK activation, COX2 and PGE2 up-regulation were due to LMW HA action through the TLR4/MYD88 mediation. In light of these results authors suggested that it is possible to hypothesize a special role linking HA-induced inflammation and lipid metabolism [62]. This experiments well demonstrated the involvement of several receptors whose modulation seems to play a key role in the inflammatory pathway.

Babasola et al. aimed to clarify the effective function of the acetyl groups of HA, at the low molecular weight, in the inflammatory response of human monocytes. Data obtained using several antibodies and receptor antagonist, as well as deacetylation, acetylation and butyrylation of HA suggested that the acetylated and the partially butyrylated lower molecular weight HA acts via the TLR-4 receptor, while a specific *N*-butyrylated lower molecular weight HA could be considered as a novel semi-synthetic anti-inflammatory compound [63].

Recent findings demonstrated that β-arrestin-1, a protein that is implicated in G-protein activity, has a role during inflammation. Starting from this basis an experiment was performed with the objective to elucidate the real function of β-arrestin-1 in murine chondrocytes stimulated with IL-1β. Results have shown that chondrocytes exposed to IL-1β produced a strong inflammatory response in terms of overexpression of TLR-4, CD44, β-arrestin-1, TAK-1, serine/threonine kinase (AKT), TNF − α, IL-6, iNOS, and NF-kB activation. The incubation of the cells previously exposed to IL-1β, with β-arrestin-1 and/or AKT and/or TAK-1-selective blocking agents markedly decrease the inflammation response. These data supported the hypothesis that β-arrestin-1-induced NF-kB translocation was activated through the AKT pathway. In addition, the use of a selective HA-blocking compound leds authors to conclude that these data deeply support the involvement of HA fragments in the inflammatory process of IL-1β-induced activation of β-arrestin-1 [64].

Since acute lymphoblastic leukemia (ALL) with mixed lineage leukemia (MLL) gene rearrangements (MLL + ALL) present high surface CD44 expression, an experiment was proposed with the aim of studying the action of HA at different molecular weight, on MLL + ALL cultured cells. Results have shown that the incubation of ultra-low-molecular-weight (ULMW)-HA with MLL + ALL inhibited their thymidine uptakes. On the contrary, in MLL + ALL cells, in which CD44 expression was abolished, no inhibition of the thymidine uptake was revealed. By these evidence and others obtained, authors concluded that HA fragments are highly produced at the site of inflammation [65]. Therefore these data are useful in clarifying the complex process of the transient inflammatory response associated with ALL remission, and the CD44 receptor modulation could be useful to study a new strategy for the future treatment of leukemia in which CD44 initiated the disease.

It has been shown that the accumulation of HA fragments in colorectal tumours is linked with lymphatic invasion and metastasis. On the basis of these findings, a study was performed in order to evaluate the potential of small HA pieces on cultured primary lymphatic endothelial cells (LECs). Data demonstrated that very small HA oligosaccharides were able to induce LECs proliferation and lymphangiogenesis in a dose dependent way that correlated with HA size and concentration. The use of knockout mice and selective inhibiting antibodies revealed that the action of HA oligosaccharides was mediated through the lymphatic HA receptor LYVE-1, and not by CD44 or TLR-4. Therefore in relation to these results, authors concluded suggesting the following key messages: HA fragments induced lymphangiogenesis primarily through the augment of LEC proliferation. Very small HA fragments are more potent than larger ones. The receptor LYVE-1, and not CD44 or TLR-4, mediated the action of HA fragments on LEC [66]. This experiment underlined the important finding that HA fragments may exert their action through different pathways and it depends on the cell type involved.

5.2. Chondrocytes

Several reports have shown that the action of HA is strictly dependent on its molecular weight. A study aimed to investigate the effect of low, high and medium molecular weight HA on TLR-4 modulation in an experimental model of LPS-induced inflammation in murine chondrocytes. The expression of mRNA and related protein levels were assayed for TLR-4, myeloid differentiation primary response protein (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF-6), NF-kB, TNF-alpha, IL-1beta, MMP-13, and inducible nitric oxide synthetase (iNOS) after LPS and HA exposition. LPS administration to chondrocytes deregulated all the considered markers, while HA treatment produced the following effects: low MW HA, by itself activated the TLR-4 receptor and NF-kB that in turn primed the inflammatory response and was able to increment the LPS effects. Medium MW HA had no effect in LPS-treated/untreated chondrocytes. On the contrary, high MW HA was able to limit the LPS action. Data obtained using antibodies

against the TLR-4 supported the evidence that TLR-4 was the target of HA [67]. In light of these results, it is reasonable to state that HA may have opposite effects on NF-kB activation, and this actions, in part mediated by TLR-4 involvement, is dependent on its state of aggregation.

Previous reports have shown that HA fragments can stimulate an inflammatory response through its interaction with the TLR-4 and the CD44 receptors. The activation of these receptors mediated the activation of the NF-kB which in turn activates the release of several pro-inflammatory cytokines. Campo et al., investigated the activity of small HA fragments on the TLR-4 and CD44 receptors in human articular chondrocytes. Data demonstrated that the addition of the HA oligosaccharides to cells incremented the CD44 and TLR-4 expression, the NF-kB activity and the released inflammation cytokines. The block of the TLR-4 and CD44 was able to reduce the inflammatory response induced by the small HA fragments [68]. Therefore the study confirm that the TLR-4 and the CD44 receptors are involved in the inflammatory response exerted by HA at very low molecular weight in human chondrocytes.

TLR-2/TLR-4 are responsible for the innate immunity activation but at the same time are involved in tissue repair and remodeling in response to endogenous products generated during inflammation. As many reports have demonstrated that small HA fragments are markedly augmented in osteoarthritic joints, an investigation was performed with the aim of clarifying if these product of HA degradation together with high mobility group box chromosomal protein 1 (HMGB-1) are responsible for the chondrocyte inflammatory responses mediated by the TLR-4 and TLR-2 activation, which involved the MyD88. Experiments were carried out using femoral head cap cartilage explants and primary knee articular chondrocytes from TLR-2/TLR-4-double-knockout, MyD88-knockout, and congenic wild-type mice. Results have shown that IL-1β, TNF-α, peptidoglycan (PGN) and purified LPS (pLPS) produced HMGB-1 increment from chondrocytes and HA fragmentation in normal chondrocytes. This increment was not observed in TLR-2/TLR-4(−/−) and MyD88(−/−) mouse cartilage explants and chondrocytes. These cells showed no capacity of response to HA at the low molecular weight and to HMGB-1 evaluated by assaying different inflammatory markers such as nitric oxide release MMP-3 and MMP-13 production. TLR-2/TLR-4, or of MyD88 null chondrocytes, limited NO production and markedly reduced the MMP-3 and MMP-13 levels. Results also demonstrated that the MyD88 was fundamental for HMGB-1 and HYAL-2, for HA degradation, to produce chondrocyte hypertrophy, that is involved in OA progression. In the light of these data, authors concluded that MyD88-dependent TLR-2/TLR-4 mediation is necessary for the action of HA fragments and suggest that the small HA pieces derived from HYAL-2 degradation and HMGB-1 activated innate immune system via TLR-2/TLR-4 and therefore are involved in the modulation of the chondrocyte behavior during OA progression [69]. This study further supports the TLR-2/4 involvement in the inflammatory pathway primed by HA fragments.

A study aimed to investigate the effect of HA fragments in an experimental model of IL-1β-induced inflammatory response in mouse chondrocytes. The obtained data showed a marked CD44, TNF-α, IL-6, MMP-13, iNOS expression and NF-kB activation in cells exposed to IL-1β. Cell Exposition with both IL-1β and HYAL augmented the inflammatory response as revealed by the inflammation parameters. The use of a selective CD44 blocking antibody and a protein that binds specifically the HA (HABP) supported the evidence that the CD44 was the target of IL-1β and HA mediated this action. The evaluation of HA levels and their molecular weight further supported the role of HA fragments [70]. These results indicated that IL-1β produced an inflammatory response through the CD44 receptor and this response was mediated by HA fragments produced from native HA degradation.

The effect of small HA oligosaccharides (4-mer HA) were assayed in an inflammation experimental model of normal mouse chondrocytes. Exposition of chondrocytes to 4-mer HA induced a marked over-expression of TLR-4,TNF-α, IL-1β, IL-6, IL-18 and NF-kB activation. The treatment of cells with specific TLR-4 small interference RNA (siRNA) supported the TLR-4 as the target of 4-mer HA action [71]. On the basis of these data, it is possible to hypothesize that HA can regulate the inflammatory response through its different molecular size and the inflammation mechanism can be primed/modulated as a consequence of the interaction with the TLR-4.

Considering the anti-inflammatory role of the adenosine, an investigation was conducted in order to study the action of an HA-blocking compound (PEP-1), that abolished the inflammation effect of HA fragments generated during inflammatory processes, with a selective A(2A)R agonist in an experimental model of mouse articular chondrocytes exposed to IL-1β. Results demonstrated that the over-expression of TLR-4, TLR-2, CD44, TNF-α, IL-6, MMP-13, and iNOS, as well as NF-kB translocation into the nucleus, produced by the IL-1β stimulation, was strongly decreased by treating chondrocytes with Pep-1 and/or the selective A(2A)R agonist. By these data, authors concluded that the inflammation mechanism can be modulated either by blocking HA fragments derived from native HA and/or by A(2A)R activation [72]. This investigation has shown the involvement of the A(2A) receptor in the complexity of the inflammation pathway, therefore it could represent a further target useful to modulate the inflammatory response.

A further study investigated the inhibition/stimulation of A(2A)R regulating the inflammatory response stimulated by HA fragments in murine chondrocytes. Exposition of chondrocytes to a specific (2)A adenosine receptor agonist, together with selective antibodies against the CD44 and TLR4 receptors significantly limited NF-kB translocation into the nucleus and decreased inflammation cytokine release induced by the HA oligosaccharides pre-treatment. The data were confirmed by exposing the cells to A(2A)R selective (A(2A)R siRNA) which augmented the inflammatory response induced by HA fragments. In addition, using an exchange protein activated by cAMP (EPAC) siRNA and a selective PKA inhibitor it has been found that EPAC was involved in the regulation of the A(2A)R activity [73]. These data support the evidence that HA degradation, occurring during the inflammation response, took part in the inflammation mechanism, at the same time endogenous adenosine, by acting via A(2A)R, attempts to limit the inflammatory response. Pharmacological strategies involving new drugs acting on HA fragments and A(2A) receptors could better reduce inflammation compared to current formulations.

It is known that in pathological states, free radicals produced pro-inflammatory small HA fragments from native HA. In this direction, a study was carried out to investigate the antioxidant effect of a selective SOD mimic, with the aim to prevent HA depolymerization in murine chondrocytes exposed to Fe (II) plus ascorbate. The oxidative burst produced an increase of hydroxyl radical/peroxynitrite levels, lipid peroxidation, and HA fragmentation. As a consequence, a marked over-expression of the CD44, TLR-4, TLR-2, TNF-α, IL-1β, MMP-13, iNOS, and NF-kB activation was observed. The incubation of chondrocytes with the SOD mimic limited free radical generation and the consequent HA degradation and NF-kB translocation in the nucleus, as well as the over-expression of CD44, TLRs and the inflammation markers. The use of a selective HA-blocking compound strongly reduced the inflammation response initiated by Fe (II) plus ascorbate [74]. Therefore it is reasonable to suppose that HA depolymerization plays a central role in the initiation of the inflammation mechanism in cartilage and the use of appropriate antioxidants can prevent the propagation of this response.

It has been proposed that β-arrestin-2, a protein implicated in G protein activation, took part in the regulation of inflammation. An experiment was planned in order to investigate the involvement of β-arrestin-2 in mouse chondrocytes activated with very small HA fragments. Data showed that the incubation of cells with HA fragments induced a strong inflammatory response. The addition of antibodies neutralizing β-arrestin-2 and/or a selective PKA blocking agent, markedly enhanced the inflammatory response, on the contrary, the addition of a selective

p38MAPK blocking compound strongly diminished inflammation. Of interest, the anti-inflammatory effect due to β-arrestin-2 seemed to be mediated partially by the direct neutralization of p38MAPK, avoiding NF-kB activation, and partially by cAMP and PKA induction starting by G protein intervention and culminated with NF-kB inhibition [75]. These data could be fundamental for the development of future anti-inflammatory drugs.

5.3. Tumor cells

Since small Ha fragments have been associated with tumor invasiveness and metastasis, a study inferred that the treatment of human melanoma cells with HA oligosaccharides was able to activate the NFk-B that in turn leads to the transcription of the detrimental intermediates, the matrix metalloprotease-2 (MMP-2) and interleukin-8 (IL-8), that, as it is known, participated in melanoma expansion. Authors verified an involvement of the TLR-4 in the activation of this pathway. In particular, they found that melanoma cells expressed TLR4 on their surface in vivo and in vitro, and using specific siRNA, they showed the activation of TLR4 by HA fragments and the mediation in the IL-8 expression in melanoma cells. In addition, it has been verified that the treatment with small HA oligosaccharide increased the motility of melanoma cells. This effect was abolished using a TLR4-specific siRNA. In light of these results, authors suggested that in melanoma disease, the TLR-4 can modulate the tumor invasiveness by activating MMP-2 and cytokine-expression [76]. Hence, these data provide a new feature on the relationship that links carcinogenesis and innate immunity receptors.

Starting by the evidence that inflammation accompanies cancer progression and that neutrophils are often infiltrated in several tumours, a study was conducted in order to clarify whether the population of neutrophils can infiltrate in the pericancer stroma of cervical, gastric, hepatocellular and colorectal carcinomas, and whether this accumulation correlates with metastases in gastric and hepatocellular carcinomas. Results demonstrated that neutrophil exposition to culture supernatants, obtained by different types of solid tumor cells (TSN), leds the cell to acquire pro-tumorigenic capacity and increased survival. In addition, it has been found that HA fragments were regularly produced by different tumours and they were capable of mimicking the action of TSN to confer long live to neutrophils and the consequent metastasic progression. TSN action was inhibited by blocking the HA-TLR4 interaction on neutrophils thus indicating that this interaction could be a key signal in tumor advancement. Authors hypothesized that HA generated during cancer activity can induce neutrophils to acquire an activated phenotype in order to drive the cancer progression [77]. Also in this case the use of new compounds capable of blocking the degradation of HA could represent future candidate drugs in the treatment of tumours.

A report aimed to study the effects of small HA pieces and HA at high molecular weight (HMWHA) in human SW-1353 chondrosarcoma cells. HMWHA treatment was able to reduce the inflammatory response exerted by IL-1β exposition by augmenting PPARγ activity and reducing cyclooxygenase-2 (COX-2), MMP-1, and MMP-13 levels. HMWHA also activated the Akt, blocked the mitogen-activated protein kinases (MAPKs), as well as the translocated NF-κB, suggesting a clear anti-inflammatory action. On the contrary, the cells incubated with the HA fragments have shown overall opposite effects compared to those obtained with HMWHA [78]. In light of these results demonstrating that HMWHA and fragments of HA exerted opposite inflammatory effects through PPARγ in chondrosarcoma cells exposed to IL-1β, it is plausible to suppose that HA degradation play a role in chondrosarcoma cancer and studies in this direction can support cancer therapies.

5.4. Skin cells

As known, during inflammation in the injured tissue, HA is depolymerised into small fragments, which are capable of activating the immune system. In a study, it was reported that little pieces of HA induced the activation of human/mouse keratinocytes with consequent increased production of beta-defensin 2 (BDF2). The BDF2 increment was due to TLR2 and TLR4 activation and involved the pathway in which c-Fos and the protein kinase C mediated the signalling. In fact,

Table 1
Production of HA fragments in various inflammatory diseases.

HA DEGRADATION IN INFLAMMATORY DISEASES
• Asthma • Atherosclerosis • Bowel diseases • Cancer • Diabetes • Immunological disorders • Neuroinflammation • Osteoarthritis • Rheumatoid arthritis • Skin wounds and fibrosis • Kidney diseases

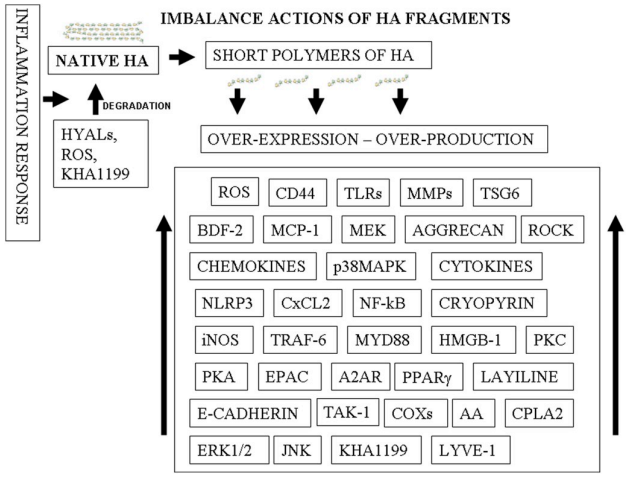

Fig. 1. The HA fragments generated by native HA breakdown through the interactions with different receptors induced upregulation of several mediators that culminate with a reinforcement and perpetuation of the inflammatory response and a consequent amplification of cellular and tissue damage.

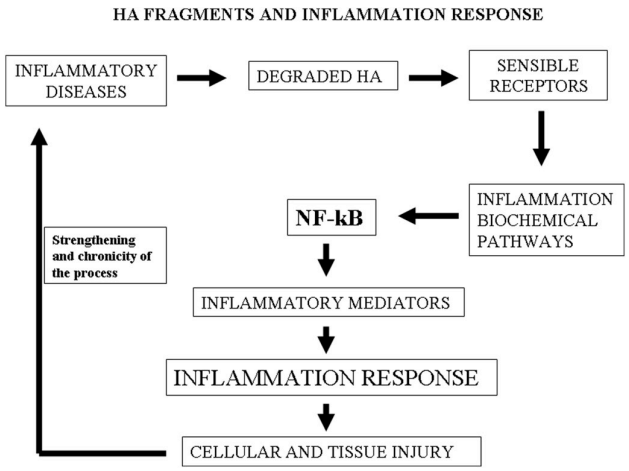

Fig. 2. General hypothetical scheme of HA fragments involvement in the inflammation pathway.

the exposition of human/murine skin to low molecular mass HA showed a marked production of BDF2 in the entire epidermal compartment. Therefore, authors suggested that the degradation of ECM components, such as HA, during damage, induced the keratinocytes to produce BDF2, with the aim of protecting cutaneous tissues as in this contest it is particularly exposed to infections [79]. Therefore these findings could be useful in the development of new topical drugs based on HA oligosaccharides with the aim of increasing the release of BDF2 by keratinocytes, in attempt to improve the self-defense of the skin against infections.

It has been demonstrated that HA fragments produced through native HA degradation can promote the mechanism involved in wound healing. The CD44 receptors seem deeply implicated in the mediation of cell response to HA of different sizes. Since this mechanism was not fully elucidated, a study was performed with the aim of clarifying the cell response to HA at different molecular weight in human dermal fibroblasts. Data showed that LMW HA increased the expression of IL-6, IL-8, CXCL1, CXCL2, CXCL6 and CCL8. On the contrary, cells incubated with HA at high molecular weight have shown the capacity to the inflammatory parameters. The use of a specific CD44 siRNA produced a significant reduction in the inflammation response induced by HA at low molecular weight. Authors concluded asserting that the inflammation response obtained in dermal fibroblasts incubated with HA of low molecular weight was able to activate the leukocytes [80]. These data further suggest a key role played by HA fragments during the wound healing.

Since several authors reported that small HA fragments (Mw $<$ 5 kDa) are able to stimulate an inflammation response, while HA pieces from 15 to 250 kDa have demonstrated controversial action. A study, using cultured keratinocytes, was conducted with the scope to clarify the link between the HA sizes and their cellular functions. Results showed a significant over-expression of TGF-β1, TNFα and IL-6 as the molecular weight of HA was reduced. The HA piece at 6 kDa resulted in the only fragment that exerted the major inflammation response. In addition, by studying the HA receptors, CD44, RHAMM and TLR4, it revealed a specific interaction with HA 1800, 1400, 500 kDa for CD44, while TLR-4 was mildly activated by 50 and 15 kDa [81]. By these results it is possible to highlight clearly that the different HA size may regulate the biological cellular response in many ways in which different biochemical pathways are involved.

5.5. Synovial cells

As cytokines are strongly involved in the intricate pathway stimulating the inflammatory response, a study was carried out with the aim of investigating the role of IL-1β on HA degradation in rabbit synovial cells. Results have demonstrated that the incubation of cells with IL-1β produced marked HA levels with the size of less than 300 kDa, and between 300 and 1900 kDa with respect to untreated cells. On the contrary, the levels of HA with size greater than 1900 kDa was very low and the HAS2, HAS3 as well as HYAL-1 and HYAL-2 were over-expressed. Based on these data authors concluded suggesting that IL-1β plays a key role in the depolymerization of HA implicated during inflammation of the joints [82]. A suggestion deriving from this study could be that of acting directly by inhibiting the production of IL-1β or blocking its receptor could be prevented the degradation of HA.

A further investigation was carried out with the scope to study the effects of the inhibition of HA depolymerization on normal mice synovial fibroblasts (NSF) and on synovial fibroblasts (RASF) obtained from mice underwent to collagen-induced arthritis (CIA). Both NSF and RASF were stimulated with TNF-α which activated the NF-kB, induced up-regulation of CD44, TLR-4, IL-1beta, IL-6, MMP-13, iNOS, and small HA fragment accumulation, although in RASF the up-regulation was of greater intensity. RASF exposition to antioxidants and selective HYAL1, HYAL2, and HYAL3 small interference RNA (siRNAs) markedly decreased TLR-4 and CD44 activity and the up-regulated inflammatory. Authors concluded supporting the evidence that the reduction of HA depolymerization during arthritis may contribute to limit TLR-4 and CD44 stimulation and the inflammation response [83]. The development of compounds able to selectively inhibit HYALs could be useful in anti-inflammatory therapy.

A further investigation was performed to study the action of another very small HA oligosaccharide (6-mer HA) on normal mouse synovial fibroblasts (NSF) or fibroblasts obtained from mice underwent to CIA (RASF). The addition of 6-mer HA both to NSF and RASF markedly up-regulated the CD44, TLR4, IL-18, IL-33 expression and activated the NF-κB, although to a greater intensity in RASF. The treatment of a fibroblasts specific protein binding HA significantly reduced 6-mer HA action in terms of inflammatory response [84]. Also for this experiment the data obtained suggest that the HA depolymerization, occurring during the inflammatory events, should be taken into account for the development of future anti-inflammatory compounds.

As the transforming growth factor-activated kinase-1 (TAK-1) and the p38-mitogen-activated protein kinase (p38-MAPK) are involved in the NF-kB activation, an experiment was performed in order to study their role in an experimental model of 4-mer HA oligosaccharide-induced inflammation in NSF and RASF obtained from normal mice or subjected to CIA, respectively. Data have shown that the incubation of NSF and RASF, previously exposed to 4-mer HA fragments, with TAK- 1 and/or p38-MAPK selective blocking compounds, strongly decreased TLR-4, TLR-2, TAK-1, p38-MAPK, TNF-α, IL- 1β, MMP-13, and iNOS activity and the NF-kB. The treatment of NSF and RASF with specific CD44 antibody also revealed that the CD44 receptor was not involved in this pathway. Authors concluded suggesting that these results gave evidence that very small HA pieces are able to prime an NF-kB-mediated inflammatory response in synovial fibroblasts, in part via TAK-1 and in part via p38-MAPK [85]. It is evident from the results that the magnitude of oligosaccharides is fundamental for the activation of receptors that trigger the inflammatory mechanism, and some oligosaccharides are able to stimulate both CD44 and TLR-2/4, others only TLR-2/4.

A recent investigation was conducted with the aim to clarify the implication of exchange proteins directly activated by cyclic adenosine monophosphate (EPAC) in a model of small HA fragment-induced inflammation in murine synovial fibroblasts (NSF). Data showed that the incubation of cells with HA oligosaccharides produced over-expression of the TLR-4, TNF-α, IL-1β, and NF-kB translocation into the nucleus. The treatment of synoviocytes with exogenous adenosine, after exposition to HA fragments, markedly limited NF-kB activation, TNF-α, and IL-1β up-regulation. The addition of NSF of cyclic adenosine monophosphate (c-AMP) and/or PKA and/or EPAC selective blocking compounds strongly reduced the anti-inflammatory action due to ADO treatment. By these results, authors concluded that both PKA and EPAC pathways are implicated in ADO-reduced NF-kB activation and EPAC appears to play a major role than PKA [86]. These results highlight the evidence that the modulation of the inflammatory mechanism can be also mediated by EPAC and PKA, thus pharmacological strategies acting on these biochemical pathways could be helpful in reducing the inflammatory response.

Finally Ollson et al., performed a new experiment with the purpose of investigating the potential activity of HA fragments in RA through the addition of different HA sizes on synovial fibroblasts and chondrocytes obtained from RA patients and peripheral blood monocytes obtained from healthy subjects. The inflammatory response was monitored by the increase of cytokine release and the TLR-4 and CD44 presence by immunocytochemistry analysis. In contrast with other previous experiments, results have shown that the addition of HA pieces to the cells, although, expressed the TLR4 and CD44, did not produce any significant up-regulation of IL-1ß, IL-6, IL-8, IL-10, IL-12 and TNF-α. In addition, HA fragments had no effect until after first stimulated with low doses of LPS. Therefore, authors state that the data obtained do not support the shared hypothesis that HA fragments are able to

prime inflammation responses and they suggested that it is unlikely that HA fragments produced during RA aggravate the disease pathogenesis [87]. The lack of results in this experiment could be due to many variable such as incubation times, cell passages, as well as the types of HA fragments used.

5.6. Lung cells

As HA degradation occurs in asthma, a study was performed with the aim to evaluate the action of glucocorticoids and long-acting β(2)-agonists (LABAs) on HA metabolism using human primary airway smooth muscle cells (ASMCs) of patients with asthma. Cells were treated with glucocorticoids and/or LABAs, and also with their selective inhibitor antagonists. In ASMCs, glucocorticoids and LABAs markedly reduced the synthesis and accumulation of GAGs. However, an increase and deposition of HA at high molecular size has been found. This result was in line with the increment of HAS-1 expression and the reduction of HYAL-1 expression. Results were confirmed using the glucocorticoid and LABAs specific receptor inhibitors [88]. Hence it can be suggested that the combination of glucocorticoids with LABAs can reduce HA degradation occurring in asthma, and thus may decrease the inflammatory response, due to HA fragments, in asthmatic patients.

As cigarette smoke (CigS) produces bronchial epithelial injury and damaged the barrier homeostasis, an investigation was conducted in human bronchial epithelial cells exposed to CigS. Results have shown a significant reduction of E-cadherin expression and decreased trans-epithelial electrical resistance. Authors hypothesized that this action was mediated by HA since the block of its production, using a specific HA synthesis inhibition, prevented this action, and the cell treatment with HA fragments had the same CigS effects. It has also been found that the HA receptor layilin is expressed in human airway epithelium and the cells after infection with lentivirus were protected against the augmented permeability due to CigS. Based on the findings authors concluded that the cigarette smoke produced HA degradation and the small fragments interact with layilin which by signalling through the Rho/ROCK produced the reduction in E-cadherin expression with consequent impairment in the epithelial cell-cell interaction [89]. Therefore the results indicated that degraded HA is deeply involved in the steps that prime the inflammation response to CigS.

5.7. Other cell types

HA oligosaccharides were also involved in atherosclerosis lesions. An investigation has examined the HA receptor for HA-mediated motility (RHAMM) and HYAL enzyme expression in carotid artery samples using vascular transplants that exhibit different atherosclerotic lesions. The data obtained showed a marked expression of HYAL-1 in the district of tissue where the inflammation was exacerbated with plaques. At the same time, was revealed that the RHAMM, which is deeply involved in the transduction of the angiogenic signals, was overexpressed on blood vessels with atherosclerotic lesions [90]. By these data it is possible to hypothesize that the degradation of HA and the consequent activation of RHAMM in atherosclerotic lesions with plaques could be partially responsible for the detrimental consequences of atherosclerotic vessel damage.

HA oligosaccharides were also able to interact with tumor necrosis factor-stimulated gene 6 protein (TSG-6) in such a way that stimulates an inflammatory response. A report investigated the interaction between HA, the TSG6 link and G1 aggrecan domain using a sophisticated biophysical analysis with confocal fluorescence. Both TSG6 link and G1 domain were able to interact with HA oligosaccharides at a well-defined molecular weight. The experiments also clarified that the HA interaction with TSG6 and G1 domain was strictly dependent on specific pH value [91]. Therefore these data suggest that TSG-6 and G1 aggrecan interact with HA through homologous domains and the difference in the pH-dependent binding could be due to differences in the distribution of amino acids in binding sites.

As HA is present in human atherosclerotic plaques a study was performed in order to elucidate if HA degradation was incremented in atherosclerotic lesions with unstable characteristics such as wide lipid core, high macrophage, and type IV collagenase activity. Results have shown that HYALs activity, the presence of HA at a low molecular weight and CD44 expression was markedly augmented in the atheromatous plaques with respect to the fibrous plaques. The type IV collagenase activity correlated with CD44 expression and HYAL using HA at a low molecular weight [92]. Thus these results highlight the evidence that both HA degradation and the increase in CD44 expression could be associated with destabilized atherosclerotic plaque potentially through the augment in the type IV collagenase and the increment in angiogenesis activity.

Several findings demonstrated a significant degradation of ECM components during inter-vertebral disc (IVD) degeneration, in this contest a study planned to evaluate the effects of HA fragments using human IVD cells. Results have shown that the incubation of IVD cells with HA fragments markedly augmented IL-1β, IL-6, IL-8, COX-2, MMP-1/13 expression, and protein production. The incubation of cells both with a specific TLR-2 siRNA or a selective TLR2 blocking antibody limited significantly the increase of IL-6 produced by the small HA pieces. It was also found that the capacity of HA fragments to increase IL-6 and MMP-3 levels was due to the activation of the MAP kinase pathway [93]. In light of these results, it is plausible to confirm that degraded HA was able to mediate IVD damage and discogenic back pain through the involvement of the TLR2 activation pathway in IVD cells.

As glomerular mesangial cells (MC) may produce HA, and during inflammation resulted a marked HA cleavage, a study was conducted in order to clarify if HA fragments can modulate the immunostimulatory response of smooth muscle cell-like MC. MC cells were incubated with HYAL in order to degrade native HA into small HA pieces of various size in the MC culture. Results demonstrated that HYAL treatment had no effect on MC cells obtained from TLR4-deficient mice. On the contrary, TLR2-deficient MC produced a significant response due to LPS-contamination of HYAL. HYAL treatment was also able to potentiate the action of TLR2−/−3/-5 agonists in MC obtained from TLR4-deficient mice not mediated by LPS contamination. Addition of the MC with heparin blunted all effects [94]. By this investigation it can be hypothesized that HA, probably, formed a pericellular gelatin barrier, which masks the surface of TLRs receptors. The barrier depth is regulated by HA metabolism and the expression of HA-receptors on the cell surface modulate the inflammatory responses through the receptor accessibility.

New evidence reports that during injury, platelets interact with the ECM of the vessel wall and then, by releasing HYAL-2, degrade HA from the endothelial cells into small fragments able to stimulate an inflammatory response in monocytes. In this contest, a study was carried out with the aim of determining the pathway through which platelets depolymerize HA in the damaged tissues. Results confirm that human platelets disgregate native HA by HYAL2 and also showed that the activated platelets produced the translocation of HYAL2, from a specific population of α-granules, to platelet surfaces in order to better exert its enzymatic action. Interestingly it was also found that patients with inflammatory bowel disease possess less platelet HYAL2 amount with respect to the healthy controls [95]. Therefore it can be assert that HA degradation by platelet HYAL2 could be a crucial step for proper tissue repair after injury and having HA fragments, generated by platelet HYAL2, a role in the progression of wound healing, could be conceivable that these functions can be impaired in IBD patients with low platelet HYAL2.

Starting by the evidence that HA fragments were able to induce an inflammatory response in different cell types, a study was ideated in order to test the potential of HA oligosaccharide in neuron-like SH-SY5Y cells and the implication of TLR-2, TLR-4, CD44 receptors, the release of α-synuclein, as this protein seems to be produced from

neurons and capable of inducing and maintaining a neuro-inflammatory response. Results showed that the incubation of cells with HA oligosaccharides over-expressed TLR-2, TLR-4, CD44, TNF-α, IL-β, α-synuclein and NF-kB activation. The treatment of cells with TLR-2, TLR-4, and CD44 selective inhibitors was able to abolish the inflammation response induced by HA fragments [96]. Hence, based on these data it is conceivable that HA metabolism plays an important role in neuroinflammatory diseases and it should be taken into great consideration during the development of new strategies against neurodegenerative disorders.

Recently, it has been reported that although HA at very low molecular weight derives from the HYALs action, HASs can also produce very small HA pieces. Starting by these findings a study was carried out in the Streptococcus equisimilis C-terminus, which contains a tandem B-X_7-B motif (K^{398}-X_7-R^{406}-X_7-K^{414}), with the purpose to evaluate the activity of 27 site-specific scanning mutations and 7 C-terminal truncations on HA synthesis and its molecular weight. Results demonstrated that HASs synthesize and regulate HA amount and mass and also they are proteins that may be uncoupled by mutagenesis of conserved cysteines [97]. Considering these results it is possible to claim that HASs possess regulatory modifications that altered the B-X_7-B motif conformation and could imitate these mutagenesis-induced actions, so that HASs are able to produce small HA fragments directly.

Of note, it has been discovered that a novel protein (KIAA1199) is able to degrade HA beyond the classic known routes. In this contest, an experiment was planned in order to verify whether KIAA1199 protein was augmented in Crohn's Disease (CD) colon fibroblasts, obtained from CD patients, and produced small HA pieces that promote inflammation response and fibrosis. Results have found marked amounts of KIAA1199 protein released and deposited in the ECM of CD fibroblasts with respect to the controls. Incubation of fibroblasts with the IL- 6 was able to augment the deposition of KIAA1199 in the ECM. In itself, IL6 was overproduced in CD cells, and the use of specific antibodies against IL6 receptor reduced the KIAA1199 protein in the ECM of CD cells. Interestingly, the addition to CD fibroblasts of a siRNA silencing KIAA1199 abolished the capacity of the cells to degrade HA [98]. Thus, these data highlight that in CD fibroblasts the amounts of KIAA1199 are up-regulated mainly through a mechanism involving IL-6 and the prime of these pathways imply a massive HA degradation and with the consequent production of HA fragments, which contributes to perpetuating gut inflammation and fibrosis.

6. Conclusions

The data here presented are related to the last decade of the in vitro novelty about the effects of HA fragments, produced by native HA during inflammatory pathologies (Table 1), on various experimental models and cell types mimicking human diseases. The data reported until now support the special role possessed by HA and particularly the deleterious action of small HA fragments that may regulate the prime of the inflammatory response and the consequent cellular imbalance during tissue damage (Fig. 1). Starting from these findings, it is possible to hypothesize that studying the mechanism of HA degradation and the action of the different sizes of HA pieces/oligosaccharides can give light to interesting new approach for contrasting the intricate pathways of the inflammation response and the detrimental consequences on cell survival. As reported, a number of experiments have used different specific and selective approaches that has achieved excellent goals using inhibiting/stimulating tools acting mainly on CD44, TLRs, HYALs, ROS, and other correlated/uncorrelated pathways (Fig. 1). Therefore future research should not only consider the molecular weight of HA fragments, but new investigations should be focused on the synthesis of selective blocking agents acting on the receptors and/or other intermediates activated by the HA fragments (Fig. 2). In this way, it would be possible to develop new biochemical strategies that could reduce HA degradation and tissue injury more effectively than the current therapies. Hence, we believe these findings should promote future studies on the effect of HA catabolism on the modulation of immunity in inflammatory conditions. However, the complexity of the processes and sometimes the ambivalent endpoints strongly stress the need for further study in this direction.

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