Role, Metabolism, Chemical Modifications and Applications of Hyaluronan

Nicola Volpi*[,1], Juergen Schiller[2], Robert Stern[3] and Ladislav Šoltés[4]

[1]*Department of Biologia Animale, Biological Chemistry Section, University of Modena & Reggio Emilia, I-41100 Modena, Italy*

[2]*University of Leipzig, Medical Faculty, Institute of Medical Physics and Biophysics, D-04107 Leipzig, Germany*

[3]*Department of Pathology, Faculty of Medicine, Al Quds University, Abu-Dies, 20002, East Jerusalem, Palestine*

[4]*Institute of Experimental Pharmacology, Slovak Academy of Sciences, SK-84104 Bratislava, Slovakia*

Abstract: Hyaluronan (hyaluronic acid, HA) is a linear naturally occurring polysaccharide formed from repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronate. Despite its relatively simple structure, HA is an extraordinarily versatile glycosaminoglycan currently receiving attention across a wide front of research areas. It has a very high molar mass, usually in the order of millions of Daltons, and possesses interesting visco-elastic properties based on its polymeric and polyelectrolyte characteristics. HA is omnipresent in the human body and in other vertebrates, occurring in almost all biological fluids and tissues, although the highest amounts of HA are found in the extracellular matrix of soft connective tissues. HA is involved in several key processes, including cell signaling, wound repair and regeneration, morphogenesis, matrix organization and pathobiology. Clinically, it is used as a diagnostic marker for many disease states including cancer, rheumatoid arthritis, liver pathologies, and as an early marker for impending rejection following organ transplantation. It is also used for supplementation of impaired synovial fluid in arthritic patients, following cataract surgery, as a filler in cosmetic and soft tissue surgery, as a device in several surgical procedures, particularly as an anti-adhesive following abdominal procedures, and also in tissue engineering. This review will provide an overview of the structure and physiological role of HA, as well as of its biomedical and industrial applications. Recent advances in biotechnological approaches for the preparation of HA-based materials, and as a component of tissue scaffolding for artificial organs will also be presented.

Keywords: Hyaluronan or hyaluronic acid, glycosaminoglycans.

1. INTRODUCTION

Hyaluronan/hyaluronic acid (HA) possesses a fairly simple, indeed common, structure defined in 1934 by Karl Meyer's laboratory in a well-designed series of experiments between its discovery in the vitreous humor of the eye [1] and the final definition of the repeating disaccharide, -D-glucuronic acid-β-1,3-*N*-acetyl-D-glucosamine-β-l,4-, as described by Weissman and Meyer in 1954 [2] (see below). However, apart from its simple backbone structure, HA is a fascinating macromolecule thanks to its physiological role in important pathological conditions and diseases at a molecular and cellular level, and its physico-chemical properties, useful in the biomedical field and biotechnological products as a biomaterial. HA is used as a therapeutic agent in veterinary medicine and it is an established aid in cataract surgery [3]. HA is also commonly used for intraarticular injections in osteoarthritis (OA) in humans (see below). Finally, HA can be analyzed in nanogram quantities in blood and other body fluids as well as in tissues. These analyses are of paramount diagnostic value in various disorders. Highly comprehensive reviews and volumes covering all aspects of HA chemistry and biology have been published recently [4-14]. This article intends to extend and update HA data and applications from the technical, as well as from a molecular and cellular point of view.

*Address correspondence to this author at the Department of Biologia Animale, University of Modena & Reggio Emilia, Via Campi 213/d, I-41100 Modena, Italy; Tel: 0039 (0)59 2055543; Fax: 0039 (0)59 2055548;
E-mail: volpi@unimo.it

2. HA STRUCTURE

2.1. Hyaluronan Structure

HA is a linear high-molar-mass natural polysaccharide composed of alternating (1→4)-β linked D-glucuronic (GlcA) and (1→3)-β linked *N*-acetyl-D-glucosamine (GlcNAc) residues (see Fig. **1**). The chemical structure of HA is fairly regular. The only deviation is the possible replacement of GlcNAc with deacetylated glucosamine residues.

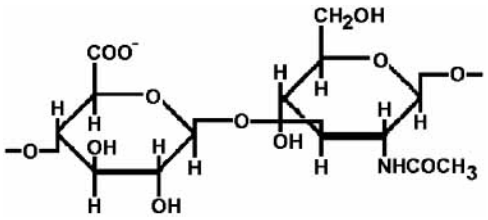

Fig. (1). Structure of disaccharide forming hyaluronan.

HA belongs to a group of substances known as glycosaminoglycans (GAGs), represented along with HA by chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate and heparin. HA is the only nonsulfated GAG, and the only GAG which is not synthesized in the Golgi apparatus.

Despite its relatively simple structure, HA behaves as an unusually stiff and complex polymer in solution and its structure in intracellular, pericellular, and extracellular environments is not yet known. Very comprehensive reviews and

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volumes covering structural aspects of HA chemistry have been published recently [4-14].

2.2. Hyaluronan Occurrence in Living Organisms

HA is omnipresent in the body of all vertebrates, including humans. An adult of an average body weight of 70 kg contains approx. 15 g of HA. Almost half of the human body's HA occurs in skin with most of the HA located in the intercellular space. Here HA immobilizes water in skin tissue and thereby changes dermal volume and compressibility. In the skin, the largest organ of the human body, HA also plays the role of a scavenger of free radicals generated by the ultraviolet rays from sunlight. In synovial (joint) fluid, the high concentration of high-molar-mass HA provides necessary lubrication for the joint and serves as shock absorber, reducing friction of the moving bones and diminishing wear of the joint.

A brief listing of the occurrence of HA in different human tissues and its content is provided in Table **1** (see also [15]). So far, rooster combs are the animal tissues with by far the highest HA content (7.5 mg/g). Besides being present in vertebrates, HA occurs extracellularly by some bacteria, e.g. strains of *Streptococci*.

In an aqueous milieu, including that of synovial fluid and vitreous humor of the eye, HA occurs as a negatively charged macromolecule. In normal human synovial fluid and vitreous humor HA macromolecules are present in a free/not protein-associated form. Due to their megadalton molar masses and extended conformations, these fluids demonstrate unique visco-elastic and rheological properties [16].

HA occurs primarily in the extracellular and pericellular matrices, although recently it has been shown to be also present intracellularly [17]. Within most soft and hard tissues, such as that of the articular cartilage matrix, HA is associated *via* a link protein with a proteoglycan (PG), aggrecan, consisting of GAGs, namely chondroitin sulfate and keratan sulfate. The HA-aggrecan aggregates have enormous molar masses of up to 100 MDa, embedded within a collagenous framework [18].

Although it was initially thought that the principal role of HA is to serve as an inert molecular filling in connective tissue, subsequent identification and study of HA-binding proteins and specific receptors has revealed that HA mediates many other functional activities [19]. HA is now recognized as playing important roles in embryogenesis, signal transduction and cell motility, and is associated with cancer invasiveness and metastasis (see below).

2.3. Hyaluronan Synthases

2.3.1. Structure and Biology of the Hyaluronan Synthases, the HAS Enzymes

The enzymes that synthesize HA are referred to as the HA synthases (HAS). The pursuit of these enzymes has had a prolonged and arduous history, largely because they are difficult to purify, are membrane-bound, and lose activity when solubilized. Discovery of the first HA synthase gene, from Group A *Streptococcus*, did not occur until 1993 [20, 21]. This 42 kDa single bacterial enzyme protein was identified, which added in strict alternating fashion GlcNAc and GlcA to the non-reducing end of the growing HA chain, using the two UDP-sugar substrates, creating alternatively the $\beta1\rightarrow3$ and $\beta1\rightarrow4$ glycosidic bonds [20, 21].

Since then, the field has expanded rapidly. In eukaryotes, HA is synthesized on the inner surface of the plasma membrane as an unadorned linear polymer. It is of such enormous size that it is coordinately extruded across the membrane out into the extracellular space as it is being synthesized [22, 23]. Such growth could not occur in the endoplasmic reticulum nor in the Golgi apparatus, where most sugar polymers are synthesized, without destroying the cell. Synthesis of such an enormous polymer, which can reach 1,000 to 10,000 kDa, containing up to 25,000 disaccharide units, could not be possible intracellularly, nor could the high viscosity be tolerated.

Early investigations documented an anomaly, whereby treating cells with very low concentrations of hyaluronidase up-regulated levels of HA synthesis [24, 25], suggesting that there must be some feed-back mechanism that signals to the cell the amount of HA polymer that has been made. It is tempting to postulate that the HA receptor, CD44, is a component of such a servomechanism.

It is now recognized that in eukaryotes, there are three HAS enzymes (Fig. **2**), encoded by three related *HAS* genes on three different chromosomes [26, 27]. The single exception is the frog, *Xenopus laevis,* in which there are apparently four such genes [28, 29]. The HAS enzymes are integral

Table 1. Occurrence of HA in Different Human Tissues/Fluids and its Content (Adapted from [9])

Tissue or body fluid	Concentration (μg/g; μg/ml)	Remarks[a]
Umbilical cord	4100	Contains primarily HA with a relatively high molar mass
Joint (synovial) fluid	1400-3600	The volume of the synovial fluid increases under inflammatory conditions. This leads to a decreased HA concentration
Vitreous body	140-340	HA concentration increases upon the maturation of this tissue
Dermis	200-500	HA is suggested as a "rejuvenating" agent in cosmetic dermatology
Epidermis	100	HA concentration is much higher around the cells that synthesize HA
Thoracic lymph	0.2-50	The low molar mass of this HA is explained by the preferential uptake of the larger molecules by the liver endothelial cells

[a]The highest amounts of HA are present in the extracellular matrix of soft connective tissues [10].

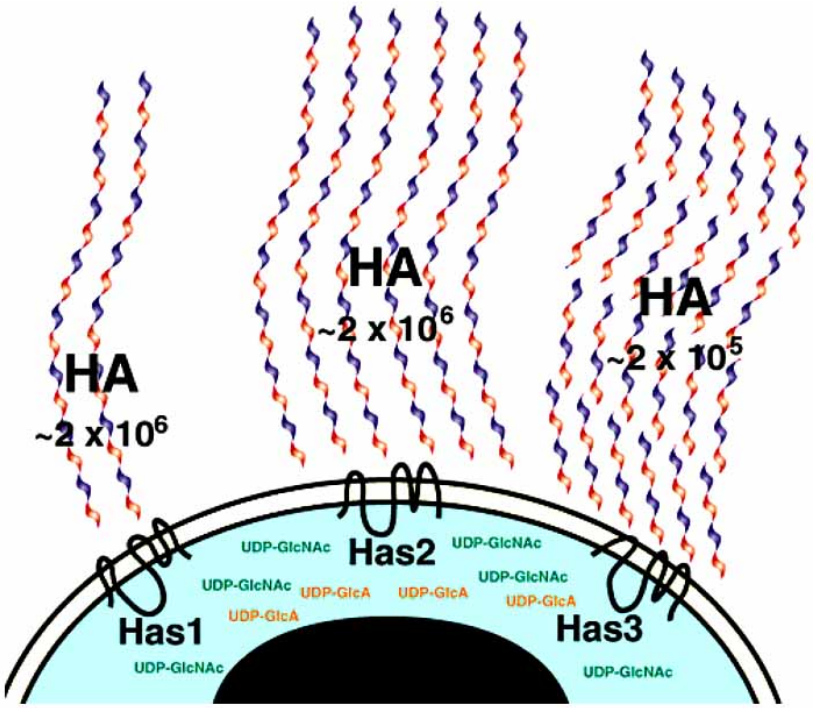

Fig. (2). Regulation of HA amount and chain length by expression of a specific HAS protein. Biochemical characterizations of the vertebrate HAS enzymes expressed in mammalian cell culture have revealed similarities and differences between the respective mammalian hyaluronan synthase enzymes. The differences are depicted in this illustration. Has1 produces small amounts of high-molar-mass hyaluronan. Has2 produces significantly more high-molar-mass hyaluronan. Has3 is the most active of the hyaluronan synthases, yet produces low-molar-mass hyaluronan chains. The physiological significance of these differences in enzymatic activity is not yet known (http://www.glycoforum.gr.jp/science/hyaluronan/).

membrane proteins. Sequence data of the HAS isoforms suggest that they contain seven membrane-associated regions and a central cytoplasmic domain possessing several consensus sequences that are substrates for phosphorylation by protein kinase C [30, 31].

Each enzyme is able to synthesize *de novo* HA chains. The products have identical structures, yet each enzyme has distinct properties [30-32]. The catalytic rate and mode of regulation for each isozyme is different [32]. HAS1 is least active, but is responsible for the synthesis of a range of polymers, from 2×10^5 Da to a very high-molar-mass HA of 2×10^6 Da, suggesting a low but constitutive level of synthesis.

HAS2 is more active, and also generates a large form of HA, greater than 2×10^6 Da. This may be the HAS enzyme that responds to stress-induced increases in synthesis, as found in shock, septicemia, inflammation, massive wounding, after major blood loss, and in burn patients. HAS2 is also implicated in developmental and repair processes involving tissue expansion and growth. HAS2 is involved in embryonic and especially in cardiac cushion morphogenesis [33], and is associated with cell migration and invasion, cell proliferation, and with angiogenesis during development. HAS2 is also widely distributed in normal adult tissues.

HAS3 is the most active HAS enzyme, and promotes the synthesis of large amounts of HA chains that also vary in size from 0.2×10^6 to 2×10^6 Da [32]. The products of HAS3 may provide the pericellular glycocalyx, and the HA that interacts with cell surface receptors. The shorter HA chains may trigger cascades of signal transduction events that modulate changes in cellular behavior. HAS3 also appears to favor the malignant phenotype [34]. However, the exact function of each of the HAS isozymes is unknown. Much of the foregoing is conjecture, and defined functions for each HAS enzyme remain an enigma.

2.3.2. The Export Mechanism

An important issue remains as to the precise mechanism of membrane extrusion that transports HA out of the cell coordinated simultaneously with its synthesis. Export of HA from bacteria such as *Streptococcus pyogenes* occurs by means of an ABC (ATP-Binding Cassette) transporter system [35]. Curiously, in bacteria, the ABC transporter system proteins are encoded at a chromosomal region immediately adjacent to the HA synthase genes [36].

A sequence homology search against human proteins reveals strong homology to a multi-drug resistance system, the transporters ABC-B (MDR-1) and ABC-C (MRP 5, multidrug resistance protein) [37]. Using several inhibitors directed against these, decreased HA production in cell culture as well as in purified membrane fractions is observed, indicating that HA export is one physiological role for these transport proteins, though they may not be the only ones available for such activities.

2.3.3. Control of the HAS Enzymes

Little is known about the regulation of the three different eukaryotic HAS enzymes. The HAS genes appear to be tissue- and cell-specific [30, 31, 38]. Differential regulation of the individual HAS genes has also been well documented [39, 40]. However, many experiments with the HAS genes were conducted in early experiments using transfection of cultured cells. It is very difficult to extrapolate such results into the real *in vivo* situation. Transfection experiments using the *HAS* gene cDNA have the obvious drawback that the protein products are not accompanied by associated membrane molecules that may modulate enzyme activity *in vivo*.

Many established and primary cell lines synthesize HA [41]. These include: a) embryonic fibroblasts, such as 3T3 and 3T6 cells, b) mouse oligodendroglioma line, G26-24, c) human lung fibroblasts, WI38, and d) primary cultures of chondrocytes, keratinocytes, synovial and dermal fibroblasts. Other cells have greatly reduced or absent HA synthetic activity, which makes them convenient for screening of putative HAS sequences in transfection experiments: a) CHO lines (Chinese hamster ovary cells), b) SV-40 transformed AGMK lines (African Green Monkey Kidney cells), c) COS-1 and COS-7 cells, and d) HEK 293, derived from human embryonic kidney.

Since that early period, a number of HAS-specific antibodies have become available, so that it is possible to conduct more precise experiments, to determine the role of each HAS in development, oncology, wound healing, and in the battery of pathophysiological processes. However, one major problem in the field remains as the absolute specificity of some of the commercial HAS antibodies now available is questionable, and a period of time must elapse until a consensus is reached.

The level of HAS activity is also sensitive to cell density. Sub-confluent, proliferating cells produce much higher levels of HA than do contact-inhibited cells [42]. This is also apparent in "scratch" cultures (a model often used for wound repair). A scratch is made in a lawn of contact-inhibited fibroblasts. The trough fills with HA, as cells proliferate at the edge and move in to repair the trough [Longaker, M.T. and Stern, R.: unpublished observations].

2.3.4. Modulation of HAS Enzymes by Growth Factors and Cytokines

HAS enzymes have distinct expression patterns controlled in part by various growth factors and cytokines [32, 34, 43-45]. Comparing the three isoforms of HAS enzymes in synovial fibroblasts in patients with rheumatoid arthritis and OA, HAS1 mRNA is up-regulated by TGF-β. The HAS3 isoform is up-regulated by IL-1β and TNF-α.

In skin-derived cells, *in situ* expression of *HAS1* and *2* genes are up-regulated by TGF-β in both dermis and epidermis, but there are major differences in the kinetics of the response, and between the two compartments, suggesting that the two genes are independently regulated. This also suggests that HA has different functions in dermis and epidermis.

Stimulation of HA synthesis also occurs following phorbol ester (PMA) and PDGF treatment. Glucocorticoids induce a nearly total inhibition of HAS mRNA in dermal fibroblasts and osteoblasts [46]. This may be the molecular basis of the decreased HA in glucocorticoid-treated skin.

2.3.5. HAS Enzymes in Cancer Biology

One of the most remarkable events that occur with malignant transformation of normal cells is the dramatic increase in HA synthesis. One of the earliest examples is the transformation of chick fibroblasts by the Rous sarcoma virus [47]. The temperature sensitive mutants that are available provided many early insights into the role of HA synthesis in malignancy, and the timing of HA deposition in the critical early events.

In the *in vivo* situation, levels of HA surrounding tumor cells often correlate with tumor aggressiveness [48]. This HA can be the product of the tumor cells themselves, or can also be contributed by the peritumor stromal cells, commandeered by tumor cells to up-regulate levels of HA synthesis. The signals produced by tumor cells to up-regulate such synthesis have been the focus of intense studies for over two decades [49, 50]. The HA synthases are the ultimate target of such signals. Levels of activity of HA synthases themselves can correlate with tumor progression, as has been documented in ovarian [51] and colon [52] carcinomas.

Despite progress in the study of HA biosynthesis, the mechanisms underlying the transformation-induced overproduction of HA have not been elucidated. Levels of HAS activity following malignant transformation were studied in a rat 3Y1 fibroblast cell line. Of three HAS isoforms, only HAS2 gene expression increases in v-HA-ras transformed cells. These have only moderate malignancy. In contrast, HAS1 and HAS2 expressions are elevated in the highly malignant cells transformed with v-src and/or v-fos. In a totally different system, however, it has been shown that HAS3 over-expression promotes the growth of prostate cancer cells [53].

High levels of HAS2 expression has the anomalous effect of inhibiting tumor growth [34], suggesting that complex relationships exist, and that proper regulation of each HAS isoform is perhaps needed for malignant transformation, tumor growth and metastatic spread.

2.3.6. Other Considerations

It is likely that the HAS proteins are part of a larger protein complex [54, 55] that together regulate enzyme activity, and coordinates interactions with other cellular components. A mini-organelle within cells may contain a complex of synthetic and degradative activities, together with regulatory proteins and hyaladherins that respond to the metabolic schemes of the cell. Such a structure would parallel a mini-organelle that has been described in association with another simple carbohydrate polymer, the glycogen granule [56]. A "hyalosome" would be an appropriate designation for a similar HA particle.

In general, HA synthase enzymes do not require primers. However, a single exception has been documented. Addition *in vitro* of HA tetramers to a bacteria-derived HA synthase system from *Pasteurella multocid,* an alga that resides within a protozoan host, stimulates biosynthesis 20- to 60-fold, compared to synthesis without primers [57, 58]. No other

primer-dependent synthesis of HA has been identified. This primer-dependent mode of HA synthesis is reminiscent of the small amount of primer required for the initiation of glycogen synthesis, another long chain linear repeating carbohydrate polymer.

The parallels among chitin, cellulose, and HA structures, all being β-chains of hexose polymers are reflected in the striking similarity in sequence between the HAS from vertebrates, cellulose synthases from plants, and chitin synthases from fungi. A primordial ancestral gene may have existed from which all the enzymes involved in the biosynthesis of polymers containing β-glycoside linkages, i.e. an ancient β-polysaccharide synthase, evolved.

2.4. Hyaluronan Degrading Enzymes, the Hyaluronidases, or HYALs

2.4.1. Classification of the Hyaluronidase Enzymes

The enzymes that catabolize HA are broadly distributed, have a wide range of pH optima, as well as an unusual range of substrate. The term "hyaluronidase" is a misnomer, since most of these enzymes, whether of bacterial or animal origin, will degrade chondroitin and chondroitin sulfates, albeit at a slower rate. The man who first described HA, Karl Meyer [1], also coined the term "hyaluronidase." He also classified them into three different groups, based on their mechanisms of action [59]. Now with genomic information available, it is apparent that Meyer's scheme was remarkably accurate.

a) Bacterial hyaluronidases are β-endo-*N*-acetylglucosaminidases. They are lyases that cleave by β-elimination, with an unsaturated bond generated during the process of cleavage [60]. This makes it possible to follow the reaction by spectrophotometry.

b) Mammalian types of hyaluronidases are also β-endo-*N*-acetylglucosaminidases but are hydrolases. They add water across the bond to be cleaved. These reactions are difficult to follow, and special assays had to be devised in order to measure the progress of the reaction [61].

c) Leeches, some parasites and crustaceans have a β-endo-glucuronidase. They are also hydrolases, and thus appear to be closer related to the vertebrate enzymes. However, to date, no sequence data are available, and they are not considered further.

2.4.2. Viral, Bacteriophage, and Bacterial Hyaluronidases

Bacteriophage isolated from *Streptococcus pyogenes* have a hyaluronidase unrelated to the bacterial enzyme. Of interest is the presence of a collagenous domain, 10 repeats of the glycine-x-y sequence, with five prolines in the x-y positions [62]. The ten collagenous repeats are the minimal size required to provide stability to a triple helical structure. The single strand of enzyme protein can interact with collagenous structures, and thus function as an adhesion mechanism as well as a virulence factor for the host of the invading bacterium, while simultaneously, helping the bacteriophage to penetrate the HA coat of the Group A *Streptococci.* This provides an excellent example of the subtle interactions that can occur between parasites and hosts in the process of evolution and survival.

A highly active baculovirus hyaluronidase was detected recently [63] suggesting that viral hyaluronidases may be more widespread than previously assumed. It can be speculated that many viruses, particularly those that attack mammalian cells, carry a wide spectrum of hyaluronidases. This is virtually an untouched area of biology, but one with considerable potential.

A bacterial hyaluronidase was identified, even before it was determined that "spreading factor" was such an enzyme [64]. The hyaluronidases of bacteria play a major role in their dissemination, with important implications for clinical medicine. They often function as virulence factors. The hyaluronidase of the syphilis bacterium, *Treponema pallidum* facilitates dissemination [65]. Antibodies to this hyaluronidase restricts tissue invasion, and in particular, prevents attachment and penetration through capillaries. The sequence of this enzyme would be of particular interest. The hyaluronidase of the gas gangrene bacterium, *Clostridium perfringens,* is a virulence factor, and together with the bacterial collagenase, assists in dissecting facial planes and in tissue destruction [66]. There are multiple hyaluronidases in the genome of *Staphylococci* [67]. It would be interesting to determine, though no longer of clinical importance, if these enzymes played a role in toxic shock syndrome. Since HA and its associated volume of solvent water can function as an intravascular volume expander, it would be interesting to establish whether Gram negative bacteria contain hyaluronidases that are the basis of the shock that accompanies Gram negative sepsis.

2.4.3. Rapid Turnover of Hyaluronan in the Vertebrate Body

There is an extraordinarily rapid turnover of HA in vertebrate tissues. This is a particularly rapid rate for a component of the extracellular matrix (ECM). It is estimated that for a 70 kg individual, there are 15 g of HA, of which there is a daily turnover of 5 g. In the bloodstream, the $t_{1/2}$ of HA is 2 - 5 min. The catabolism of HA is indeed a constantly ongoing and rapid process. Usually, the HYAL enzymes are assumed to be the predominant catabolic mechanism involved in this process throughout the vertebrate body.

However, HA catabolism can take place by one of two mechanisms, by enzymatic degradation, or scission by reactive oxygen species (ROS). Both mechanisms occur simultaneously, but nowhere in literature is there an estimate of the relative amounts that each contributes. This continues to be a major flaw in our understanding of HA degradation, and of all the pathophysiology that involves turnover of this extraordinary molecule. In surveying tissues such as the brain, lung or small intestine, these enzymes are expressed at exceedingly low concentrations. In particular, joint synovial fluid contains no detectable hyaluronidase activity. However, in joint fluid, there is a very high turnover of HA, with a $t_{1/2}$ of approximately 12 hours, even without any detectable enzyme being present. Chemical catabolism of HA is a very well established phenomenon. It is generally accepted that HA turnover, in addition to enzymatic mechanisms, can also be due to chemical degradation. It is rare, however, for chemical and enzymatic reactions to be considered in the same study.

The lymph nodes have been found to have a considerable capacity for extraction and catabolism of HA [10]. Comparison of lymph before and after passage through the nodes shows an extraction of as much as 90%, which occurs in the lining cells of the lymphatic sinuses, comparable to those of the blood vessels in liver and spleen. Studies of peripheral lymph have also revealed: 1) that its content of HA is still much lower than that of the tissues from which it is derived; 2) that it contains, nevertheless, very large polymers similar to those in the tissues, which suggests that they are displaced from the tissues hydrodynamically rather than by diffusion and do not require any prior degradation; 3) that these polymers are preferentially eliminated in the lymph node, consistent with the higher affinity for larger polymers demonstrated in experiments on the hepatic HA receptor.

When examined closely, there are major differences in the breakdown products between the two pathways. Under physiological conditions, vertebrate metabolism occurs in the presence of O_2. The actions of O_2, with the attendant oxidation by free radicals or by hydroxyl radicals are powerful participants in body chemistry. Under certain pathological conditions, acute inflammatory cells, the polymorphonuclear leucocytes, infiltrate tissues. They carry the myeloperoxidase enzymes, and there is a burst of oxidation reactions early in the inflammatory response. A battery of oxidants results from the myeloperoxidase reaction, including H_2O_2 and Cl^- ions that form e.g. hypochlorous acid. All of these products are very aggressive reactants, and powerful players in HA degradation. Peroxynitrite is a second and additional component of these reactions. This entire mixture causes rapid degradation of HA. However, what is important to consider is that the fragments generated by enzymatic reactions are highly different from those resulting from ROS.

The products of hyaluronidase degradation of HA, apart from chain length, have a chemical structure that is identical to that of the parent polymer. However fragments generated by chemical means differ greatly from the parent chain. The products of O_2 metabolism are incorporated into the chains and modify their structure. Such HA fragments contain aldehydes and hydroperoxides at their reducing termini. Both are extremely reactive. Aldehydes condense spontaneously with amines, generating Schiff bases with amino acids, with peptides, and with proteins. Hydroperoxides are also a source of cross-links for HA chains.

It is necessary to differentiate strictly and to establish under what conditions fragments are generated when evaluating the results of *in vitro* experiments. It is extremely important therefore to consider that fragments can differ. Fragments prepared under oxidative or stress conditions are entirely different from those prepared in a reducing environment. It is important to provide the precise conditions under which experiments are performed. But this is rarely done. Conditions such as more acidic, or more alkaline conditions, whether ultrasonic methods are being used, and whether in a reducing environment, whether 2-mercapto-ethanol or dithiothreitol is used in the culture medium, at what concentration, whether ascorbic acid is present in the medium, whether divalent cations, or EDTA or EGTA are present. Each of these conditions can generate fragments that differ widely.

Another proviso is that commercial preparations of HA starting materials are available from a wide variety of sources. However, companies are unwilling to disclose under what detailed conditions their HA preparations were made, claiming that this is proprietary information. Some commercial HA powders are white, while others are yellowish, and were probably prepared in the presence of iron. The consequences of the differences in all such experiments are considerable, and may explain the great havoc currently present in HA literature.

2.4.4. Structure and Biology of the Eukaryotic HYAL Enzymes

The first publications describing hyaluronidase activity appeared over 80 years ago [68-70]. Since then, over 9,000 articles have appeared. The Catalan biologist, Duran-Reynals F., discovered a "spreading factor" in testicular extracts that was only later identified as an enzyme [71].

Isolation and characterization of the enzymes that degrade HA in somatic tissues was accomplished only recently [72]. They had been long neglected [73] largely because of the many difficulties encountered in working with them. They occur in exceedingly small amounts, with very high but unstable specific activities. They require the constant presence of protease inhibitors and detergents during the purification procedures to maintain activity. Once this was understood, progress was rapid, and much information accumulated.

In humans, the hyaluronidases constitute an enzyme protein family with a high degree of sequence homology. There are six genes tightly clustered at two chromosomal locations with hyaluronidase-like sequences. The three genes, *HYAL1*, *HYAL2*, and *HYAL3* coding for HYAL1, HYAL2, and HYAL3, are on chromosome 3p21.3 [74, 75]. They are organized in an extraordinarily complex and overlapping manner in an area densely packed with transcribed genes [76]. An example of this complex packing is a sequence coding for an *N*-acetyltransferase partially imbedded in an intron of *HYAL3*. Polycistronic transcription and tissue co-expression of these genes occur, suggesting that some unknown but probably important physiology is occurring here.

Of the cluster on chromosome 3p, it is presumed that HYAL1 and HYAL2 constitute the major hyaluronidases of somatic tissues. HYAL2 is anchored to the plasma membrane by a glycosylphosphatidylinositol- (GPI-) link. HYAL2 cleaves high-molar-mass HA to a limit product of approximately 20 kDa [77, 78], while HYAL1 appears to be a lysosomal enzyme, cleaving HA predominantly into tetrasaccharides [71, 74, 75]. HYAL3 is widely expressed, but its function is unknown. There have been major difficulties in documenting its enzymatic activity [79, 80].

The three genes HYAL4, PHYAL1, and SPAM1 (Sperm Adhesion Molecule-1) are clustered in a similar fashion on chromosome 7q31.3, coding respectively for HYAL4, a pseudogene transcribed but not translated in the human, and PH-20. This chromosomal pattern is highly suggestive of two ancient gene duplications, followed by en masse block duplication of the resulting three genes.

PH-20 is the enzyme that facilitates penetration of sperm through the cumulus mass surrounding the ovum, and is also necessary for fertilization [81, 82]. It is a multifunctional enzyme protein, with a separate domain that binds to the

zona pellucida. With the more sensitive technique of polymerase chain reaction (PCR) analysis, PH-20 can be detected in other sites in the male reproductive tract [83], the female genital tract [84], as well as in certain malignancies [85, 86]. Curiously, most other species, other than *Homo sapiens* have a seventh *HYAL* sequence at that site. This may explain why the PH-20 knockout mouse is fertile [87].

The pseudogene, PHYAL1, contains an aberrant stop codon, and while not translated into active enzyme in the human, does appear to be translated in other species.

The hyaluronidases involved in HA catabolisms in somatic tissues are HYAL1 and HYAL2. These β-endoglycosidase enzymes initiate the breakdown of HA. Completion of HA degradation to individual sugars is assisted by the lysosomal exoglycosidases, β-glucuronidase and β-*N*-acetyl-glucosaminidase.

A problem that ultimately must be resolved is why very low or non-apparent enzymatic activities, using conventional assays, can be detected for HYAL3 or for HYAL4. Increasing and decreasing in levels of *HYAL3* and *HYAL4* transcription and translation have been reported in response to changing tissue culture conditions or to cytokines [88, 89] suggesting that they do have some as yet unknown, but probably important biological functions.

All of these enzymes must have functions other than their hyaluronidase activities. Just because we have named them enzymes does not indicate that they are only enzymes. Enzymes are blithely unaware of and indifferent to what we name them. They may have other functions, and perhaps some that are even more important. These enzymes and products of enzyme-like sequences may function as receptors, for example, as already documented [90], or as adhesion or even as anti-adhesion molecules. There is still much to be learned from the hyaluronidase gene family.

2.4.5. Putative HYAL Inhibitors

In early studies, inconsistencies in hyaluronidase data were attributed to the presence of hyaluronidase inhibitors. Several sporadic attempts have been made since then to identify such inhibitors [91, 92]. During purification of the HYALs from tissue extracts, the number of units of activity increases sharply after the initial steps, attributed to the separation of inhibitor from the enzyme. However, this continues to be an unexplored area of biology.

A major proviso must be inserted at this point. As aforementioned, HA degradation can occur by two mechanisms, by enzymatic reaction or by chemical scission. The proportion contributed by each mechanism is unknown. True inhibitors of the hyaluronidase enzyme reaction clearly exist. However antioxidants, reducing agents, and scavengers of free radicals can prevent chemical scission of HA chains. Such reagents can masquerade as hyaluronidase inhibitors. They do retard HA chain scission, but as it is difficult to distinguish between the two catabolic reactions, it is perhaps even more difficult to distinguish between the two classes of inhibitors. And once again, there is confusion in the literature as to what constitutes an inhibitor. Particularly among the weak inhibitors described from plant extracts, it may be that they function as anti-oxidants, rather than as enzyme inhibitors, or perhaps as both.

2.5. Hyaluronan Degradation by the Action of Reactive Oxygen Species

HA (as well as to a similar extent other GAGs, such as chondroitin sulfate and keratan sulfate) are susceptible to degradation by different ROS. This leads in particular to the diminution of the molar mass, i.e. to scissions of the glycosidic linkages. Here, we will focus primarily on ROS with *in vivo* relevance [18] whereas ROS that are exclusively generated under *in vitro* conditions will not be considered. Finally, neither degradation under acidic or alkaline conditions nor degradation induced by ultrasonics nor thermal degradation of hyaluronan will be considered. These degradation pathways were recently summarized [93].

Before details of HA degradation can be discussed, however, the (biological) pathways leading to ROS generation have to be introduced briefly. It should be noted that there is often confusion in the literature regarding the terms ROS and "free radicals" [18]. Each "free radical" is an ROS, however not every ROS is a free radical because these species are defined by the presence of an unpaired electron. HOCl and H_2O_2, for instance, are ROS, but they do not represent free radicals because they lack an unpaired electron. Therefore, the more general term ROS will be primarily used in this overview.

ROS are generated in different cell types under stress situations (for instance following bacterial invasions as well as with injuries) and many cells possess enzymes enabling them to produce ROS. However, as ROS are massively generated under inflammatory conditions, this review will focus on these conditions. Macrophages and particularly polymorphonuclear leukocytes (PMNs, also often called "neutrophils") primarily contribute to ROS generation under inflammatory conditions [94].

A rather simplified overview on the generation of the most important ROS, by e.g. neutrophils, is given in Fig. (**3**). The first step of this complex reaction pattern is catalyzed by the enzyme NADPH oxidase. This enzyme [95] catalyzes the reduction of molecular oxygen to superoxide anion radicals ($O_2^{\bullet-}$), whereby the required electrons are supplied from the oxidation of NADPH [96]:

$$NADPH + 2\ O_2 \rightarrow NADP^+ + H^+ + 2\ O_2^{\bullet-}$$

Please note that in addition to NADPH oxidase there are many further ways to generate $O_2^{\bullet-}$ under *in vivo* conditions [97]. Superoxide anion radicals dismutate either spontaneously or much faster in the presence of the enzyme superoxide dismutase into H_2O_2 and oxygen:

$$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 is the substrate for the enzyme myeloperoxidase (MPO) that is nearly exclusively localized in neutrophils and monocytes [98]. MPO, a heme peroxidase, exhibits a rather complex action pattern. Beside other reactions, MPO oxidizes (pseudo)halides to (pseudo)hypohalous acids, whereby the generation of hypochlorous acid (HOCl) and hypothiocyanite is particularly important in immune defense as well as in saliva, respectively [99].

Finally, hydrogen peroxide that exhibits *per se* rather low reactivity, may be converted into further much more reactive

oxygen species, particularly if low valent transition metal ions (such as Fe^{2+} or Cu^{+}) are present:

$$H_2O_2 + Fe^{2+} \rightarrow HO^- + HO^\bullet + Fe^{3+}$$

This is the famous "Fenton reaction." This reaction is particularly notorious because more than a hundred years after its first description, there is no complete agreement as to whether actually hydroxyl radicals or primarily perpheryl species are generated [100]. The role of pheryl and perpheryl species was reviewed comprehensively by Quian and Buettner [101]. This ongoing debate about the products of the Fenton reaction is one major reason why radiation chemistry is still widely used: under radiochemical conditions, the type and the yield of the generated radicals is highly defined [18].

Of course, reactions may also occur between the individual radicals leading to the generation of further ROS species, as for example, peroxynitrite (Fig. **3**). Since it is impossible to consider the potential role of all oxidants being formed by activated neutrophils or other cells, this review will focus on selected ROS species and their reactions with HA.

2.5.1. Superoxide Anion Radicals

Superoxide anion radicals ($O_2^{\bullet-}$) are in a pH-dependent equilibrium with the superoxide radical ($HO_2^\bullet$). However, as the pK_a value of this acid-base equilibrium is about 4.8 [102], $O_2^{\bullet-}$ is the only relevant species at physiological pH (7.4). Therefore, the term "superoxide anion radical" will be exclusively used throughout this paper.

Reactions of $O_2^{\bullet-}$ with many biological substrates were studied in detail by radiation chemists over many years and a comprehensive overview is given in [103]. Basically, the reactivity of $O_2^{\bullet-}$ is very low and to the best of our knowledge, there is so far no convincing evidence showing that $O_2^{\bullet-}$ *per se* (and not subsequent conversion into more reactive species) reacts with HA. In the majority of cases where reactions between HA and $O_2^{\bullet-}$ are postulated, the contribution of transition metal ions cannot be completely ruled out [104]. This holds particularly true if the reactivity of complex tissues or body fluids is analyzed, where no detailed analysis of the content of transition metals is made in advance. For instance, cartilage specimens [105] are incubated with $O_2^{\bullet-}$-generating systems, and the amount of uronic acids released into the supernatant is used as a measure of the induced deleterious effects. As scavengers of $O_2^{\bullet-}$ decreases the extent of uronic acid release, it is concluded that $O_2^{\bullet-}$ are responsible for the observed effects [105]. However, it is more likely that $O_2^{\bullet-}$ provides a source of other, more reactive ROS. There are reports that the degradation of HA can be strongly reduced if the activity of superoxide dismutase (SOD) is inhibited or if knock-out mice that are not capable of expressing SOD are investigated [106].

Despite their low reactivity, however, the simultaneous presence of $O_2^{\bullet-}$ enhances the deleterious effects of other ROS, such as HOCl, on HA. Although not completely understood, this finding is interpreted as HOCl and $O_2^{\bullet-}$ acting synergistically to induce fragmentation of HA [107].

2.5.2. Hydrogen Peroxide

Superoxide and superoxide anion radicals are known to dismutate to hydrogen peroxide and oxygen according to the reaction:

$$HO_2^\bullet + O_2^{\bullet-} + H^+ \rightarrow H_2O_2 + O_2 \text{ and}$$

$$HO_2^\bullet + HO_2^\bullet \rightarrow H_2O_2 + O_2$$

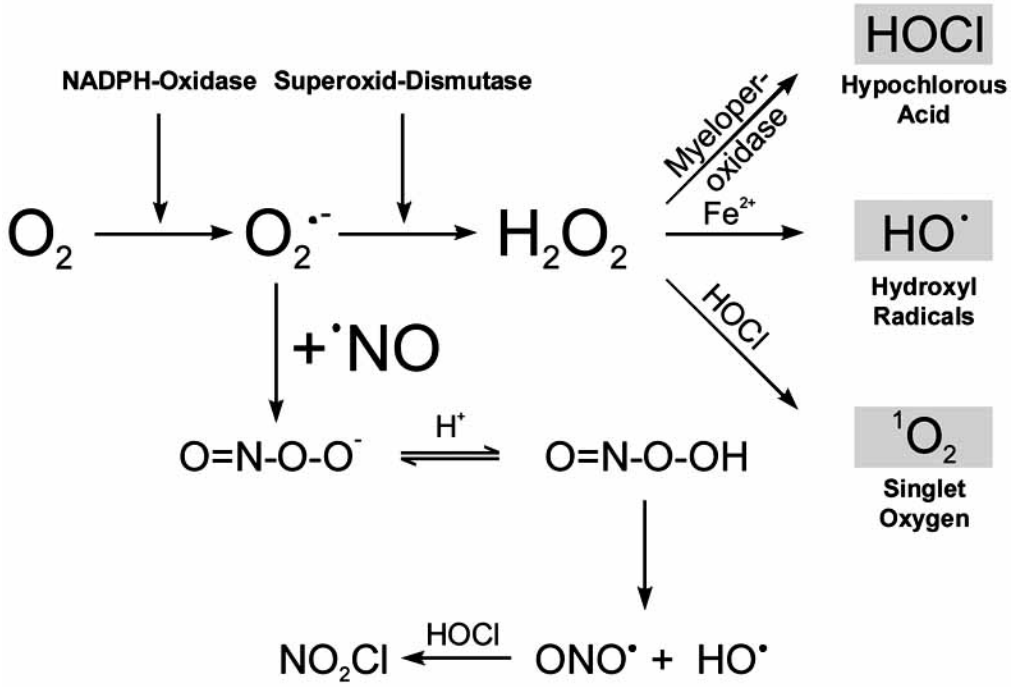

Fig. (3). Scheme of the generation of different reactive oxygen species. The enzymes that catalyze the individual reactions are also provided in the figure. Reprinted with permission from [18].

The second-order rate constant of the overall dismutation is about 2×10^5 M^{-1} s^{-1} at pH 7.4, but may be significantly enhanced in the presence of SOD by 3-4 orders of magnitude. Comparably, the reaction by which H_2O_2 is generated primarily, H_2O_2 production is inevitable if $O_2^{\bullet-}$ is generated in advance [93].

Similar to $O_2^{\bullet-}$, H_2O_2 also exhibits low reactivity. Although the standard reduction potential of the redox couple $H_2O_2/2H_2O$ is rather high with 1.32 V at pH 7.0 [108], H_2O_2 promotes only a few selected *in vivo* reactions due to electronic restrictions. The most important role of H_2O_2 is most probably to act as the source of more reactive oxygen species, for instance, by the above mentioned Fenton reaction [109, 110].

Accordingly, there are so far no reliable reports that absolutely pure H_2O_2 reacts with HA and the results obtained with complex tissue samples are not very reliable [111] because the contribution of transition metals cannot be completely excluded. Nevertheless, it is reported that high concentrations of H_2O_2 (in the hundred millimolar range) react with the PGs of cartilage. It is also reported that HA strands, but not CS or KS, are affected by H_2O_2 [112].

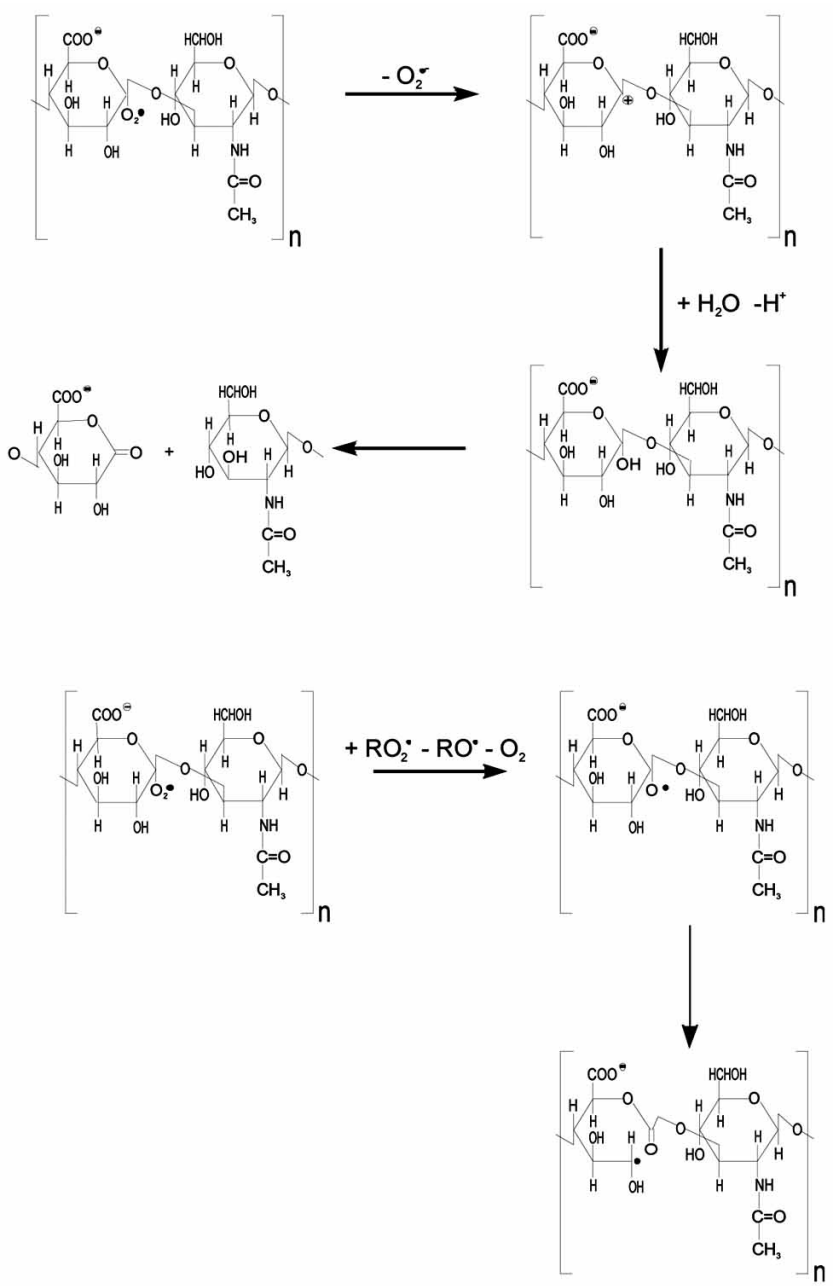

Fig. (4). Different pathways showing the degradation of hyaluronan under the influence of hydroxyl radicals. Reprinted with permission from [18].

The presence of transition metals is a particular problem if cartilage specimens are investigated, because this tissue binds multivalent cations like Fe^{2+} very efficiently due to its strong negative charge density [113]. It has been shown by means of NMR that the effect of pure H_2O_2 on cartilage tissue is very weak, but can be enhanced dramatically in the presence of ferrous ions [114].

Both findings, however, agree with the result of a former study: H_2O_2 inhibits the PG biosynthesis by interacting with the central atoms of different enzymes, but is unable to destroy native cartilage aggregates [115].

2.5.3. Hydroxyl Radicals

Hydroxyl radicals are the most reactive oxygen species on earth. Due to their high reactivity, they "live" only a few nanoseconds in a biological environment after being generated [116]. The standard reduction potential of the redox couple $HO^{\bullet}/H_2O$ is 2.31 V at pH 7.0 [108], representing the highest value ever found in biological systems. Thus, $HO^{\bullet}$ reacts unspecifically with all biomolecules with second-order rate constants of about 10^9-10^{10} M^{-1} s^{-1}, i.e. most reactions are diffusion-controlled [116] and hydroxyl radicals react with all targets passing by. The free diffusion pathway of hydroxyl radicals is, accordingly, just 5-10 times their molecular size [117].

Although in this review, reactions of $HO^{\bullet}$ in oxygenated solutions will be discussed exclusively, it should be noted that reactions also occur under anaerobic conditions as well as in the solid state. These effects were comprehensively investigated by using glucose as well as GlcNAc and GlcA, the monomeric constituents of HA, as model compounds. A detailed discussion of the radiation-induced fragmentation of common monosaccharides is available in the excellent book by von Sonntag [118].

The first step of the reaction between all carbohydrates and $HO^{\bullet}$ radicals is the abstraction of one hydrogen atom ($H^{\bullet}$) under the formation of the corresponding alkyl radical. This reaction is diffusion controlled [$k \sim 10^9$ M^{-1} s^{-1}]. In the next step, O_2 is added to the alkyl radicals under the formation of the corresponding peroxyl radicals. This reaction is also diffusion controlled [118]. In the case of HA [119], the formation of the initial peroxyl radicals (Fig. **4**) is followed by the elimination of $O_2^{\bullet-}$ or alkoxyl radicals and the formation of a large variety of open-chained products.

This is of course accompanied by a reduction of the molar mass that may be easily assessed by e.g. viscometry [120-122]. The mechanism shown in Fig. (**5**) holds equally if $HO^{\bullet}$ radicals are generated by water radiolysis or by the transition metal catalyzed decomposition of hydrogen peroxide. The complex mechanism of HA degradation induced by $HO^{\bullet}$ radicals was studied in detail by Hawkins and Davies and the interested reader is referred to this work [123].

2.5.4. Hypochlorous Acid

HOCl is generated under the influence of the enzyme myeloperoxidase (MPO) from hydrogen peroxide and chlo-

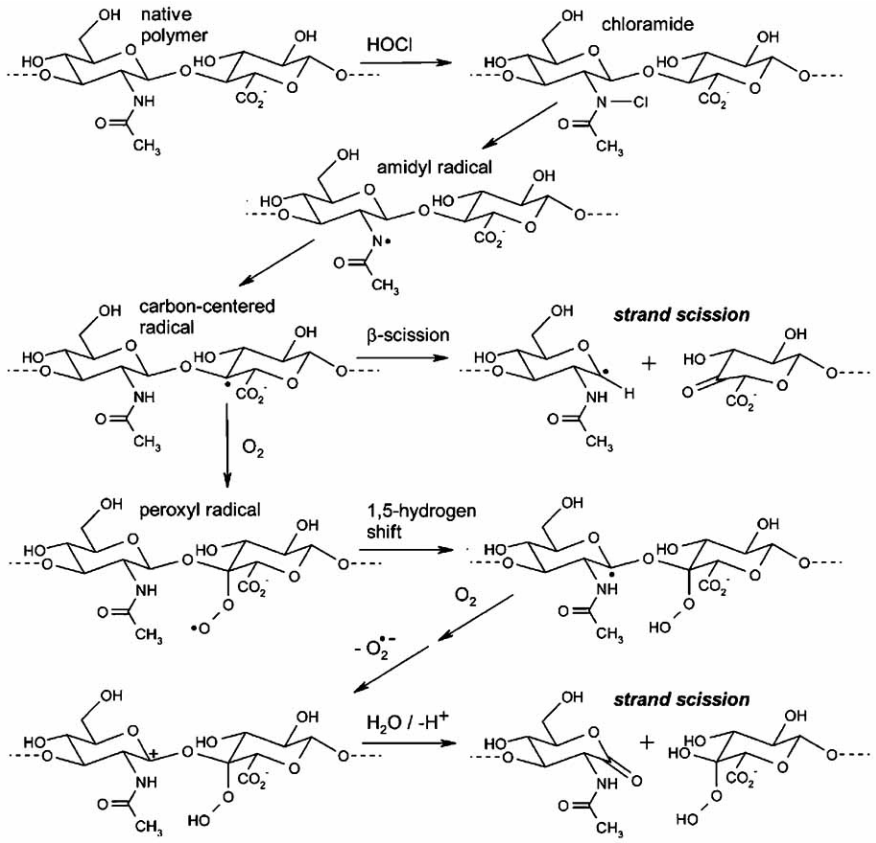

Fig. (5). Degradative pathway of HA initiated by HOCl action on the *N*-acetyl group. Reprinted with permission from [107].

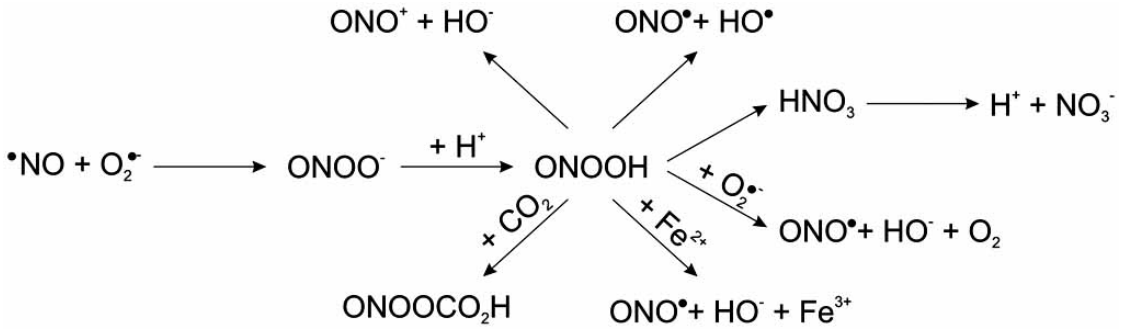

Fig. (6). *In vivo* generation of peroxynitrite ($ONOO^-$) as well as important decay mechanisms of this compound. For details see text.

ride anions. HOCl represents a very weak acid with a pK_a value of 7.53 [124]. Thus, nearly equimolar amounts of HOCl and ClO^- are present under physiological conditions, whereby the free acid, HOCl, is much more reactive than its salt.

It should be noted that MPO has strong cationic properties and is, therefore, positively charged at physiological pH. It is most likely that MPO is bound to negatively charged molecules such as HA as well as to additional GAGs. Therefore, HOCl production is most pronounced in the vicinity of acidic components. Accordingly, HOCl is regarded as a key event of tissue destruction during inflammation [111].

Two different reactions of HOCl with HA have been described so far (Fig. **5**). (a) HOCl reacts with the *N*-acetyl side chains and (b) causes a cleavage of the glycosidic bonds. The chloramide generated initially is a transient product and decomposes with the generation of acetate that can easily be identified by ^1H NMR spectroscopy [125], carbon-13 NMR spectroscopy [126], as well as by some additional methods. The proposed pathway was verified a few years later by the detection of carbon- as well as nitrogen-centered radicals by means of ESR spectroscopy [127] (Fig. **5**).

This pathway possesses considerable relevance since the same chlorinated products as well as elevated acetate levels are also detectable in the synovial fluids from patients with rheumatic diseases [128]. Additionally, a considerable diminution of the molar mass of HA under the influence of either HOCl or $MPO/H_2O_2/Cl^-$ can be detected in purified HA [129, 130] or HA in synovial fluids [131].

2.5.5. Nitric Oxide (•NO)

•NO is generated under *in vivo* conditions by nitric oxide synthase (NOS) that occurs in different forms and is present in many cells. Beside $O_2^{\bullet-}$, •NO belongs to the most important of the ROS produced by e.g. chondrocytes, and contributes to the generation of more reactive species [132].

Although •NO is much more established as a molecule with second messenger as well as regulatory functions [133], it is also shown that •NO is capable of reducing the viscosity of HA solutions in the presence but not in the absence of O_2, suggesting the involvement of Fenton chemistry [134]. This is further supported by the observation that pure •NO is a rather slow-reacting compound, whereas its reaction product with $O_2^{\bullet-}$, peroxynitrite ($ONOO^-$) is a very reactive compound.

2.5.6. Peroxynitrite ($ONOO^-$)

Peroxynitrite is generated under *in vivo* conditions by the diffusion-controlled reaction between superoxide anion radicals and nitric oxide [135, 136]:

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^-$$

$ONOO^-$ is a highly reactive compound and may easily decompose into a number of different species, the most important of which are summarized in Fig. (**6**).

Treatment of high-molar-mass HA with peroxynitrite [137, 138] unequivocally leads to a diminution of the molar mass of HA, to be seen in the differences in electrophoretic mobility as well as in the reduced viscosity [139]. From these data, a random cleavage mechanism is suggested [137]. Somewhat later [140], it was shown by more sophisticated kinetic data analyses that there is no bimolecular reaction between ONOOH and HA, but degradation is primarily caused by the unimolecular decay of peroxynitrite into additional ROS. However, no HA characteristic oxidation products may be detected by using MS or NMR spectroscopy [141]. However, it is commonly accepted nowadays that the effect of peroxynitrite on HA is mediated by radicals [138].

2.5.7. Singlet Oxygen

Singlet oxygen is generated under *in vivo* conditions by the reaction of HOCl with H_2O_2 and as a result of photooxidations, especially under the influence of UV light. The spontaneous dismutation of $O_2^{\bullet-}$ is discussed as another potential source for $^1O_2$. However, data on the later mechanism are contradictory, since singlet oxygen is efficiently quenched by $O_2^{\bullet-}$ [142].

$^1O_2$ is able to degrade polysaccharides as shown for HA by viscometry and circular dichroism (CD) [143]. Using ESR spectroscopy, four different radical species are observed, but it remains quite unclear to what extent HO• (generated as "by-products" under the utilized experimental conditions) might also be contributing to the observed effects [144].

3. PHYSIOLOGICAL ROLES OF HA AND IMPLICATIONS IN PATHOLOGICAL PROCESSES

3.1. Physiological Role of HA

HA is the major hydrodynamic non-protein component of joint synovial fluid (SF) and many tissues and organs, and its

unique visco-elastic properties confer remarkable shock absorbing and lubricating abilities to tissues and fluids. Simultaneously, its macromolecular size and hydrophilicity serve to retain fluid in the joint cavity during articulation. HA restricts the entry of large plasma proteins and cells into fluids but facilitates solute exchange between the synovial capillary-ies and cartilage and other joint tissues. In addition, HA can form a pericellular coat around cells, interact with proinflammatory mediators, and bind to cell receptors where it modulates cell proliferation, migration, and gene expression. All these physicochemical and biological properties of HA have been shown to be molar mass-dependent.

3.1.1. Functions Related to Physicochemical Properties of HA

The carboxyl groups of HA are fully ionized at extracellular pH and osmotic activity is very high in relation to HA molar mass. As a consequence, there are effects on the distribution and movement of water influencing water homeostasis. Secondary hydrogen bonds form along the axis of the polysaccharide, creating a twist in the chains, imparting some stiffness, and generating hydrophobic patches that permit association with other HA chains. Nonspecific interaction with cellular membranes and other lipid structures then may occur [145]. The stiffness of the HA polymers promotes an extended random-coil configuration ensuring the occupancy of enormous molecular domains. Alone or in conjunction with collagen fibers and other macromolecular elements of the extracellular matrix, this ultimately reduces the mobility of HA itself and determines its permeability to other substances, whether transported by diffusion or driven by hydrodynamic bulk flow.

The capacity of steric exclusion of other (macro) molecules is another attribute of the molecular meshwork generated by HA. For example, about 15% of the total water volume is unavailable to albumin at the normal HA content of synovial fluid [3]. The degree of exclusion increases with molecular size, and explains in part why the largest plasma proteins are reduced in the extravascular fluid space to an even greater degree than albumin.

None of the above-mentioned effects is restricted to HA, although they unequivocally make the major contribution to the structural properties and functions of extracellular matrix in many areas. Among these are synovial fluid, the renal medulla, and parts of the gut and other soft tissues where HA is the dominant GAG largely free from specific structural binding.

The most distinctive property of HA is its visco-elasticity in the hydrated state. Both the viscosity and the elasticity may vary with the rate of shear or oscillatory movement. For example, the viscosity of a 1% solution of HA, having a molar mass of $3\text{-}4\times10^6$ Da, is about 500000 times that of water at low shear rate, but can drop 1000-fold when forced through a fine needle. As a consequence, rapid movement reduces HA viscosity, which is reflected in the force required to overcome internal friction. This also increases the elasticity, which stores energy and permits recovery from deformation [3]. This phenomenon is familiar to physicians as "pitting" edema in normally soft subcutaneous tissues. The fluid is slowly displaced by sustained digital pressure and gradually returns upon release. After brief and rapid indentation to the same depth, the tissue immediately recovers its original form.

The anomalous viscosity of HA solutions suggests that it should be an ideal biological lubricant, at least by reducing the workload during rapid movements. HA is abundant in the fluids of synovial joints and tendon sheaths. It is also found in smaller amounts in the fluids of several "cavities", in the pleura, pericardium and peritoneum, and in less well-defined planes of tissue movement, such as those between muscle bundles and skin. Notably, it persists between individual fibers, spindles and septa in skeletal and cardiac muscle, but disappears with maturation in the slow-moving smooth muscle fibers of gut and vessel walls. The lubricant role of HA in the soft tissue lining of joints is well established, but its contribution to the hard weight-bearing cartilaginous surfaces is less clear. A very thin film can maintain separation of surfaces bearing a high static load [146], but reduction of friction on cartilage probably relies on a complex interplay of its surface with HA, utilizing a particular glycoprotein of synovial fluid, and phospholipid vesicles [147, 148].

Finally, both viscosity and elasticity properties are positively related, in a complex way, to molar mass and the concentration, a point that must be considered in the surgical and medical uses of various preparations of HA for viscoprotection and viscosupplementation (see below) [12].

3.1.2. HA is Required During Mammalian Embryogenesis

Gene targeting reveals for HA synthase-2 (HAS-2) an essential role during mid-gestation in the mouse [149]. This is related to the fact that the HAS-2 enzyme is the major source of HA during development. In fact, mid-gestational mouse embryos still possessing functional HAS-1 and HAS-3 genes but lacking HAS2 show just 3% of wild-type levels of HA. Thus, neither HAS1 nor HAS3 are sufficient to replace the requirement for HA, nor are they up-regulated in response to the absence of HA in such mice [150].

3.1.3. HA is Essential in Matrices to Expand the Extracellular Space

The mouse phenotype lacking HAS2 is remarkably similar to that of the heart defect mouse which results in embryonic lethality in the homozygous state [39]. Recent studies indicate that the expanded extracellular matrix present in the developing heart requires both versican, a HA binding PG, and HA for stabilization [150, 151]. Clearly, intermolecular interactions between proteins with HA binding motifs are essential in forming and stabilizing HA-rich matrices, comparable to the requirement for heavy chains of inter-alpha-inhibitor, in expansion of the HA-rich matrix in the cumulus oophorus [152]. Many other HA binding molecules are present in HA-rich matrices in the developing heart, such as fibulin, and, to date, their role in matrix deposition or stabilization is not known.

3.1.4. HA Role in Stimulation of Intracellular Signaling Pathways Resulting in Cell Migration and Invasion

Studies of tissues genetically deficient in HA derived from the HAS-2 null mouse embryo have produced particularly informative and unambiguous insights into the role of HA in cell migration and transformation [39]. In fact, under-

standing the role of HA is complicated by it being so ubiquitous, together with the possible activity of hyaluronidases in HA removal. The resulting oligomer degradation products have major pro-inflammatory activities [153]. Cells in HA-rich matrices, particularly in embryonic tissues, often exhibit marked migratory activity. This is historically attributed to the ease of penetrating a hydrated HA-rich matrix. However, a complementary mechanism is suggested by the capacity of HA to activate intracellular signaling pathways stimulating cell migration and invasion [154]. For example, the embryonic heart is extensively invaded and remodeled by migrating cells. Furthermore, exogenous HA stimulates cell migration during cardiac development [155, 156] and exogenous hyaluronidase affects ventricular function *ex vivo* [157]. Because atrioventricular septal defects are the most common sporadic congenital heart defect [158], considerable interest has also been focused on the molecular mechanisms of cushion morphogenesis [159].

The programmed transformation of endothelial cells to mesenchyme in atrioventricular canal explants is totally deficient in HAS-2 null embryos [39], and this defect is specifically attributed to HA. Thus, HA is required for two pivotal developmental events in the heart: matrix expansion and initiation of cell migration [39]. Because activity of the G protein Ras is implicated in atrioventricular transformation, signaling pathways resulting in Ras activation normally triggered by HA might be deficient in the HAS-2 null embryo [39]. In fact, one well characterized signaling pathway resulting in Ras activation and involving HA occurs *via* a family of receptor tyrosine kinases (ErbB) [154].

Finally, in the presence of HA, endothelial organization and migration are intact and this places ErbB and Ras activation at the stage of epithelial-mesenchymal transformation. This is termed "activation," characterized by cellular shape change and loss of cadherin-mediated junctions, and "invasion", when the activated endothelial cells adopt a mesenchymal phenotype, invade the subjacent cardiac jelly, and activate a mesenchymal gene expression profile for migration [39].

3.1.5. HA in Ovulation and Fertilization

Soluble factors produced by cumulus cells during the preovulatory period are essential for the oocyte to acquire the ability to be fertilized and to sustain normal embryonic development [160]. When the ovulatory gonadotrophin surge occurs, cumulus cells retract their cytoplasmic projections and lose intercellular contact with each other and with the oocyte. However, they are subsequently embedded in the HA network and remain closely associated with the oocytes. Several studies suggest that HA is present in the outer third of the mouse and hamster zona pellucida, and even in the perivitelline space of opossum, pig and human oocytes [161].

The accumulation of HA creates a spongy, elastic and reversibly deformable matrix that facilitates the extrusion of the oocyte at ovulation. When the follicle wall is ruptured, the expanded cumulus cell-oocyte complex deforms considerably [162], facilitating its capture by the fimbria of the oviduct and its transport to the site of fertilization.

The extracellular matrix of the ovulated cumulus cell-oocyte complex may present a physiological barrier for penetration by functionally or enzymatically deficient spermatozoa. In fact, a good correlation has been observed between the ability of spermatozoa to penetrate a highly viscous solution of HA and both sperm motility and fertilization efficiency [163]. Therefore, this procedure is used in clinics for sperm preparation or evaluation of functional sperm competence. The extracellular matrix of the cumulus oophorus is more complex than a simple HA solution, with specific molecules that link HA strands and limit their extension. In spite of this, spermatozoa take only about 2 min to pass through the cumulus cell layer and only a few seconds to penetrate the zona pellucida [161]. This must largely depend on the hyaluronidase activity of a GPI-anchored protein, namely PH-20, which is present on the plasma membrane of the sperm head [164].

By the above-illustrated observations, it is now clear that HA participates in many steps during the process of ovarian development, ovulation, and fertilization.

3.2. Intracellular HA as Mediator of Inflammatory Processes

HA was isolated for the first time in rat brain nuclei where it accounted for nearly 1/3 of the total GAGs [165]. This was a surprising finding since HA was thought to be present only as an extracellular macromolecule, because of its expansive, large size, and its polyanionic properties. Several further studies supported the hypothesis of intracellular HA with the identification of intracellular HA-binding proteins (IHABPs), including RHAMM, P32, CDC37 and IHABP4 [166, 169].

3.2.1. HA and Mitosis

Evanko and Wight show that HA accumulates intracellularly in the perinuclear region of aortic smooth muscle cells during premitotic and mitotic stages [170]. Furthermore, HA accumulates at the mitotic spindle suggesting associations with intracellular structures. At this time, cells also formed an extended pericellular matrix to enable cell detachment and rounding, and for mitosis. More recently, the intracellular hyaladherin RHAMM has been found to maintain spindle pole stability [171]. Intracellular HA may provide a more compliant milieu for facilitating the process of nuclei separation and subsequent cell division, and it may also influence RHAMM function in maintaining spindle pole stability.

3.2.2. Endoplasmic Reticulum Stress may Induce the Production of the HA Cables

When the demands on the ER exceed its capacity, cells can undergo ER stress and in an effort to restore homeostasis, cells selectively increase the expression of numerous ER-resident proteins, including molecular chaperones, and proteases involved in protein degradation [172-174]. Furthermore, there is a global inhibition of protein synthesis to reduce the workload of the ER, and apoptosis can also occur. Recent studies have indicated that ER stress contributes to the pathogenesis of chronic inflammatory conditions [5]. Several factors, such as tunicamycin, an inhibitor of *N*-linked glycosylation of proteins, thapsigargin, an inhibitor of a sarcoplasmic reticulum ATPase, and A23187, a calcium iono-

phore, are able to induce ER stress in smooth muscle cell cultures and also activate the production of the HA cable structures. Furthermore, cycloheximide, a potent inhibitor of protein synthesis, also initiates the production of HA cables [5]. This indicates that the response is not likely to be dependent upon initiating new protein synthesis, such as up-regulation of the relevant HA synthase.

3.2.3. HA Promotes Adhesion of Monocytes In Vitro

HA cable structures can be formed from the coalescence of smaller HA strands, often arising from different cells, to which monocytes tightly adhere. However, they do not adhere to HA patches, indicating that the cables have unique structures that are somehow recognized by non-activated monocytes [5]. These results indicate that cells respond to various conditions, such as ER stress, cycloheximide treatment, viral infection, and exposure to double-stranded RNA, by producing HA cable structures that bind leukocytes. A common consequence of all these conditions is an overall inhibition of protein synthesis. When protein synthesis is attenuated, a non-protein moiety such as HA would be an ideal "signal" for labeling cells as "stressed". Moreover, it is likely that HA synthase(s) in conjunction with appropriate ancillary proteins serve as an alarm system that cells may employ when protein synthesis is diminished. The resulting HA can be packaged into structures that serve as a distress signal engaging leukocytes recruited into the tissue after an inflammatory stimulus.

3.2.4. HA in Inflammatory Processes

A pronounced filamentous-appearing HA matrix in the interstitial connective tissue between the gastro-intestinal crypts, normally not present, has been observed in a relatively moderate stage of inflammation from the colon of a Crohn's patient [5]. This matrix is infiltrated with closely apposed inflammatory cells providing strong evidence that the adhesion and activation processes observed *in vitro* have direct relevance for these inflammatory processes. A correlation between HA production and a marker for ER stress in inflammatory responses *in vivo* was also found [5]. These results show the importance of two fundamental processes that require CD44: (a) phagocytosis to remove the new HA-based matrix synthesized by cells in response to the noxious agents [175], and (b) signals to stop both the production of more matrices the resident cells and the recruitment from the vasculature of more macrophages into the tissue.

3.3. HA in Cancer

The formation of metastasis, the growth and spread of malignant cells from solid tumors to distant sites, is dependent upon the degree of neovascularization provided by host blood vessels. Without such blood vessels, solid tumors fail to invade tissues or metastasize, and often die [176, 177]. The degree of tumor neovascularization is associated with metastasis and clinical prognosis [178, 179]. Prevention of tumor neovascularization may be beneficial in reducing or preventing tumor spread, and may aid in clinical prognosis [180, 181].

3.3.1. Tumor Neovascularization

The cellular mechanisms involved in tumor neovascularization appear to be similar to other angiogenic processes involving (a) the dilation of existing blood vessels, (b) local activation of endothelial cells, (c) local disintegration of the basal lamina, (d) migration of endothelial cells in the direction of the angiogenic signal, (e) formation of solid cords, (f) proliferation of endothelial cells in the middle zone of cords, (g) formation of a lumen, (h) formation of capillary loops, (i) appearance of pericytes around new capillaries, and (j) development and deposition of a new basal lamina [176, 177, 182]. The regulatory steps in this complex sequence of events have not been fully determined, but there is strong evidence that a cascade is occurring and that modification of any one of these steps is sufficient to prevent neovascularization [177].

Blood vessels are generally quiescent, and the stimulus to undergo neovascularization is likely to come from an exogenous source: Most probably, it is tumor-cell-derived or tumor-cell-induced, and acts specifically upon endothelial cells. Furthermore, tumor fragments have been shown to be angiogenic and to induce a capillary network within 48 to 72 hours [183] even if tumor cells may not be the only source of angiogenic signals within a tumor.

3.3.2. Mediators of Angiogenesis

Several molecules are able to stimulate angiogenesis under normal and pathological conditions including vascular endothelial-cell growth factor (VEGF), the family of fibroblast growth factors (FGF), platelet-derived endothelial-cell growth factor (PD-ECGF), the family of TGF, TNFα, prostaglandins, angiogenins, as well as ECM macromolecules themselves, including proteoglycans and glycosaminoglycans [177, 180, 184].

3.3.3. HA is an Angiogenic Factor

HA plays a dual role in angiogenesis [185]. High-molar-mass HA has been implicated in the differentiation and migration of many cell types and is thought to attract progenitor cells to sites of differentiation. Native HA also inhibits blood-vessel invasion. In contrast, degradation products of HA, i.e. oligosaccharides composed of 3 to 10 disaccharide units, are able to stimulate angiogenesis [186, 187]. These HA oligosaccharides specifically act on endothelial cells *in vitro,* where they stimulate cell proliferation and migration, the two key events associated with the formation of capillary sprouts [185].

In a number of invasive tumors, neovascularization occurs adjacent to a region of desmoplasia very rich in HA. Some studies have demonstrated that the levels of HA increase dramatically in the extracellular matrix of human breast tissue during carcinoma infiltration [188]. HA is believed to account for up to 40% of total GAGs in breast-carcinoma samples, and increased levels of GAG have also been demonstrated in colon carcinoma [189]. Furthermore, the degree of invasiveness and metastasis of some tumors has been specifically linked to elevated levels of HA [185]. Finally, increased HA levels are also found to be associated with angiogenesis in non-tumor situations, such as during fetal development in which a 10-fold increase in HA levels of endothelial cells has been observed [190].

An important consequence of HA oligosaccharide exposure to cells may be their ability to stimulate type-I and type-VIII collagen synthesis [185]. As it is well known, collagens

are involved in angiogenesis, and metabolic reduction of type-I and type-IV collagen synthesis inhibits capillary formation [191].

The action of any angiogenic mediator is likely to work *via* cell-surface receptors. HA is known to bind to specific cell-surface receptors, and one of these is CD44 [192]. Non-invasive bladder-carcinoma cell line HU-456 has low expression of HA receptors, whereas, on the contrary, highly invasive cells express many HA receptors [185]. Thus, the capacity of endothelial cells to bind HA may be an important first step in tumor invasiveness.

How the message initiated by cell-surface binding of HA is transmitted to the cell nucleus to produce an angiogenic response by endothelial cells is not yet well understood. HA oligosaccharides rapidly initiate the transient expression of several immediate early response genes, including *c-fos, c-jun* and *jun*-B [185]. On the contrary, high-molar-mass HA does not induce such an expression of response genes, but it is able to inhibit HA-oligosaccharide-induced gene expression in a dose-dependent manner. Probably, both types of ligands compete for the same receptor.

Early response genes are transcription factors and cellular oncogenes playing important regulatory roles in normal cell proliferation. They are also thought to be involved in the uncontrolled proliferation observed in tumorigenesis. The products of *c-jun, jun*-B and *c-fos* and related genes are DNA-binding proteins that associate to form a complex which binds to the same DNA-binding site, such as activator protein 1, a transcription factor massively involved in the initiation of cell proliferation [193]. In addition, *c-fos, c-jun* or *jun*-B play an important role in the initiation of extracellular-matrix degradation prior to cell migration as well as initiating cell proliferation.

Tumor growth and metastasis is a highly dynamic situation wherein extracellular matrix molecules are continuously synthesized, degraded, and replaced. As previously discussed, the leading edge of some invasive tumors is rich in high-molar-mass HA which may aid cellular expansion and invasion [185]. Since the angiogenic nature of HA is available only after partial degradation, it is unlikely that neovascularization and HA degradation will occur at the leading edge of these tumors. In fact, an examination of the new capillaries clearly indicates that the blood vessels extend from parent vessels towards the centre of the tumor mass, not towards the leading edge [194]. This observation suggests that high or low HA content need not necessarily correlate with tumor metastasis. It is more likely that metastasis will correspond with the molar mass of the extracellular matrix components and their subsequent effects on cellular migration, proliferation and differentiation [185]. Tumor growth and metastasis may occur when the leading edge invades new tissue with subsequent synthesis of new HA, and the high-molar-mass HA left behind is degraded, collagen synthesis is up-regulated, and neovascularization is initiated.

3.3.4. Mechanisms of Hyaluronan Accumulation in Cancer

To date, little is known about the relative importance of factors that determine the content of HA in malignant tissues, but some of the hypotheses are illustrated.

HA synthesis correlates with the level of HAS mRNA, suggesting that transcriptional regulation is an important determinant of the net HAS activity [195]. Furthermore, HAS expression is often increased by several growth factors like EGF, keratinocyte growth factor (KGF), PDGF [196], and both growth factors and their receptors are often over-expressed in cancers. Cancer cells may stimulate the adjacent stromal cells to produce a new HA rich tissue structure favorable for tumor growth [197, 198], and stromal cells secrete factors that enhance cancer cell migration into the new matrix.

As is well known, the rate of HA catabolism depends on multiple reactions that occur sequentially and in parallel with each other. The catabolism of native, high-molar-mass HA can be triggered by a few cleavages induced by hyaluronidases [199] or ROS (see subchapter 2.5). Slightly fragmented HA either becomes endocytosed and completely degraded by local cells [200] or diffuses into lymph vessels which carry it to specific, high capacity uptake receptors in the lymph nodes and liver endothelial cells [73]. High levels of ROS, such as superoxide anion radicals and $^{\bullet}NO$ can combine to form peroxynitrite, a powerful oxidant that attacks various macromolecules (see subchapter 2.5). For example, in breast cancer cells, protein nitrotyrosines, a signature of peroxynitrite action, is inversely correlated with cell-associated HA [195], suggesting that peroxynitrite may enhance the turnover of cell surface HA.

As described above, tumor stroma is an unusual type of connective tissue with HA accumulation as a specific feature [195]. Recent analyses of the stromal cells have revealed a novel alternative to explain its distinct nature. Several chromosomal probes revealed a frequent loss of heterozygosis in the stromal cells adjacent to the malignant tumor epithelium [201]. Therefore, the accumulation of HA may not only be due to stimulation of the normal stroma, but reflect the existence of a novel, transformed connective tissue that serves the needs of the tumor. The exact origin of these genetically altered stromal cells remains uncertain.

4. BIOLOGICAL AND BIOTECHNOLOGICAL SOURCES OF EXPERIMENTALLY USED HA

A comprehensive overview of the different sources from which HA can be isolated for biological and biotechnological purposes and the contribution of potential impurities was published recently [202]. The most relevant sources of HA and the estimated contents of HA are listed in Table **1**.

4.1. Biosynthesis of HA in Vertebrates and Bacteria

HA is synthesized at the inner plasma membrane from "activated" UDP-D-glucuronic acid and UDP-*N*-acetyl-D-glucosamine [9]. Both are connected with each other at the reducing end of the growing chain. This process is catalyzed by a membrane-located enzyme, the HA-synthases [31]. The rate of HA synthesis is most marked in dermal fibroblasts and epidermal keratinocytes.

The involved enzymes are very similar, i.e. the microbial HA-synthase shows a marked homology with the HA-synthase of vertebrates [203]. This is often explained by a lateral gene-exchange from the animal host to the bacterium.

However, a convergent development with typical bacterial enzymes as alginate synthase is also discussed.

4.2. Isolation of HA from Biological Sources

The current worldwide market for HA is estimated to be of the order of more than one billion USD due to its vast and even increasing applications in cosmetics, medicine, and many other fields [204]. As indicated above, bovine eyeballs, rooster comb, umbilical cord and connective tissues such as skin or cartilage are particularly rich in HA. The high HA content of skin is, for instance, also important for leather manufacturers but HA is actually unwelcome in this case. In fact, HA must be carefully removed from crude tissue by several washing steps. Without this, leather would be susceptible to drying and would become hard and brittle.

If HA is sampled from animal material, e.g. the rooster comb that is available in huge amounts as a waste material in slaughterhouses, it is very important that bacteria growth is carefully prevented during collecting, transport and extraction of the tissue. This is a rather challenging task and one of the reasons why HA isolated from tissues may have an unexpectedly low molar mass [205]. Despite these problems, a lot of HA is still sampled from such biological sources (particularly from rooster combs but also from bovine eyes and shark skin), although this tendency is currently on the decrease. In the past this material was even used for medical applications, however, only subsequent to considerable purification (see below) [206].

In a few pioneer studies, Balazs [207] suggested the use of formalin, glutaraldehyde and glyoxal as preserving additives during the sampling and purification process of HA. In addition to their direct preserving effects, these aldehydes lead to cross-linking of the proteins that are still present in the crude HA preparations or even bound to the HA. This results in rather high-molar-mass preparations. For instance, "Hylan A" is of the order of 4-10 MDa, whereas "Healon" (Pharmacia & Upjohn, Inc., Peapack, NJ) has a mean molar mass of about 2.5 MDa. The yield of HA per kilogram of rooster comb is about 3 g, i.e. about 0.3 % of the initial wet weight.

Although the purification of HA as explained above has been approved by the Food and Drug Administration (FDA), HA isolated from tissues often contains residual proteins that may easily lead to immune reactions if used for medical purposes. Therefore, the so-called "Non-Inflammatory Fraction of Na-Hyaluronate (NIF-NaHA)" [207] has to be additionally purified. Similarly, a residual content of DNA may lead to the induction of proinflammatory cytokines [208]. Therefore, obtaining very pure HA from these animal tissue sources is rather difficult and, thus, the use of animal sources for sampling HA is in continuous decline [205].

4.3. HA Manufacturing by Biotechnology

There are some pathogenic bacteria with a so-called HA-capsule and the HA secreted by microorganisms such as certain attenuated strains of *Streptococcus zooepidemicus*, *S. equi*, etc. is currently offered by many companies, with production of up to several tons per year. The HA-capsule was described for the first time in 1937 by Kendall and Heidelberger [209] upon their investigation of mucoid *Streptococci*. These bacteria comprise, for instance, *Streptococcus pyogenes* that are human pathogens as well as *Pasteurella multocida* that are animal pathogens.

Bacteria form a HA capsule that represents a kind of a "mimetic tool" due to the incapacity of the immune system of the host to differentiate encapsulated *Streptococci* from body cells covered with a matrix of HA. Therefore, the HA capsule may be regarded as protection of the bacteria against phagocytosis [210]. It is also suggested that the HA capsule enhances the mobility of the microorganism through the extracellular matrix of the host, due to its physicochemical properties. Finally, it may also be possible that the HA capsule enhances the adhesion and, therefore, the colonization of the host by *Streptococci* by selective binding to host-specific HA-binding proteins.

The HA capsule was initially exclusively of scientific interest [211] but its commercial interest dramatically increased with the increase in the number of applications. For about 20 years, much of the required HA was obtained from the HA capsule of bacterial strains in a special fermentation process. This HA is also often termed "Non-Animal-Source-Hyaluronan" (NASHs) or "fermentative HA" and is identical to the HA fraction obtained from vertebrate tissues but free from harmful substances such as hepatitis, HIV- or bovine spongiform encephalopathy (BSE)-pathogens. The separation of simultaneously generated lipoteichonic acid and polyanionic nucleic acids from the HA is only a minor problem. The HA isolated from these sources possesses molar masses in the range of several MDa [212]. Nevertheless, the risk of mutation of the bacterial strains, possible co-production of various toxins, pyrogens, immunogens, etc., hampers the broader application of fermentative HA in clinical practice. This is also the reason why HA samples originating from rooster combs are still currently preferred for human treatment, in cases when the HA material is designated for injection, e.g., into the knee joint.

Although there are increasing indications that the production of high-molar-mass HA might also be possible from a recombinant (gram-negative) *Escherichia coli* strain [http://www.hyalose.com], *S. equi subspez. zooepidemicus* is today most often used for the production of HA. These bacteria are cultivated under sterile conditions at 36°- 40°C and HA is formed, even under completely anaerobic conditions.

Rather typical of these bacteria is the production of about 30-80 g/l lactic acid accompanied by about 3-6 g/l HA. The achievable yield of HA depends on the requested molecular sizes and the yield of total HA decreases if a higher molar mass is needed. It is very important that the simultaneously generated lactic acid (which is very toxic for the bacteria) is neutralized by the addition of strong bases. The time of fermentation is limited because the increasing content of HA leads to an increased viscosity that compromises the supply of nutrients to the bacteria.

The above-mentioned HA capsule is initially bound to the bacteria. At later phases, however, the capsule is removed from the cells and afterwards freely floating in solution. Using the described fermentation process, molar masses of HA of about 0.5-2.5 MDa can be obtained and the yield of

HA correlates closely with the time of fermentation. Very weak degradation of HA is observed in the stationary growth phase that correlates with very small activities of hyaluronate lyase.

4.4. Purification of Microbial HA

Subsequent to cultivation, the highly viscous culture media are diluted with water and the bacteria removed by filtration or centrifugation. The remaining HA-containing solution is afterwards subjected to several purification and concentrating steps, whereby contaminating proteins are normally removed by protease treatments, followed by ultrafiltration or dialysis, using molar-mass cut-offs of the applied membrane in the order of 20-100 kDa, in order to remove the proteins but to retain all the HA.

Afterwards, the HA is normally precipitated from the aqueous solution with solvents (miscible with water) such as ethanol, acetone or isopropanol. Alternatively, polyelectrolyte complexes containing quarternary pyridinium or ammonium salts as well as cationic tensides may also be used. The precipitated HA is subsequently removed by filtration and dried in a vacuum or lyophilized. During the complete process, great care must be taken to avoid degradation of the HA. In particular, the dissolved HA macromolecules must avoid getting in contact with metals, or be subjected to shear stress that might result in diminution of the molar mass. If sufficient care is taken, about 75-80% of the initially formed HA may be recovered.

5. BIOMEDICAL APPLICATIONS OF HA AND DERIVATIVES

5.1. Viscosurgery and Viscoprotection

The concept and early experiments using visco-elastic HA solution, as well as the development of the first purified preparation to replace the vitreous body after surgery and its use as a "soft tool" to manipulate the retina in retinal detachment surgery or its use as a visco-elastic protector of the corneal endothelium in corneal transplantation, were the work of Balazs [12]. Together with co-investigators and a group of eye surgeons, he carried out the first clinical trials in the late 1960s and early 1970s. By the end of the 1970s, the use of the highly purified NIF-NaHA (Healon®; manufactured by Biotrics, Inc., Arlington, MA and later by Pharmacia AB, Uppsala, Sweden) was well established [207]. In the early 1980s this product was launched on the US market [AT/BF/Healon® Monograph-89-03-16, Pharmacia, Uppsala, Sweden]. Healon®, which brought about the beginning of viscosurgery, has been classified as one of the most versatile soft instruments in ophthalmology.

5.1.1. Viscosurgery

The main requirements for a solution to be used intraocularly at surgical interventions can be classified as follows (see also Table **2**): a) high viscosity at low shear rate to maintain space and manipulate tissues, b) moderate viscosity at medium shear rates to allow easy manipulation of surgical instruments and intraocular lenses within the polymer solution, c) very low viscosity at high shear rates to minimize the pressure needed to expel the solution through a thin cannula, d) high degree of elasticity to protect intraocular tissues, especially the endothelial cells of the cornea, from contact with surgical instruments, and e) the pH and osmolality within the ranges 6.5-8.5 and 200-400 mOsm.

Along with Pharmacia AB, the company that introduced several Healon® formulations, other companies are at present marketing various HA solutions, which compete for the attention of eye surgeons. Today, the extraction and/or replacement of a damaged lens can be classified as a routine ophthalmologic intervention (Fig. **7**). Damage to ocular tissues might be hereditary-based or even occur during different pathophysiological events. For example, cataract accounts for approximately 42% of all blindnesses and, due to the alarming prevalence of *Diabetes mellitus* worldwide, the diabetic cataract attracts ever-increasing attention [213, 214].

Since 1979 when Healon® was first introduced into human ophthalmology, it has been applied in many millions of eyes [Healon® (sodium hyaluronate) – Technical Information and Clinical Experience. Brochure 98-619, Pharmacia, Uppsala, Sweden]. Today Healon® is used as a soft surgical instrument for cataract extraction, intraocular lens (IOL) implantation, keratoplasty, glaucoma surgery, trauma and posterior segment surgery throughout the world.

5.1.2. Viscoprotection

The highly visco-elastic HA, Healon®, available for therapeutic use has also proved to be very efficient in alleviating discomfort in "dry eye syndrome". Although HA is not present in tears, in many aspects sodium hyaluronate is similar to mucin, a major component of tears. Mucin with a mean molar mass of about 2 MDa shows, similarly to HA, typical visco-elastic and shear-thinning behavior. This glycoprotein plays an important role in the lubricating, cleansing, and water-retaining properties of tears. The usefulness of an HA solution as a tear substitute resides in its water-entrapment capacity (hydration) and its function as a visco-elastic barrier between the corneal and conjunctival epithelia and noxious environmental factors (dust, smoke, etc.) [12] (Table **2**). During eye blinking, the HA eye drops are elastically deformed but not removed from the surface of the eye with blinking movements (the normal blinking frequency is about 12 blinks per minute).

Table 2. Concentrations and Mean Molar Masses of HA Products Used in Ophthalmologic Applications

Medical use	Concentration (mg/ml)	Molar mass (MDa)
Eye surgery – *viscosurgery*	10-30	0.5-4 (0.1-7)[a]
Tear substitute – *viscoprotection*	1-2	2-4

[a]See [2].

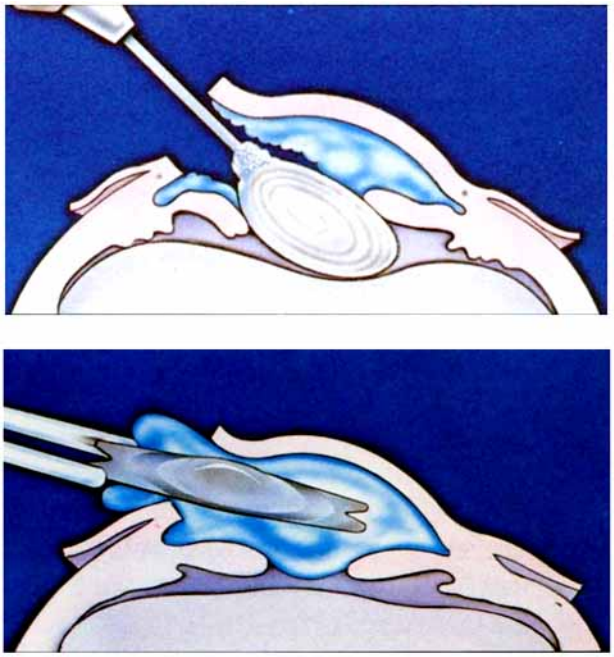

Fig. (7). The use of Healon® during viscosurgery creates and maintains a deep anterior chamber, which provides adequate space for surgical manipulation during extraction of cataractous lens (upper panel) as well as during IOL implantation (lower panel).

5.2. Viscosupplementation

Osteoarthritis (OA) is one of the most common forms of arthritic diseases. It affects more than 10% of the world population. OA occurrence is progressive with age and, if not cured, it may result in severe disabilities. It has been claimed that 50% of people over 65 years of age has evidence of OA of the knee and nearly 100% of the over-75 population report changes in at least one joint [18 and references cited therein].

Non-pharmacological treatments are focused on patient educational/self-management programs, which include e.g. exercises, weight loss, etc. [215]. Pharmacological therapies are briefly outlined in Table **3**. While the majority of orally and topically applied drugs primarily suppress symptoms such as pain, glucosamine and/or chondroitin sulfate are believed to very probably support the reconstitution of the damaged cartilage matrix.

The idea of intraarticular application of HA has been supported by the fact that the synovial fluid in OA joints lacks sufficient shock absorption and lubrication properties mostly due to the presence of HA of low mean molar mass, and an insufficient amount of viscous high-molar-mass HA [216]. Thus, the so-called "viscosupplementation", a series of HA injections, has been designed to change the character of the joint fluid. Although the mechanism of action of HA injection(s) into the OA (knee) joints is not yet fully established (see subchapter 5.6), it is claimed that viscosupplemented high-molar-mass HA increases the joint fluid viscoelasticity, stimulates the production of endogenous HA, inhibits the effects of inflammatory mediators, decreases cartilage degradation, and promotes cartilage matrix synthesis.

Briefly, the term viscosupplementation relates to a procedure that involves the injection of gel-like HA-based substance(s) into the joint to supplement the viscous properties of SF. It is a well known fact that under healthy/physiological conditions at low strain rates, SF behaves as a viscous fluid while at high strain rates, HA macromolecules become entangled, which causes them to behave more like an elastic solid matter.

Viscosupplementation has been studied for a possible OA treatment since the 1970s. Currently, HA injections (sodium HA solutions) are approved for the treatment of OA of the knee in those patients who have persistent pain or are unable to tolerate conservative treatment or joint replacement. HA solutions are considered as treatments or therapies, not drugs. Viscosupplementation for other joints (e.g. shoulder) is currently being investigated.

To date, five FDA approved HA-based products have been marketed in the United States. They are Hyalgan® and Synvisc® both approved in 1997, Supartz®, in 2001, and Orthovisc® and Euflexxa® approved in 2004. While Hyalgan® and Supartz® injection treatment is applied once weekly for 5 weeks in total, Orthovisc® is administered for a maximum of 4 weeks, and Synvisc® and Euflexxa® formulations are applied once weekly for 3 weeks. The main component of all injections is an ultra-pure high-molar-mass HA. Synvisc® along with HA contains also a Hylan®-type hydrogel component, i.e. chemically cross-linked HA. Thus, the Synvisc® injection formulation (synonym "Hylan G-F 20") represents a mixture of 80% HA fluid with 20% Hylan® gel [12].

Viscosupplementation appears to have a slower onset of action than intra-articularly injected steroids, however, the effect seems to last longer: the effects of HA appear to begin after 4 to 12 weeks and last up to one year. Intraarticular injections of a high-molar-mass HA results in significant decrease of pain accompanied by improved joint function. Beneficial effects have been found to last much longer than the residence time of the applied HA [12] suggesting that intraarticularly applied viscosupplementing material may stimulate synthesis of natural biopolymers [217]. Contraindications to intraarticular HA include joint or skin infection

Table 3. Medication Therapy for OA [See also 253]

Orally taken drugs	NSAIDs[a], COX-2 inhibitors[b], glucosamine[c] and/or chondroitin sulfate
Topically applied remedies	e.g. Capsaicin
Intra-articular therapeutics	Glucocorticoids, HA solutions[d]

[a]Non-steroid anti-inflammatory drugs; [b]Cyclooxygenase-2 inhibitors; [c]*N*-acetyl-D-glucosamine; [d]Intra-articular use of HA has been approved in Italy and Japan since 1987, in Canada since 1992, in Europe since 1995, and in the United States since 1997.

and allergy to chicken or eggs if HA preparations derived from rooster combs are used [218].

There appears to be some rationale for combining steroid administration and HA injections. Intraarticular application of steroids and HA might be a good combination therapy. The effect of steroids occurs at an earlier stage (at 4 to 6 weeks), whereas the effect of HA is delayed but lasts longer. Comparison of HA injections with corticosteroids suggests that the effect of the former lasts longer but the latter works faster. Moreover, steroids may be more effective for joint effusions or for other forms of acute inflammation.

HA of higher mean molar mass probably stimulates synovial cells to produce endogenous HA to a greater extent than low-molar-mass preparations. However, one should take into account that use of solutions containing either a very high-molar-mass HA or a chemically cross-linked HA gel may be problematic. Such formulations for injections may be compromised due to certain obstacles occurring during their preparation, e.g. their membrane ultrafiltration sterilization.

To meet the demand for application of a material of higher molar mass, a novel procedure has been recently patented [219-221]. The methodology comprises an intra-articular injection of two high-molar-mass HA derivatives that would associate *in situ via* a host-guest "complexation," along with an appropriate low-molar-mass drug competitor. On injecting such a concoction, the drug molecules should initially completely block the process of association. The "complexation" of macromolecular components *in situ* will thus take place only after the drug is cleared from the articular space. The two macromolecular components are: 1) HA whose polymeric backbone carries an appropriate substituent that plays the role of host, e.g. a β-cyclodextrine oligosaccharide [222], and 2) HA substituted with an appropriate organic guest moiety, e.g. an adamantyl residue [223]. In the function of the competing agent, the use of a non-steroid anti-inflammatory drug (NSAID) such as that of naproxen has been proven to be advantageous [224]. Other potentially applicable competitors could be selected from the group of drugs that inhibit pain (e.g. flurbiprofen, piroxicam) or an appropriately selected glucocorticoid could be applied.

Thus, the two associating HA polymeric components premixed along with an appropriate drug serve as the tri-component injection formula. Upon the injection of such a concoction into the site of application, the drug molecules initially completely block the process of association. However, upon elimination (excretion) of the drug from the tissue environment, the desired *in situ* self-association of the polymer components will occur (Fig. **8**).

This original patented methodology [220] has been implemented also by other investigators [225].

5.3. Mechanism of Action of HA in Inflammatory Joint Disease

It is well known that HA binds to specific receptors, cluster determinant CD44, intracellular adhesion molecule ICAM-1, receptor for HA-mediated motility RHAMM, which are expressed by a wide range of cells, including those implicated in the pathology of OA, inflammatory cells, synoviocytes, and chondrocytes [226-230]. It also strongly interacts with a variety of HA-binding proteins (hyaladherins), such as link protein, which is part of aggrecan and versican complexes in cartilage and forms pericellular matrices around many types of cells, including chondrocytes [230]. The finding of specific cell receptors for HA supports a pharmacological mechanism of action for this natural polysaccharide in OA, and due to the wide distribution of these receptors on the surfaces of many types of cells, it also accounts for the diversity of its effects.

5.3.1. Pharmacological Basis for the Therapeutic Application of HA in OA

The binding of HA to receptors, notably CD44 and RHAMM and their various isoforms, has been reported to trigger a variety of intracellular signal events, such as protein phosphorylation cascades, cytokine release, and stimulation of cell cycle proteins [227, 230, 231]. The result of these receptor interactions by HA is to stimulate transduction and

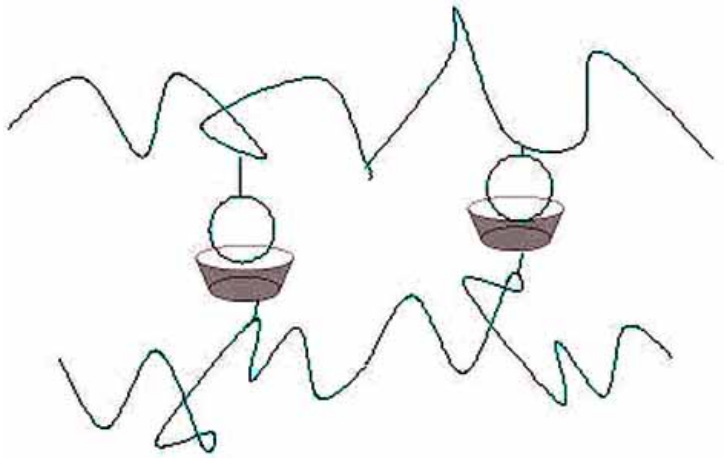

Fig. (8). The association of the two HA derivatives leading to formation of a polymeric network with enhanced visco-elastic properties applicable to e.g. viscosupplementation, ophthalmic surgery, tissue regeneration, use as drug carrier, *etc.* [223]. The two macromolecular chains are: Top: adamantyl-substituted, Bottom: cyclodextrin-derivatized hyaluronans.

other signaling pathways able to modulate cell functional activities manifested primarily by cell migration, proliferation, and endocytosis.

Studies on the effects of HA samples on CD44 cellular signaling have shown contradictory results depending on the size of the HA macromolecules used. Relatively low molar mass HA samples, 2.8×10^5 and 4.7×10^5 Da, promoted chemokine gene expression by macrophages, whereas a commercial preparation of HA having a molar mass of 6.1×10^6 Da has no such effect [232]. These data suggest that relatively low-molar-mass HA fragments produced during joint inflammation may perpetuate this process by enhancing expression of gene products by resident macrophages. Furthermore, it is proposed that the protective effect afforded by the lower molar mass HA samples on these cells is mediated *via* the increased expression of granulocyte macrophage colony-stimulating factor after CD44 activation.

The mechanisms responsible for these contrasting molar mass-dependent effects of HA samples on different cell types are, in part, related to the clustering and cross-linking of CD44 receptors on their plasma membranes. It is known that HA oligomers of 20-30 sugar units can interact with more than one CD44 receptor. Moreover, these HA chain fragments can displace macromolecular HA from its multiple binding sites [230]. It is possible that HA samples within a specific size range could provoke a pattern of CD44 clustering and cross-linking on binding, able to trigger an intracellular signal, whereas the larger HA molecules may occupy these multiple CD44 linking sites but prevent receptor cross-linking and inhibit cellular reactions [230]. Because the HA present in normal connective tissues is generally of high molar mass, it would seem to make biological sense for it not to continuously stimulate an active response from the cells it surrounds.

Despite existing extensive literature that has clearly shown that molecular size characteristics of HA are important determinants of its biological activity, there has been limited recognition of this fact in clinical practice. It generally has been accepted that the higher molar mass preparations would be superior to HA samples of lower size because the former would better fulfill the requirements for effective viscosupplementation (see Chapter 5.2.). In fact, many of the HA preparations currently used to treat OA have molar mass in the order of 1.0×10^6 Da and most of these are reported to be efficacious.

5.3.2. Effects of HA on Inflammatory Cells In Vitro

HA possessing a molar mass of 7×10^5 Da inhibited leukocyte migration, chemotaxis, and adhesion in a concentration dependent manner [233]. These observations were confirmed by studies suggesting that high-molar-mass HA in synovial joints provides a physical barrier to entry of polymorphonuclear cells into the cavity [234]. The high-molar-mass HA preparation (1.9×10^6 Da) is the most effective inhibitor of chemotaxis and, to a lesser extent, phagocytic functions. It was proposed that the effects of HA on the activated neutrophils occur *via* interactions with their CD44 cell receptors.

During the inflammatory process, polymorphonuclear leukocytes mediate both enzymatic and oxidative free radical reactions, leading to tissue damage. The HA-exhibited reactive oxygen species scavenging activity thereby protecting joint tissues from oxidative damage is molar mass-dependent [230]. It is suggested that HA forms a meshwork around the cells which is anchored to their HA receptors, thereby reducing the effective interaction between the radical generating species and the cell membrane. Furthermore, HA with an average molar mass of 9.2×10^5 Da is more effective than a lower molar mass HA sample against mitogen-induced lymphocyte proliferation [235]. Two mechanisms for HA effects are proposed: a) by reducing cell interactions and b) by the binding of mitogen enhancing factors released by the stimulated lymphocytes.

5.3.3. Effects of HA on Synovial Fibroblasts In Vitro

Human synovial fibroblasts from OA joints respond by up-regulating or down-regulating endogenous HA synthesis when cultured in the presence of exogenous HA, depending on its concentration and molar mass [236]. It is hypothesized that HA synthesis is mediated by interaction of this polymer with surface receptors on these fibroblasts able to promote a signal to increase synthesis by binding to HA within a certain molecular size range. These data suggest that synovial type B cells, the major source of synovial HA, may be capable of evaluating their extracellular HA environment and translating such information into the expression of genes able to lead to the *de novo* synthesis of HA.

5.3.4. Effects of HA on Chondrocytes and Articular Cartilage In Vitro

Adult rabbit articular chondrocytes cultured in the presence of human interleukin-1β and HA (molar mass between 5×10^5 and 2×10^6 Da), decrease their capacity to produce PGE_2 in a concentration-dependent manner [237]. The interleukin-1β induced production of superoxide anion radicals by bovine chondrocytes is also decreased by HA, confirming that HA fractions are capable of modulating the response of articular chondrocytes to inflammatory mediators *in vitro*. This anti-cytokine activity of HA is also shown with respect to the interleukin-1β or tumor necrosis factor-α mediated release of proteoglycans from the extracellular matrix of immature rabbit chondrocytes and bovine explant cultures [230]. In fact, it is reported in a study on proteoglycan synthesis in bovine articular cartilage explants in the presence and absence of IL-1β that HA penetrates the cartilage and accumulates in the pericellular matrix of chondrocytes, thereby maintaining HA synthesis [238].

The turnover of components of the extracellular matrix of cartilage is largely determined by the fine balance between the activity of matrix metalloproteinases (MMPs and the aggrecanases) and their respective inhibitors (TIMP-1, -2, -3, -4) [239]. Exposure of IL-1α-stimulated bovine chondrocytes to HA results in a marked concentration- and molar mass-dependent enhancement of TIMP-1 release, but has minimal effect on the production of stromelysin (MMP-3) [240].

In an *ex vivo* culture model of OA, HA is able to attenuate nitric oxide radical mediated chondrocyte apoptosis [241]. It is also reported that the anti-*fas*-induced apoptosis of cultured human chondrocytes is reduced when cultured in the presence of HA [242]. The anti-apoptotic effect of HA is

significantly decreased by the addition of both anti-CD44 and anti-ICAM-1 to the cultures. Furthermore, the combination of the two antibodies has an additional inhibitory effect. These data suggested that the anti-apoptotic activity of HA is mediated by its binding to specific cellular receptors.

The known stimulatory action of fibronectin fragments on the enhanced release of proteoglycans in culture is abrogated in the presence of HA [243]. Proteoglycan levels in the tissues are preserved by enhancement of their synthesis, although aggrecanase activity is not completely suppressed. It is proposed that the protective role of HA under these experimental conditions is achieved by its blocking of fibronectin entry into cartilage and, thus, its contact with chondrocytes. However, the stimulatory effects of HA on proteoglycan synthesis appears to be *via* an independent mechanism [230].

5.3.5. Anti-Inflammatory Effects of HA in Animal Models

HA modulates acute and chronic inflammation in a dose related manner in animal models and this effect has been hypothesized to be related to the modulation of the early stages of the immune process [244]. In this study, arthritis in the rat was suppressed by using HA administered subcutaneously. Because the rat OA model is driven by T-lymphocyte activation, the authors suggest that HA is able to modulate the early stages of immune processing (cellular antigen presentation and/or recognition).

The analgesic effects of HA have also been investigated in animal models of pain. HA is effective in reducing the induced hyperalgesia at a concentration range of 2.5-10.0 mg/mL [230]. It is also noted that other polysaccharides, i.e. methylcellulose and oversulfated chondroitin polysulfate having similar viscosity or polyanionic properties as HA, and used at the same doses, exhibit no analgesic activity in these pain models. The authors suggest that this HA analgesic effect is mediated by its ability to bind pain producing peptides or prevent their interaction with their corresponding pain receptors. However, further studies suggest that HA is somehow influencing pain transmission in this animal model by interacting with CD44 receptors and possibly other pain producing pathways that are presently unknown.

5.3.6. Effects of HA in Animal Models of OA

Intraarticular injections of HA in a dog model of OA are reported to reduce osteophytes and other pathologic modifications in cartilage, synovial membrane, and menisci [245]. Reduced proteoglycan degradation together with improved biochemical scores of cartilage damage are also noted in the HA-treated animals. Furthermore, it is also suggested from the morphological data that cells of destabilized joints are more viable and metabolically more active after intraarticular HA treatment, and that this effect is maintained for a long time after treatment.

In another study using an adult canine model [246], HA is injected into the joint at weekly intervals for a total of 5 injections. The results led to the conclusion that administration of HA preparation into the OA joints decreases cartilage hypertrophy as indicated by proteoglycan levels. It is suggested that the analgesic effect conferred by HA administration allowed animals to bear more weight on the unstable joint.

An important study on the effects of different molar mass-HA samples on synovia changes in a dog model was undertaken by Asari *et al*. [247]. The synovial fluids were analyzed for volume and PGE_2 content while the synovia were examined for cell proliferation, vacuolar degeneration, HA distribution, and heat shock protein (HSP72) expression. Clear differences were noted between the effects of the two HA preparations used. The smaller 8.4×10^5 Da HA preparation was more potent in reducing inflammatory changes in the synovium, particularly with regard to maintaining the abundance and intensity of HSP72, a protein marker of cell viability. Significantly, the stronger anti-arthritic activity noted for the lower molar mass-HA correlated with the extent of its penetration into the synovium. This effect was consistent with studies which showed that trans-synovial flow and flow buffering by synovial HA in rabbit joints is dependent on chain length [230]. The movement of HA across the synovial intima of the joint would appear to be limited by the presence in this tissue of a gradient of sulfated GAGs and collagens. These matrix components would be expected to restrict the diffusion of macromolecules, such as HA through the tissue, as a function of their charge and size.

Rabbit models of OA have been extensively used and these studies have supported a beneficial effect of HA with respect to rapidly proceeding disease, providing evidence that one of the mechanisms of action of HA in protecting cartilage is *via* the down- regulation of pro-inflammatory cytokines and MMP activities in synovial tissues [230].

Open and arthroscopic meniscectomies are common orthopedic procedures frequently accompanied by premature cartilage degeneration and the onset of OA irrespective of the amount of meniscus excised. Tissue degeneration and the onset of OA are thought to arise from high focal and shearing stresses imposed on articular cartilage and subchondral bone caused by excision of the weight-bearing meniscus. Meniscectomy in adult sheep induces pathologic changes in their stifle joints, which parallel those described for human OA joints [230]. Because of these similarities and the reproducibility of this procedure in this animal model, it has been extensively used to investigate the effects of intraarticular HA preparations on gait, serum biochemical markers, cartilage, bone, and synovial tissue metabolism. Two different HA preparations were able to improve the clinical signs of OA in sheep [248]. The improved weight-bearing on OA joints observed in both HA-treated groups was associated with increased morphological scores in cartilage and enhanced osteophyte development at the joint margins. It is proposed that the increased weight-bearing on the joints afforded by HA treatment is responsible for the increased osteophyte formation. Moreover, the biosynthesis of ^{35}S-PGs by cartilage explants derived from joints of animals injected with the higher molar mass-HA is less than that for the corresponding cartilages of animals who received the lower molar mass analogue [249]. The molar mass of the HA in the collected synovial fluids was determined by gel-permeation chromatography coupled with multi-angle laser-light scattering photometry. The results of this study indicate that HA increases the molar mass of endogenous HA in synovial fluid collected from the OA joints, in contrast with saline injections.

The effects of intraarticular administration of HA on cartilage integrity and release into synovial fluid of keratan sulfate peptides in an ovine model of early OA induced by meniscectomy were also studied [250]. In the control animals, keratan sulfate peptide levels increase progressively in synovial fluid as well as in the HA treated group, but the difference is not statistically different from the controls. Furthermore, cartilage at necropsy of HA-injected joints shows less damage than similar regions of saline-treated animals.

The effect of repeated injections of HA on a sheep model of OA temperomandibular joint disease was also tested [251]. The control group showed severe OA changes in the condyle, deviation in form from normal morphology, and marked marrow fibrosis. The HA-treated group showed less deviation from normal condylar morphology. There was also a significant difference in OA changes between HA-treated and control temperomandibular joints, with the HA-treated group having less severe changes.

The pathological changes in the synovium of an ovine model of OA and evaluation of the effects of two HA preparations were tested in a recent study [252]. Aggregate scores of pathological change were higher in OA joint synovia compared with controls, with individual measures of subintimal fibrosis and vascularity predominantly affected. The depth of intimal fibrosis was also significantly higher in meniscectomized joints. Intraarticular treatment with HA (Hyalgan) decreases aggregate scores, vascularity and depth of fibrosis. HA sample (HYADD) treatment decreases vascularity, intimal hyperplasia and increases high-molar-mass HA synthesis by synovial fibroblasts. CD44, connective tissue growth factor (CTGF) and iNOS expression are increased in the synovial lining of OA joints compared with normal joints, but there is no significant modulation of this increase by either HA preparation. Both HA samples reduced aspects of this pathology thus providing a potential mechanism for improving joint mobility and function in OA.

6. CONCLUSIONS

In this overview, issues such as HA structure, occurrence in living organisms and metabolism, biological sources and biotechnological approaches for its production, physiological role and its implications in pathological processes, along with biomedical applications have been evaluated in terms of mechanism of action and structure-activity relationships. All available information has been assembled so as to provide some support for our knowledge of HA properties as a naturally occurring macromolecule and as a drug.

The study of this fascinating natural polysaccharide will certainly extend into the future with so many questions left unanswered, such as a) improved preparation and its chemoenzymatic synthesis, b) new HA-based drugs with various molar masses and improved properties, c) new HA derivatives conjugated with different molecules, d) new therapeutic applications, (e) new biomaterials, and (f) development of an improved understanding of its physiology and pathophysiology through glycomics.

The preparation of HA from tissues creates concern, particularly after the recent appearance of prion- and virus-based diseases and its production by microorganisms. This may lead to possible co-production of various toxins, pyrogens, and immunogens. As a consequence, alternative routes, such as defined, recombinant mammalian cell lines capable of being cultured in large-scale fermentations, or synchronized chemo-enzymatic synthesis of monodisperse HA polymers, offer an exciting alternative to the present preparation methods.

New therapeutic applications might include the use of HA and HA-derivatives for acceleration of wound-healing, treatment of cancer and vascular disease, and in anti-arthritis therapy. These new activities will require the engineering of HA with appropriate pharmacokinetics and pharmacodynamics, and optimally oral bioavailability properties.

There has also been considerable interest in the use of biochemical markers released into synovial fluid, serum and urine as a means of assessing the extent of cartilage and bone breakdown in arthritic joints. Furthermore, under normal conditions, HA concentration depends on the tissue, organs and species and intense local changes have been reported to be associated with many diseases. Typical examples are many cases of malignancy, where a significant modification of HA concentration occurs. In these cases, separation techniques must be applied to delineate possible alterations of HA, with particular regard to its fine chemical structure, such as molar mass, and utilized for the precise diagnosis of the pathophysiological status. Obviously, conclusive diagnosis may be achieved when the analytical results are in harmony with the clinical findings. As a consequence, more studies are required to establish the utility of the HA content for direct or differential diagnostic purposes. In the near future, automation and miniaturization will be necessary for high-throughput structural elucidation of HA and fragments in glycomics, in which the implementation of microfluidic devices and chip-based technology will play a central role, generally applicable to any biological system under well-designed conditions.

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ABBREVIATIONS

ABC = ATP-binding cassette

AGMK = African green monkey kidney

BSE = bovine spongiform encephalopathy

CD = cluster determinant

CHO = Chinese hamster ovary

CTGF = connective tissue growth factor

ECM = extracellular matrix

EDTA = ethylenediaminetetraacetic acid

EGTA = ethylene glycol tetraacetic acid

ER = endoplasmic reticulum
ESR = erythrocyte sedimentation rate
FDA = Food and Drug Administration
FGF = fibroblast growth factors
GAG(s) = glycosaminoglycan(s)
GlcA = D-glucuronic acid
GlcNAc = *N*-acetyl-D-glucosamine
GPI = glycosylphosphatidylinositol
KGF = keratinocyte growth factor
HA = hyaluronan or hyaluronic acid
HAS = HA synthases
HEK = human embryonic kidney
HSP = heat shock protein
ICAM = intracellular adhesion molecule
IHABPs = intracellular HA-binding proteins
MMP(s) = matrix metalloproteinase(s)
MPO = myeloperoxidase
MDR = multi-drug resistance
MRP = multidrug resistance protein
NADH = nicotinamide adenine dinucleotide fosfate
NASHs = non-animal-source-hyaluronan
NIF-NaHA = non-inflammatory fraction of Na-hyaluronate
NMR = Nuclear Magnetic Resonance
NOS = nitric oxide synthase
NSAID = non-steroidal anti-inflammatory drug
OA = osteoarthritis
ONOO = peroxynitrite
PCR = polymerase chain reaction
PD-ECGF = platelet-derived endothelial-cell growth factor
PDGF = platelet-derived growth factor
PG(s) = proteoglycan(s)
PMA = phorbol 12-myristate 13-acetate
PMN = polymorphonuclear leucocytes
RHAMM = receptor for hyaluronan-mediated motility
ROS = reactive oxygen species
SF = synovial fluid
SOD = superoxide dismutase
SPAM = sperm adhesion molecule
TGF = transforming growth factor
TIMP = tissue inhibitor of metalloproteinases
TNF = tumor necrosis factor
UDP = uridin difosfate
VEGF = vascular endothelial-cell growth factor.

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