Towards a molecular understanding of arthritis

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Several different agents including free radicals, oxidizing compounds and proteases are believed to play a role in the onset of arthritis. The evidence and underlying chemistry presently available for each destructive agent are presented.

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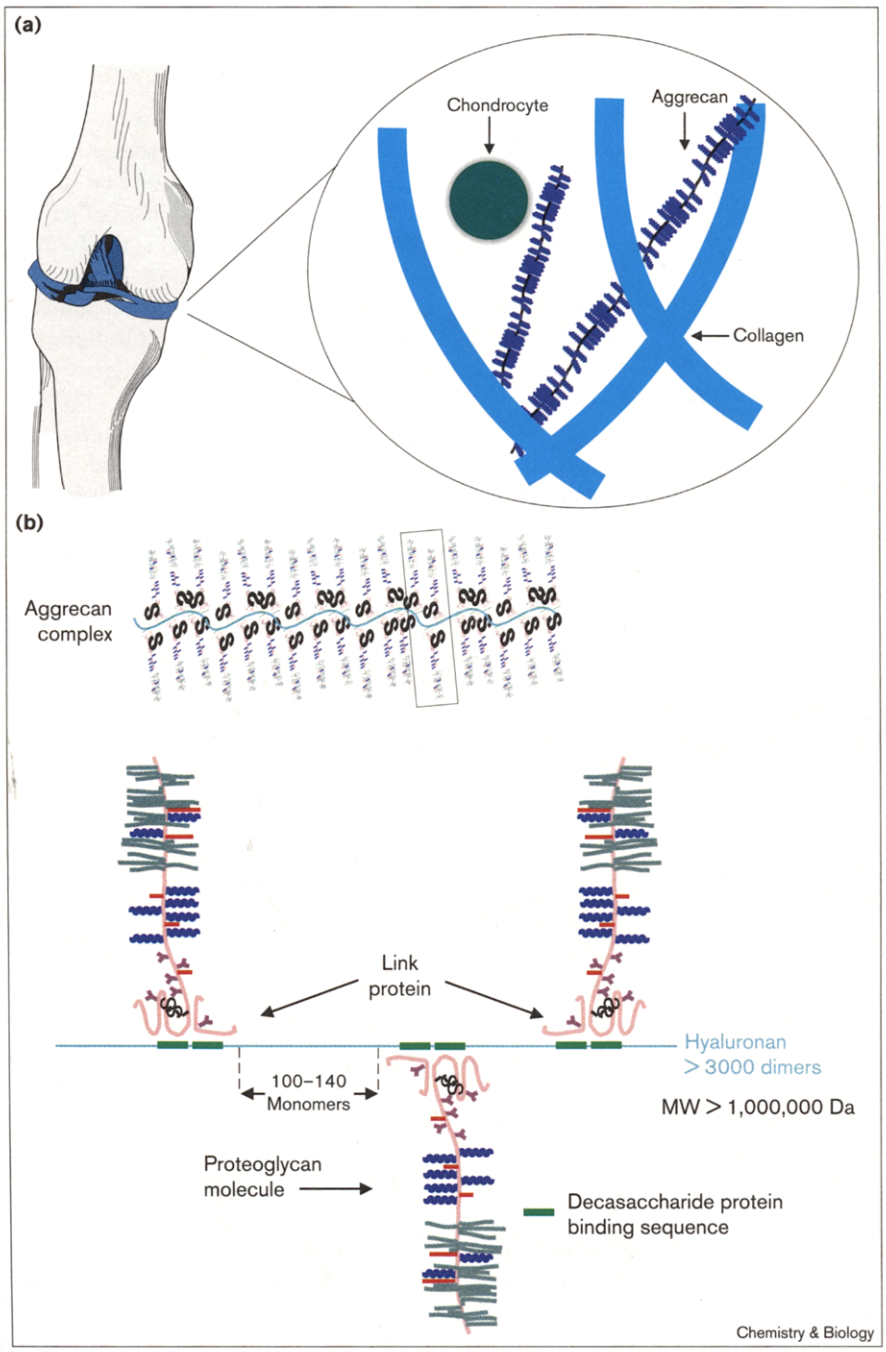
Introduction

Arthritis is the general term for a family of diseases characterized by joint inflammation, pain and swelling that lead to permanent joint damage and a loss of flexibility and mobility. More than 40 million people suffer from some form of arthritis, making it not only a leading cause of disability in the United States, but also a heavy burden on healthcare resources, especially geriatric medicine [1–4]. Of the many conditions characterized as arthritic disorders (e.g., osteoarthritis, rheumatoid arthritis, lupus, gout, bursitis, rheumatic fever and Lyme arthritis), osteoarthritis and rheumatoid arthritis account for most diagnoses. The National Institutes of Health report arthritis as the second most common diagnosis, after chronic heart disease, leading to long-term Social Security disability payments in the USA. Although risk factors certainly increase with age, arthritis afflicts individuals of every age, race and gender. More than one half of the people suffering from arthritis are under 65 years of age, with greater prevalence seen among women than men [2–4]. The Centers for Disease Control report higher proportions of patients suffering activity limitations attributable to arthritis among African Americans, North American Indians and Native Alaskans than among Caucasians and Asians/Pacific Islanders [5].

Osteoarthritis (OA), the most common form of arthritis, involves the destabilization of the normal balance between the degradation and the synthesis of articular cartilage and subchondral bone within a joint [6]. As articular cartilage degrades over time, its smooth surface roughens and bone-against-bone contact ensues, producing the inflammation response symptomatic of this 'wear and tear' disease. Although a variety of genetic, developmental, metabolic and traumatic factors may initiate the development of osteoarthritis [7], its symptoms (joint pain, stiffness and curtailed function) typically evolve slowly, and patients experience periods of relative calm alternating with episodes of inflammation and pain [8]. Because diagnosis requires X-ray analysis of the affected joints following the onset of pain, joint damage generally occurs prior to clinical diagnosis. Roughly one third of all white North American and Northern European adults exhibit features of osteoarthritis in at least one joint [7].

Rheumatoid arthritis (RA), an autoimmune disease of unknown etiology characterized by chronic synovitis and cartilage destruction, affects 1% of the total population [9]. This crippling disease, associated with such conditions as scleroderma, lupus, polymyositis, Reiter's syndrome and juvenile rheumatoid arthritis, involves the release of digestive enzymes within the joint as a result of prolonged

Figure 1



(a) The left section of the figure shows macroscopic representation of cartilage within a joint. The inset shows components of cartilage. Cartilage consists of chondrocytes embedded in an extracellular matrix of aggrecan and collagen. The collagen network traps proteoglycan aggregates (aggrecan), forming large polyanions capable of water sequestration. The resulting gel-like properties of cartilage cushion and protect bones within a joint. **(b)** A schematic diagram of proteoglycan aggreagate (the boxed area is enlarged to show detail). Core protein fragments bearing glycosaminoglycan chains arrange themselves laterally at regular intervals along the hyaluronan backbone via noncovalent interactions with link proteins.

inflammation of the synovial membrane. These enzymes lead to the production of a series of antibodies responsible for the degradation of cartilage, ligaments, tendons and bone [8,10]. The presence of one such antibody in the bloodstream, termed rheumatoid factor, often precedes the onset of outward symptoms as a diagnosis of rheumatoid arthritis. Nearly a quarter of all RA patients never develop this factor, however, and this antibody may be present in individuals who do not have RA, complicating its diagnosis [10]. Because of its systemic nature, RA can affect a variety of organs, including the heart, blood vessels, lungs and eyes, and can cause overall muscle aching and stiffness, fatigue, anemia and low-grade fever [8].

Despite the differences in diagnosis, etiology and outward symptoms, rheumatoid and osteoarthritis maintain one defining common characteristic: the degradation of articular cartilage. Cartilage and synovial fluid, persisting at the

articular surfaces on the bones of all movable joints, serve to cushion and lubricate the joint, enhancing the support, protection and smooth mechanical operation of the bones involved. Cartilage is a connective tissue consisting of chondrocytes embedded in an elastic ground substance or matrix composed of collagen and a proteoglycan gel (Figure 1) [11]. The type II collagen found in cartilaginous tissues incorporates a triple helix of hydrophilic chains that binds matrix macromolecules critical to cartilage stability. Cartilage elasticity stems from the proteoglycan gel, comprised mainly of aggrecan units assembled along a hyaluronan skeleton. Aggrecan, the major proteoglycan of cartilage, contains many carbohydrate chains (keratin- and chondroitin-6-sulfate specifically) attached to a protein core that fastens itself to the hyaluronan backbone via a small link protein (Figure 2). The collagen network traps the proteoglycan aggregates to form large polyanions capable of sequestering water in the matrix, thereby conferring the gel-like properties of aggrecan and the cushioning properties of cartilage [12]. Hyaluronic acid (HA, also referred to as hyaluran), the principal component of synovial fluid, also retains water, effecting gel formation and subsequent joint lubrication.

In arthritic joints, the chemical components of cartilage and synovial fluid degrade, reducing their ability to retain water and limiting their protective properties. A detailed understanding of these processes is a necessary prerequisite in the development of therapeutic and preventive methodologies to control disease progression. Research indicates that fragments of core protein, keratin sulfate, chondroitin sulfate, collagen and hyaluronan exist in increased concentrations in arthritic cartilage, as do matrix metalloproteinases that serve to degrade cartilaginous proteins [13] (Figure 3). Examination of the components of arthritic cartilage from chemical, biochemical [14–16] and genetic [17–19] perspectives has suggested possible mechanisms by which cartilage and synovial fluid degradation occur. Investigation of the potential factors involved in osteoarthritis implicates the participation of free radicals, nitric oxide (NO), and matrix metalloproteinases, one or more of which may trigger the depolymerization of HA and the release of proteolytic enzymes responsible for the destruction of constitutive cartilaginous proteins [6]. This review focuses on the investigation of these factors as they relate to the depolymerization of HA and the potential mechanisms by which this degradation might occur at the molecular level.

Effect of free radicals on HA structure

Research implicates the degradation of HA as a major cause of arthritis [20–23]. Serving as the skeleton upon which the proteoglycan complex assembles, HA is a key structural component of articular cartilage, although it accounts for only 1% of its composition (Figure 4). In addition, the link and core proteins utilized in the formation of proteoglycan gel require an HA decasaccharide in order for complexation of aggrecan to the HA backbone to occur [24,25]. As a result, the degradation of HA serves a dual role in the destruction and disintegration of cartilaginous tissues. Appropriately, a voluminous body of work focuses on the elucidation of HA degradation, the consensus being

Figure 2

A schematic diagram of the proteoglycan monomer. Negatively charged glycosaminoglycans (e.g. keratin sulfate and chondroitin-6-sulphate) covalently bind to core protein to form the proteoglycan monomer. Approximately 140 of these proteins noncovalently bind at intervals of 300 Å along a central filament of hyaluronic acid (HA).

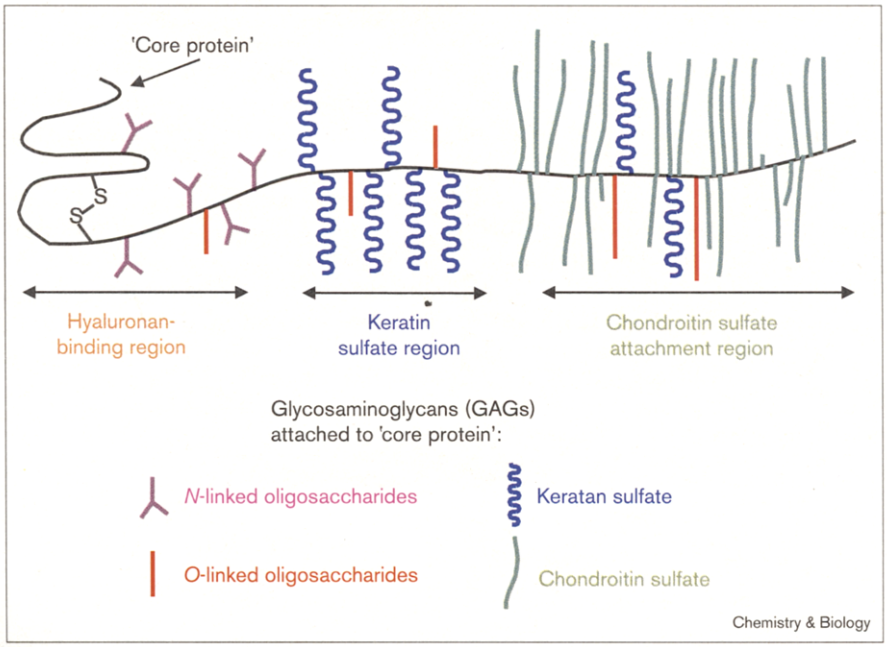
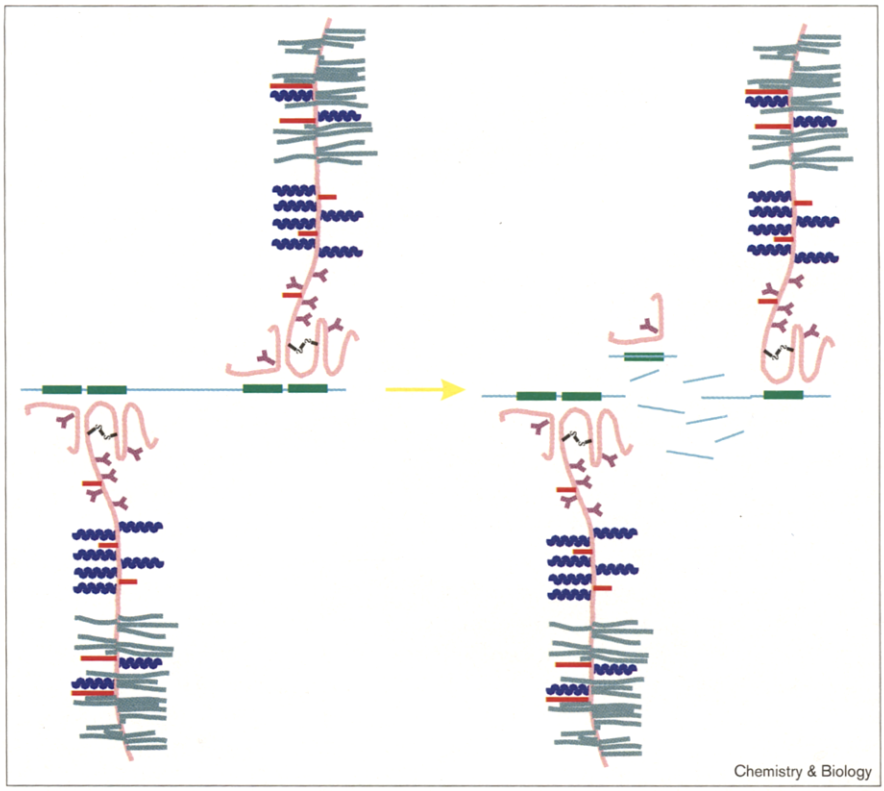


Figure 3



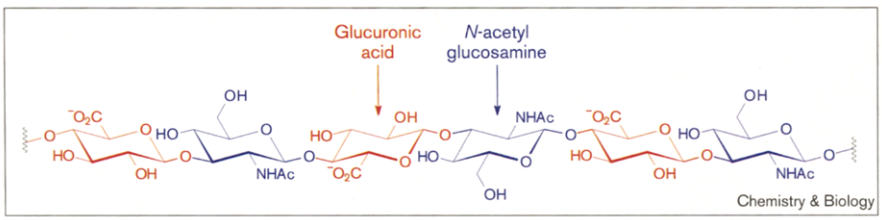
The degradation of aggrecan. The depolymerization of HA results in collapse of the structural scaffold of the aggrecan complex, reducing its ability to retain water and diminishing its gel-like properties.

that free radicals participate, although the means of participation and the species involved remain in question. Species hypothesized to participate in the depolymerization of HA are reactive oxygen species (ROS; e.g., hydrogen peroxide, hypochlorous acid, singlet oxygen, superoxide radical and hydroxyl radical) generated in the presence of Fe^{2+}, xanthine and xanthine oxidase (HX/XO) and myeloperoxidase (Figure 5) [20]. Although these systems actively engage in carbohydrate degradation processes, the conditions required for the production of ROS *in vivo* remain elusive.

Exposure of polycyclic aromatic hydrocarbons to UV light produces reversibly photooxidizable peroxide species that liberate singlet oxygen, a ROS capable of HA degradation. Lapcik and Schurz [26] utilized the singlet-oxygen sensitizing system anthracene-1-sulfonic acid (A-SO_3H) to investigate the degradation process provoked by UV radiation. They exposed aqueous solutions of HA to UV radiation both in the presence of and in the absence of sensitizer. Although those solutions containing sensitizer lost all elasticity, those without sensitizer showed remarkable decreases in viscosity as well, indicating the dramatic degradation of HA by UV radiation both with and without sensitizer. In fact, high-performance liquid chromatography (HPLC) analysis demonstrated degradation of HA after just 60 minutes of UV exposure.

In an effort to identify the intermediate radicals involved in the UV depolymerization of HA, Lapcik *et al.* [27] used electron paramagnetic resonance (EPR) spin-trapping experiments to study the radicals generated by degradation.

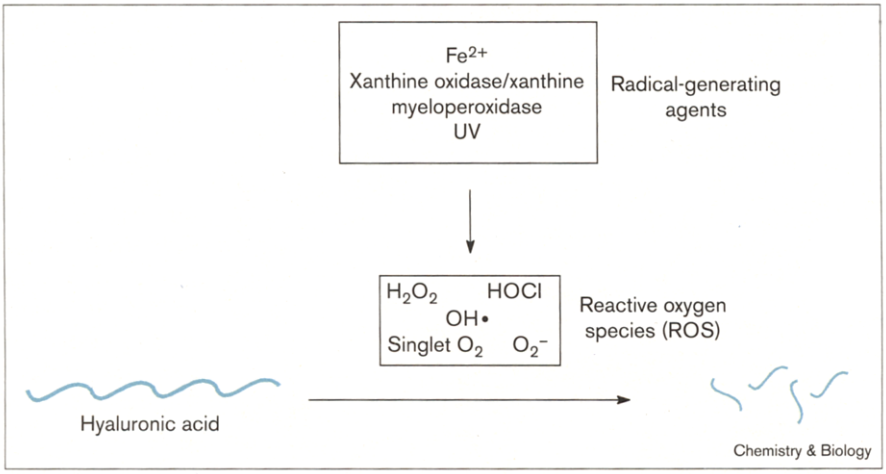
Figure 4



Hyaluronic acid, showing the repeating glucuronic acid and *N*-acetyl glucosamine residues.

Figure 5

Depolymerization of HA.



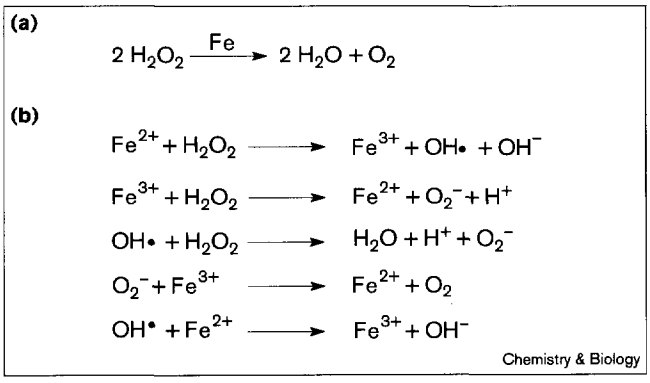
Although the authors identified four different radical species (three from sidechain groups, one from a ring carbon), they did not assign any of these species to the abstraction of a specific proton. Instead, they proposed that the carbon-centered radicals form first, initiating HA degradation; they offer no proof in support of this hypothesis. Because the concomitant use of UV radiation and a sensitizer generates hydroxyl radicals in aqueous solution, it follows that this system provokes HA depolymerization. The work of Lapcik *et al.* [27] work, therefore, more appropriately serves to demonstrate the role that radicals play in HA degradation and how EPR can be used to characterize radical products generated by depolymerization.

Most HA depolymerization studies employ hydroxyl radicals as the reactive oxygen-containing species. Initial studies demonstrated a decrease in viscosity of HA solutions upon exposure to solutions containing iron (II) cations [28–30]. Iron (II) cations generate hydroxyl radicals via the Fenton, or Haber–Weiss, reaction first observed by Fenton in 1894 (Figure 6). The tiny amount of iron present in synovial fluid is sufficient to initiate Fenton chemistry, as the reaction requires only catalytic amounts of iron for hydroxyl radical production. In 1998, Miyazaki *et al.* [31] observed a significant decrease in the viscosity of HA solutions stored in stainless-steel sample containers over several days. This observation led to the discovery that HA degradation can occur as a result of contact with metallic surfaces — trace amounts of iron can leach from the solid-state metal into solution.

Historically, the oxidation of 80% of the ascorbic acid in the synovial fluid of patients with RA led researchers to believe that ascorbic acid might participate in HA depolymerization [32]. Experiments have shown that HA degrades in the presence of ascorbic acid, and that it degrades at a faster rate in the presence of iron and ascorbic acid. In both cases, the addition of desferrioxamine, an iron chelator, completely inhibited HA degradation. This result indicated that ascorbic acid does not degrade HA; rather, the acid contains trace amounts of iron contaminants responsible for HA depolymerization [33–35]. Kvam *et al.* [36] proposed that the ascorbic acid reduces iron (III) to iron (II), increasing the rate of the Fenton reaction and thus the rate of HA degradation. Other metals shown to catalyze HA degradation include copper and manganese, present in the metalloproteins found in the extracellular matrix [37,38].

Hawkins and Davies [39] have employed electronic spin resonance (ESR) to extensively study hydroxyl radical attack on HA, as well as on related carbohydrate polymers and their constituents. Under Fenton conditions at pH 4, hydroxyl radical attack on glucuronic acid and *N*-acetyl glucosamine generated a variety of products. The authors

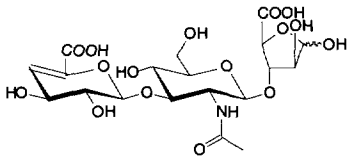
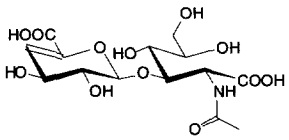
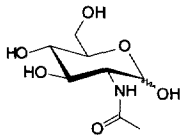
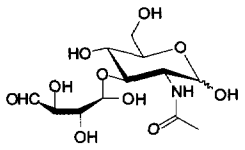
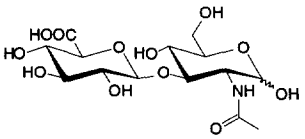
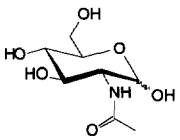
Figure 6



(a) Fenton reaction (net equation). **(b)** Sequential progression of the intermediate reactions involved in the Fenton reaction. Note the production of hydroxyl radical in the first step.

Table 1

End groups isolated from HA depolymerization under Fenton conditions.

Compound	% found
Reducing terminal residue	
	24
	21
	51
Nonreducing terminal residue	
	8
	20
	45

observed random proton abstraction for glucuronic acid and nearly random proton abstraction for *N*-acetyl glucosamine, with the exception of the *N*-acetyl methyl carbon and C(2), neither of which demonstrated radical formation. Any fluctuation in pH led to the disappearance of spectral features for glucuronic acid, indicating probable radical rearrangement, whereas for *N*-acetyl glucosamine, a decrease in pH led to the prolonged maintenance of its spectral features, demonstrating some increased stability. An equimolar mixture of glucuronic acid and *N*-acetyl glucosamine exhibited completely random attack with no preference shown for one sugar over the other; spin-trapping experiments on HA demonstrated the same pattern. These results suggest that at least some initial radicals undergo base-catalyzed rearrangement reactions resulting in strand-cleavage and the formation of low-molecular-weight fragments of HA.

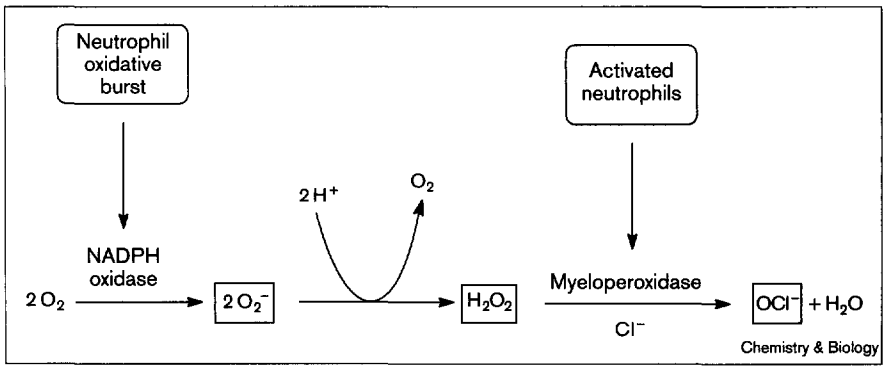
Schiller *et al.* [40,41] employed nuclear magnetic resonance (NMR) spectroscopy to monitor the breakdown of monosaccharides, HA and cartilage after subjection to irradiation or Fenton conditions. Irradiation of monosaccharides induced the formation of formate and malondialdehyde as by products, whereas HA predominantly showed cleavage of the glycosidic linkages leading to depolymerization. Exposure of bovine nasal cartilage to Fenton conditions generated increased concentrations of *N*-acetyl groups and formate, indicating depolymerization and degradation respectively. No attack on articular collagen was observed. Other preliminary studies suggested a close correlation between myeloperoxidase activity in RA patients (see below) and the content of NMR-detectable degradation products (e.g., *N*-acetyl groups and acetate). These data help support the predominant role of hypochlorous acid (HOCl) in HA degradation as HOCl induces a breakdown of glycosidic linkages leading to an increase in the appearance of *N*-acetyl groups in NMR spectra. Additionally, Schiller *et al.* [40,41] proposed that HOCl also reacts with *N*-acetyl groups of monomeric and polymeric carbohydrates to give acetate as a final product via a transient, chlorinated product. These observations, in combination with the aforementioned studies, led to their conclusion that HOCl serves as the reactive species *in vivo*.

The end groups of degraded HA offer additional clues as to the mechanism of depolymerization. Uchiyama *et al.* [42] subjected HA to Fenton conditions and further digested the resultant fragments with chondroitinase AC-II, which yielded the smallest oligosaccharides bearing modified terminal groups in addition to the disaccharide formed from the internal sequences of the fragments. HPLC isolation and NMR structure elucidation of the terminal units on each fragment indicated the presence of three different end groups for both the reducing and nonreducing termini (Table 1). These results also indicate that the polymer is randomly attacked during degradation, supporting the earlier work of Hawkins and Davies [39].

The xanthine/xanthine oxidase system (HX/XO) also has the potential to cause HA depolymerization. Found primarily in the liver, xanthine oxidase reacts with xanthine to create uric acid and in the process forms oxygen-derived free radicals (ODFRs), such as hydrogen peroxide and the superoxide radical, that can degrade HA (Figure 7). Viscosity measurements performed by Kvam *et al.* [36] indicated that HA depolymerizes rapidly in the presence of xanthine and xanthine oxidase, with increased rates of depolymerization observed after the addition of iron (III), suggesting Fenton conditions. This study also

Figure 7

The purine degradation pathway, showing the generation of ROS (boxed).



investigated the effect of ODFRs on chondrocytes, finding that ODFRs not only disrupt the glycolytic pathway, but also degrade the cellular membrane, leading to cartilage damage.

Polymorphonuclear leucocytes (PMNs), phagocytic cells that participate in the immune response, represent another class of potential radical-generating agents. Their respiratory burst releases myeloperoxidase, which works in conjunction with hydrogen peroxide and a halide as an antimicrobial system within the body. This system in turn generates hydrogen peroxide and HOCl (Figure 8). Lindvall and Rydell [43] were able to produce hydroxyl radical in the presence of high concentrations of hydrogen peroxide and sodium chloride in a test myeloperoxidase system. Varying the concentrations of hydrogen peroxide and sodium chloride and alternately eliminating each component from the system indicated that, although sodium chloride enhanced degradation to some extent, the system did not require hydrogen peroxide for the production of radicals and degradation of HA. In addition, potassium iodide and potassium bromide, as well as desferrioxamine and mannitol, a radical scavenger, inhibited the degradation process, indicating the presence of iron and the involvement of Fenton conditions. The observation that chloride ions enhanced HA degradation indicated HOCl participation, but they concluded (on the basis of radical stability) that hydroxyl radicals probably serves as the destructive agent. These results illustrate a need for further studies on the effect of HOCl on HA depolymerization.

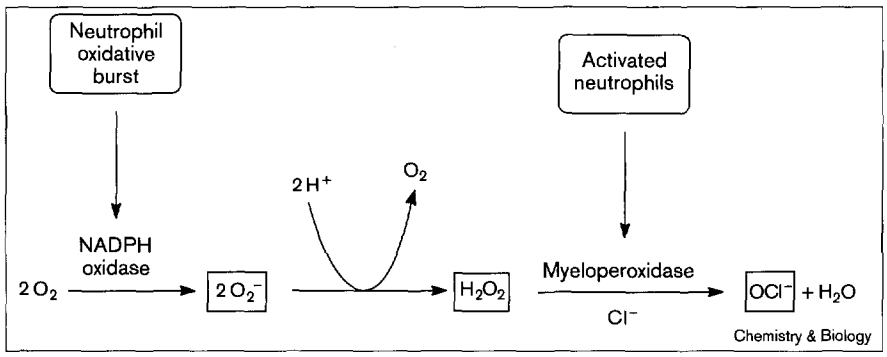
Stimulated neutrophils and myeloperoxidase generate HOCl through the reaction of hydrogen peroxide with chloride ions. The accumulation of neutrophils and the high concentration of myeloperoxidase in arthritic synovial fluid would indicate that HOCl plays a definite role in HA degradation *in vivo*. Viscometry and HPLC experiments have shown that HOCl degrades HA [44,45], whereas a study of the effect of HOCl on HA and pig articular cartilage shows that NaOCl attacks the *N*-acetyl groups of HA which degrade to acetate, causing depolymerization [46,47]. Researchers observed no breakdown of the sugar rings to produce formate and little effect on collagen.

In their recent report, Hawkins and Davies [48] studied the effect of HOCl on HA using electron paramagnetic resonance (EPR) and UV spectroscopy, and showed that HOCl initially reacts with the *N*-acetyl groups of HA. Although they did not observe radical trapping upon exposure of glucose, galactose and glucuronic acid to HOCl, *N*-acetyl glucosamine showed attack at the *N*-acetyl methyl and cleavage at C(2). ESR data suggested that attack occurs exclusively at the *N*-acetyl glucosamine residues and that initial radical formation occurs on the *N*-acetyl group, which rearranges to yield a carbon-centered radical that achieves polymer fragmentation.

A comparative study of the possible radical-generating systems involved in HA degradation would serve to elucidate the reactive species *in vivo*. Saari *et al.* [49] used HPLC to monitor the effect of xanthine and xanthine oxidase (HX/XO), hydrogen peroxide and HOCl on synovial fluid HA and human umbilical cord HA *in vitro*. In synovial fluid, HX/XO and hydrogen peroxide caused depolymerization and HOCl did not; the HX/XO system caused a dramatically greater degree of depolymerization than that found in arthritic synovial fluid, however, indicating that HX/XO is not an important contributor to HA depolymerization. In contrast, all of the agents caused depolymerization of purified umbilical cord HA, leading the authors to conclude that HOCl performs a scavenging action on synovial fluid HA by consuming hydrogen peroxide, which forms hydroxyl radical in the presence of iron (II).

NO has recently emerged as a possible factor in cartilage damage and arthritis [50]. NO is a key signaling molecule *in vivo*, produced by endothelial cells, macrophages, neutrophils, hepatocytes, chondrocytes, synoviocytes and certain classes of neurons. Independent studies have

Figure 8



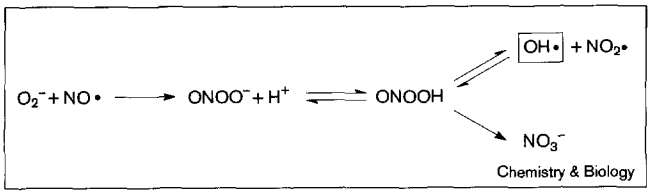
The myeloperoxidase pathway showing the generation of ROS.

shown increased concentrations of nitrate and nitrite — the decomposition products of NO — in arthritic synovial fluid, implicating NO involvement in the development of arthritis [51,52]. The synovial capillaries, infiltrating leucocytes and mesenchymal cells all serve as potential sources of NO in the joint. Because of the short half-life of NO and the amounts of NO produced by endothelial cells, the most likely sources are the neutrophils, lymphocytes, mast cells and macrophages in inflamed joints, in addition to the articular chondrocytes and synovial fibroblasts capable of NO synthesis [53,54]. NO gas reduces the viscosity of HA solutions under aerobic but not anaerobic conditions, suggesting the potential involvement of Fenton chemistry [50]. Further studies suggest that superoxide interacts with NO to form a potent oxidizing agent, and that the presence of iron will either enhance or inhibit this reaction depending on the relative concentration of each species (Figure 9) [55]. Connor *et al.* [56] have shown that the inhibition of NO synthase (NOS) will suppress the development of arthritis in animal models. Examination of cartilage and synovial biopsy samples from osteoarthritic patients showed increased expression of inducible NOS (iNOS) in chondrocytes, indicating NO participation [57]; the role and mechanism of action of NO in arthritis are still largely unknown, however.

Degeneration of other matrix components

Cartilaginous proteins also serve as targets for degradation in arthritis. Heterogeneity and differences in molecular weight help to characterize the noncollagenous proteins extracted from normal and osteoarthritic cartilage [58]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases required for cellular migration, tissue remodeling and turnover [59]. Tissue inhibitors of matrix proteases (TIMPs) regulate the action of MMPs; the dysfunction of this regulation can result in a variety of disorders including cancer, autoimmune disease and arthritis. MMPs in cartilage serve to balance out the synthesis of new cartilage in matrix turnover; sometimes a disruption in this balance of cartilage synthesis and degradation occurs, and degradation exceeds the rate of matrix formation. This catalyzes the breakdown of articular cartilage leading to arthritis. MMPs in the extracellular matrix include collagenases and gelatinases, which cleave collagen, collagen fragments and proteoglycans such as aggrecan. In both osteoarthritis and rhematoid arthritis, these enzymes exist at elevated levels, implicating their participation in disease [13,60,61]. Two preferred sites for MMP attack exist close to the HA-binding site in proteolysis of both aggrecan core protein and link protein [62–64]; attack at either of these sites disrupts HA binding and causes the loss of proteoglycans from the matrix. Researchers are currently investigating the potential of MMP inhibitors for the treatment and prevention of arthritic disease.

Figure 9



The interaction of NO with superoxide produces OH radical, a ROS.

Free radical generating systems can induce damage in collagen as well [65–70]. Collagen in arthritic cartilage exhibits increased cross-linking and decreased sensitivity to degradation by chemical agents. *In vitro*, collagen exposed to Fenton conditions at high iron concentrations exhibited signs of degradation, whereas at lower iron concentrations, the collagen proved more susceptible to proteolysis, presumably due to the destabilization of the triple helix [71].

Conclusions

Viscometric, spectroscopic, chromatographic and enzymatic investigations strongly suggest that free radicals, NO and MMPs participate in the processes of cartilage degeneration. Myeloperoxidase, xanthine/xanthine oxidase and iron (II) systems produce reactive oxygen species (hydrogen

peroxide, hypochlorous acid, singlet oxygen, superoxide radical and hydroxyl radical) capable of degrading HA. Analysis of depolymerization products suggests random radical attack upon the HA backbone, with little indication as to the conditions required for the initial production of radical species. The presence of NO by-products in arthritic synovial fluid implicates its participation in cartilage degradation as well. Inhibition of NO synthase in animal models suppresses the development of arthritis, whereas NO gas has been shown to decrease the viscosity of HA solutions. Imbalances in MMP activation effects the degradation of aggrecan and collagen, depriving cartilage of its gel-like character and protective properties.

Many questions remain as to the molecular events leading to synovial inflammation and cartilage degeneration. The crucial role of HA degradation in the onset of osteoarthritic disorders has spurred the development of a series of high-molecular weight HA formulae (e.g. ARTZ®, Hyalart®, Hyalgan®, Opelead® and Synvisc®) for use in viscosupplementation therapy [8]. Although these therapies appear to reduce pain and augment joint lubrication, these agents remain susceptible to the radical-initiated depolymerization processes that plague naturally occurring HA. Although effective in the short term, their long-term effectiveness has yet to be established.

Despite the lack of a complete, molecular-level picture of cartilage degeneration processes, sufficient data exists to help guide further development of novel therapeutic agents and synthetic matrix replacements for use in the treatment of arthritic disorders. For example, Tanaka *et al.* [6] report that activation of protein kinase C, a signaling protein found in chondrocytes, prevents stress induced osteoarthritic changes in cartilage. Furthermore, they show that increased mechanical stress on a system decreases proteoglycan synthesis and PKC activity. Examination of this correlation may lead to a model for the development of therapies capable of activating proteoglycan synthesis *in vivo*. A more molecular-level approach might involve the inhibition of MMPs, as the ability to regulate cartilage turnover would serve to maintain proteoglycan concentration within the matrix.

From a chemist's perspective, the future challenges associated with the treatment of osteoarthritic disorders are twofold: the continual refinement of the pathways resulting in matrix degeneration, and the design and construction of new, biocompatible materials for use in replacement therapies. Biomaterials such as artificially engineered, highly branched forms of HA may be capable of augmenting the lubricative properties of the natural matrix without succumbing to the oxidative stress responsible for natural HA degradation. Future efforts in chemistry will undoubtedly continue to address these issues until new, clinically effective long-term therapies are generally available.

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