Catabolism of hyaluronan: involvement of transition metals

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ABSTRACT

One of the very complex structures in the vertebrates is the joint. The main component of the joint is the synovial fluid with its high-molar-mass glycosaminoglycan hyaluronan, which turnover is approximately twelve hours. Since the synovial fluid does not contain any hyaluronidases, the fast hyaluronan catabolism is caused primarily by reductive-oxidative processes.

Eight transition metals – V 23, Mn 25, Fe 26, Co 27, Ni 28, Cu 29, Zn 30, and Mo 42 – naturally occurring in living organism are essential for the control of various metabolic and signaling pathways. They are also the key elements in catabolism of hyaluronan in the joint.

In this overview, the role of these metals in physiological and pathophysiological catabolism of hyaluronan is described. The participation of these metals in the initiation and propagation of the radical degradation hyaluronan is critically reviewed.

KEY WORDS: hyaluronan catabolism; synovial fluid; joint; transition metals; peroxidation; oxidative stress

Biogenic elements

Virtually each of the chemical elements plays some role in the Earth’s living systems, however, only 24 elements account for the vast majority of material in these systems. These elements are divided into (i) six major biogenic elements, namely carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus, (ii) five minor biogenic elements, sodium, potassium, magnesium, calcium, and chlorine, and (iii) thirteen biogenic trace elements comprising manganese, iron, cobalt, copper, zinc, boron, aluminum, vanadium, molybdenum, iodine, silicon, nickel, and bromine.

Transition metals

Thirty eight chemical elements, with the atomic numbers 21 to 30, 39 to 48, 72 to 80, and 104 to 112, are termed transition metals (cf. Table 1). Of these the following eight – vanadium, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum – belong to the biogenic trace elements. Thus V 23, Mn 25, Fe 26, Co 27, Ni 28, Cu 29, Zn 30, and Mo 42 could be classified as “biogenic transition metals”.

In most cases, the valence electrons of the transition metal, i.e. those electrons used in formation of the bonds with other elements, are present in two outermost orbitals. For this reason, transition metals may exist in several oxidation states (characterized by the oxidation numbers). For the biogenic transition metals, these states/numbers are listed in Table 2.

Transition metals may form complexes with both charged and neutral ligands. A variety of different oxidation states as well as the coordination by ligands provide redox and/or catalytic activity to transition metals, which serve as the catalytic centers of enzymes (oxidases, dehydrogenases).
Most biogenic transition metals participate in the control of various metabolic and signaling pathways. However, their versatile coordination chemistry and redox properties allow them to escape the control mechanisms, such as homeostasis, transport, compartmentalization, and binding to the designated tissue and cell constituents (Valko et al., 2005).

When these metals are liberated from their catalytic sites within enzymes and sequestered abnormally by other ligands, the consequences can be deleterious for the environment. Under such circumstances, irreversible oxidative changes to both the metal-complexing ligand itself and the neighboring molecules that intercept the reactive intermediates of the initial reaction, may initiate a cascade of oxidative stress events (HaMai et al., 2001).

Moreover, transition metal ions have a specific feature, the so-called "isoelectronics". The manganese ions are known to require vanadium; deficiency in vanadium results in their reduced growth and impaired reproduction. Coordination of iron with biomolecules involves the participation of its d orbitals. Since molecular oxygen using its electrons in antibonding π* orbitals can ligate to iron by overlapping its d orbitals, iron may serve as a bridge between the biomolecule and oxygen. The "flexibility" of iron not only means its ability to vary the oxidation state, but also implies its capacity to change electronic spin properties and relative redox potential in response to interaction with different coordinating ligands. When ascorbate is the coordinating biomolecule/ligand, it acts as both the iron chelator and reductant. Hence ascorbate upon binding with Fe(III) reduces iron to the Fe(II) ion, which forms a coordination complex ascorbate-Fe(II)-oxygen (Valko et al., 2005). This so-called Udenfriend’s oxidative complex/system is a very efficient reagent, used by organic chemists, to hydroxylate aromatic compounds, saturate hydrocarbons to alcohols, olefins to epoxides, etc. (Udenfriend et al., 1954). With copper, the so-called Weissberger’s system – ascorbate-Cu(I)-oxygen (Weissberger et al., 1943; Khan and Martell, 1967) – may generate hydrogen peroxide (cf. Scheme 1) (Fisher and Naughton, 2003; 2004; 2005). Due to H₂O₂ decomposition through Fenton-like reaction, the Weissberger’s system belongs to the most efficient generators of hydroxyl radicals (Soltés et al., 2006).

In the case when Udenfriend’s or Weissberger’s systems do not have additional oxidizable substrate, one speaks of "ascorbate auto-oxidation" (Buettnner and Jurkiewicz, 1993), where the resulting reaction product is dehydroascorbate (DHA). However, in the simultaneous presence of oxidizable substrates, the reaction products comprise both oxidized/decomposed substrates (Soltés et al., 2007).

### Vanadium

The oxidation states of vanadium include +5, +2, +3, and +4; that of +1 is rarely observed (Table 2). Vanadium is an essential element of some enzymes (e.g. nitrogenases in nitrogen-fixing micro-organisms). Rats and chickens are known to require vanadium; deficiency in vanadium results in their reduced growth and impaired reproduction.

| Period | Group | Transition Metals |
|--------|-------|-------------------|
| Period 4 | 3 (III B) | Sc 21, Ti 22, V 23, Cr 24, Mn 25, Fe 26, Co 27, Ni 28, Cu 29, Zn 30 |
| Period 5 | 4 (IV B) | Y 39, Zr 40, Nb 41, Mo 42, Tc 43, Ru 44, Rh 45, Pd 46, Ag 47, Cd 48 |
| Period 6 | 5 (V B) | Hf 72, Ta 73, W 74, Re 75, Os 76, Ir 77, Pt 78, Au 79, Hg 80 |
| Period 7 | 6 (VI B) | Nb 81, Mo 82, Tc 83, Ru 84, Rh 85, Pd 86, Ag 87, Cd 88 |
|          | 7 (VII B) | Nb 89, Mo 90, Tc 91, Ru 92, Rh 93, Pd 94, Ag 95, Cd 96 |
|          | 8 (VIII B) | Re 97, Os 98, Ir 99, Pt 100, Au 101, Hg 102 |
|          | 9 (VIII B) | Re 103, Os 104, Ir 105, Pt 106, Au 107, Hg 108 |
|          | 10 (VIII B) | Os 109, Ir 110, Pt 111, Au 112, Hg 113 |

* La – lanthanide series, ** Ac – actinide series

The transition metals (in shaded boxes) – vanadium, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum – belong to biogenic trace elements. The numbers in the first line under the element symbol represent number of electrons in the innermost (closest to the nucleus) orbitals, while those in the second line correspond to the electrons in the valence/binding orbitals.
Administration of oxovanadium compounds was shown to alleviate Diabetes mellitus manifestations in certain animal models and humans.

Manganese

The common oxidation states/numbers of manganese are +2, +3, +4, +6, and +7, although oxidation states +1 and +5 have also been observed (Table 2). Compounds, in which manganese exists in higher oxidation states are powerful oxidizing agents. Potassium permanganate (oxidation number +7) is used in medicine as an efficient disinfectant. Mn(III)-acetate is a relatively powerful oxidizing agent; the Mn(III)/Mn(II) standard reduction potential $E^\circ$ equals +1.5 V.

Manganese ions function as cofactors for a number of enzymes, of which Mn-containing superoxide dismutase (Mn-SOD) is one of the most essential enzymes required for almost all organisms living in the presence of oxygen. They use Mn-SOD to deal with the toxic effects of superoxide anion radicals ($O_2^{•–}$), formed by the one-electron reduction of dioxygen. Exceptions include Lactobacilli bacteria, which for the task of $O_2^{•–}$ detoxification use a different non-enzymatic mechanism involving Mn(II) complexed directly with polyphosphate. Further classes of enzymes, which may have manganese “cofactors”, include oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases.

The human body contains about 300 ppm of manganese; the recommended daily intake of this essential element is 3–9 mg (Roth, 2006). The low manganese turnover observed in patients with rheumatoid arthritis may cause disturbances in the synthesis of mucopolysaccharides observed in experimental animals with manganese deficiency (Niedermeier and Griggs, 1971). A heavy exposure to manganese can, however, cause chronic manganism – a form of Parkinson’s disease.

Manganese ions are paramagnetic and hence they are detectable by EPR spectroscopy. Six-band EPR spectrum of Mn(II) has been detected in most natural products.

| Element      | Oxidation states/numbers | Electronegativity (Pauling scale) | Ionization energies: 1st, 2nd, 3rd [kJ/mol] |
|--------------|--------------------------|-----------------------------------|---------------------------------------------|
| Vanadium     | V, II, III, IV (I)       | 1.63                              | 650.9, 1414, 2830                           |
| Manganese    | II, III, IV, VI, VII, (I, V) | 1.55                              | 717.3, 1509, 3248                           |
| Iron         | III, IV, (V, VI)         | 1.83                              | 762.5, 1561.9, 2957                         |
| Cobalt       | II, III, (IV)            | 1.88                              | 760.4, 1648, 3232                           |
| Nickel       | II, III, (I, IV)         | 1.91                              | 737.1, 1753, 3395                           |
| Copper       | II, I, (III, IV)         | 1.90                              | 745.5, 1597.9, 3555                         |
| Zinc         | II                       | 1.65                              | 906.4, 1733.3, 3833                         |
| Molybdenum   | VI, II, III, IV, V       | 2.16                              | 684.3, 1560, 2618                           |

*The most (2nd column) and the less (3rd column) common oxidation states/numbers.*

| Table 2. Oxidation states (oxidation numbers) of the biogenic transition metals. |

Iron

The Fe(III)/Fe(II) reduction potential at pH 7 equaling +0.48 V (Koppenol, 1994) predetermines iron as one of the major candidates in the production (and metabolism) of free radicals in biological systems. Contrary to ferric salts, which at neutral pH precipitate forming oxyhydroxide aggregates, ferrous compounds are soluble, though unstable, since they tend to react with dioxygen yielding $O_2^{•–}$ and Fe(III).

In mammals, the iron distribution is heavily regulated because Fe(II) ions represent a risk/toxic factor. Excessive iron intake can result in iron overload disorders, such as hemochromatosis, while diminished absorption of this essential biogenic element from the duodenum or deficit of this metal in food may lead to anemia.

The average-weight human body contains approximately 4–5 g of iron firmly bound/complexed in metalloproteins such as the oxygen carrier – hemoglobin, the enzymes containing heme prosthetic groups – catalase, cytochromes,
and in the iron transport/storage proteins – transferrin, ferritin, and hemosiderin.

Transferrin, a glycoprotein, has two separate binding sites to which Fe(III) attaches extremely tightly. Under physiological conditions, the transport protein – transferrin – present in the bloodstream is, however, loaded only to ca. 30% of its total iron-binding capacity, so that the amount of “free” iron salts available in plasma should be negligible.

Ferritin is a high-capacity and low-affinity storage protein with the capacity of about 4,500 atoms of iron per one protein macromolecule. Iron from ferritin can be removed by the action of some “bioreductants”, such as ascorbate. However, in tissues only trace amounts of iron are thought to remain “free” (non-chelated) or loosely chelated. Evidence was provided that iron may be present in tissues in the micromolar range complexed/bound to ATP, AMP, GTP, and possibly citrate (Koppenol, 1994).

Cobalt
Cobalt is essential to humans. This metal ion is the central component of cobalamin (Vitamin B₁₂).

Nickel
The most common oxidation state of nickel is +2, although in Ni-complexes oxidation states +1, +3, and +4 have also been observed. Solubilized Ni(II) ions in aqueous media at physiological pH are hydrated to a hexahydrate [Ni(H₂O)₆]²⁺ cation.

The main Ni-transporting protein in blood is albumin, though nickeloplasmin also transports these metal ions. The role of nickel in enzymes such as urease, glyoxalase, and a cation.

Molybdenum
Molybdenum is present in several enzymes such as aldehyde and sulfite oxidases. The purine catabolism, involving the oxidation of xanthine to uric acid, is catalyzed by xanthine oxidase – a Mo-containing enzyme.

As can be concluded from the above facts, biogenic transition-metal ions are sequestered by proteins: iron in transferrin and ferritin, copper in ceruloplasmin, etc. However, a small amount of low-molar-mass metal chelates/complexes is likely to be present at all times due to the transfer of metals from storage proteins to metalloproteins and the turnover of these proteins (Koppenol, 2003; Halliwell and Gutteridge, 1990).

Copper
An average adult human body contains about 80 mg of copper; the recommended copper intake for adults is 0.9 mg/day. When copper is first absorbed in the gut, it is transported to the liver tightly bound to albumin.

Copper is stored in hepatocytes bound to metallothionein. In the bloodstream, copper is bound/carried within a plasma protein – ceruloplasmin. Each ceruloplasmin macromolecule, which molar mass is around 134 kDa, complexes/bind up to eight copper ions, of which two can liberate relatively easily (Shukla et al., 2006). Redox-active copper is the principal constituent in a variety of enzymes, including cytochrome c oxidase and CuZn-superoxide dismutase (CuZn-SOD).

Copper toxicity is derived from its ability to accept and donate single electrons. It has been claimed that cuprous/cupric ions catalyze the production of hydroxyl radical in a manner analogous to Fenton chemistry. This increase in unmediated reactive radicals, generally termed oxidative stress, is an active area of research in a variety of diseases where copper may play an insidious role.

In high amounts, copper can be poisonous and even fatal to organisms. This metal has been implicated in the pathogenesis of neurodegenerative disorders, such as Alzheimer’s and Parkinson’s diseases, as well as amyotrophic lateral sclerosis. In Wilson’s disease, body tissues, mostly the liver and brain, retain too much copper. d-Penicillamine and oral Zn-supplements have been used for treating patients with Wilson’s disease (Gaetke and Chow, 2003).

Zinc
Zinc is a component of many metalloproteins such as metalloenzymes and metallothionein proteins. Chelatable redox-inert Zn(II) may serve as an antioxidant by preventing binding of pro-oxidant cuprous ions at tissue sites.

The adult human body contains approximately 1.5–2.5 g of zinc. The recommended dietary allowance of zinc is 11 mg for males and 8 mg for females, with higher amounts recommended during pregnancy and lactation. Brain development is stunted by zinc insufficiency in utero and in youth.

Zinc is excreted mostly in feces (12–15 mg/day) and lesser amounts (0.5 mg/day) are eliminated in urine.

Non-enzymatic peroxidation – a radical chain reaction
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Metal ions – especially those of iron – are capable of redox cycling by accepting [Fe(III) + 1e⁻ → Fe(II)] or donating [Fe(II) – 1e⁻ → Fe(III)] electrons. These capabilities are closely related with the catalytic participation of iron in reactions producing oxygen-derived reactive species.

In a wide variety of in vitro systems, Fe(II) salts and/or non-enzyme chelated/complexed ferrous cations (e.g. Fe(II)-EDTA) were shown to enhance radical damage by increasing the production of oxygen-derived reactive species.

Initiation reaction(s)
In a completely peroxide-free system, the initiation of a peroxidation sequence refers to the attack of any species that has sufficient reactivity to abstract a hydrogen atom (H⁺).
radical) from the (bio)substrate involved in a radical chain reaction. $O_2^{•−}$ is insufficiently reactive to abstract the $H^•$ radical. The protonated form of $O_2^{•−}$, i.e. $HO_2^•$, although present in a trace amount, is more reactive and appears to be capable of abstracting the $H^•$ radical (Halliwell and Gutteridge, 1990). Alternatively, complexes of Fe(II) ions and dioxygen are also assumed to yield reactive species of unknown nature, which are subsequently able to oxidize fatty acids in lipids (LH) and thus generate peroxyl lipid radical – $LO^•$, which, due to the reaction $LOOH + Fe(II)$, may propagate the lipid peroxidation as

$$LOOH + Fe(II) \rightarrow LO^• + Fe(II)$$

that is subsequently followed by reaction 5.

An important fact should be noted, namely that due to a high tendency of ferrous ions to be oxidized (the Fe(III)/Fe(II) reduction potential is equal to +0.48 V at pH7 (Koppenol, 1994)), practically none of the Fe(II) salts/complexes is free of Fe(III) cations. Although Fe(III) should not trigger lipid peroxidation, due to ubiquitous contamination of lipids with the hydroperoxides LOOH, addition of Fe(III) initiates a radical chain reaction since its reaction with hydroperoxides generates LOO• radicals according to the reaction 8.

If the potential of the Fenton reaction to take place is not unambiguously confirmed, one can come to a false conclusion that upon reacting with hydrogen peroxide any (biogenic) transition metal in lower oxidation state ($M^{n+}$) would generate hydroxyl radicals by the reaction $M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + •OH + HO^•$. However, most researchers agree with the statement that under normal/physiological conditions in vivo only the iron(I)-dependent formation of $•OH$ radicals may actually occur (Halliwell and Gutteridge, 1990).

**Joint – role of hyaluronan**

A joint is formed by the ends of two (or more) bones connected by connective tissues. The function of joints in the human organism is to ensure mutual motion of the adjacent bones in plane (bending $x \leftrightarrow y$) as well as in space (rotation $x \leftrightarrow y \leftrightarrow z$). The bone ends linked in a joint are covered with a thin soft layer – the cartilage, which matrix is being permanently restructured/rebuilt by the embedded chondrocytes.

Every joint is surrounded by a fibrous tissue capsule called synovium. The primary function of synovium is the production of synovial fluid (SF), which reduces friction and wear of the joint. The main components of this “lubricating” fluid are a filtrate of blood plasma and a high-molar-mass polysaccharide – hyaluronan (HA), extruded by the type II synoviocytes, the cells localized within synovial membrane.

HA (Figure 1) is a linear high-molar-mass natural polysaccharide formed from disaccharide units containing $N$-acetyl-$\beta$-glucosamine and $D$-glucuronic acid. The chemical structure of HA is rather regular, the only deviation
being a possible replacement of $N$-acetyl-$d$-glucosamine by deacetylated glucosamine residues. In an aqueous milieu, HA is represented by negatively charged hyaluronate macromolecules with extended conformations, which impart high viscosity/viscoelasticity to its solution (Hardingham, 2004). In human beings, HA is abundantly present in almost all body fluids and tissues. In synovial fluid and vitreous humor, HA macromolecules reaching megadalton molar masses, are present in a “free form”, i.e. not associated with proteins. In the articular cartilage matrix, however, HA is associated via a link protein with a proteoglycan – aggrecan – consisting of collagen and glycosaminoglycans, namely chondroitin sulfate and keratan sulfate.

In a healthy human being, the synovial fluid containing along with the blood plasma filtrate also the entangled macromolecules of HA (1.4–3.6 mg/mL; Kogan et al., 2007), represents a gel-like colloidal medium. The nutrients, including oxygen supply, upon crossing the synovial barrier permeate through the viscous colloid to the avascular articular cartilage where they are utilized by embedded chondrocytes. On the other hand, the chondrocyte catabolites (should) cross both the viscous fluid and synovial membrane. It can thus be concluded that within the articular gel-like fluid, the process of “mixing” at the increased mobility of the joint, may significantly affect joint homeostasis.

**Hypoxia and re-oxygenation of the joint**

Taking into consideration that cartilage does not contain any teleneurons, regulation of chondrocytes should be of chemical nature. In the relaxed state – for example, at night – chondrocytes experience a decreased oxygen supply (a status termed “hypoxia”). However when the status changes to an enhanced mobility during the morning, joint SF receives elevated supply of oxygen (a situation termed “re-oxygenation”). Such increased content of oxygen can be, however, deleterious for the homeostasis of the chondrocytes – the cells that in adults lack mitotic activity.

As “normal” SF contains no hyaluronidase activity, it has been inferred that oxygen-derived reactive species/metabolites are involved in a self-perpetuating process of hyaluronan catabolism within the joint (Grootveld et al., 1991). This process has been considered responsible for the short – about twelve hours – half-life of native HA macromolecules in SF.

To understand how to keep a radical reaction active/self-perpetuating, its propagation stage should first be analyzed. Let us assume that a peroxyl-type macromolecular radical (AOO•) exists within SF. Taking into account the relatively high reactivity/affinity of the unpaired electron on the oxygen, the following particular reaction step can be assumed

$$\text{AOO}^+ + \text{HA} \rightarrow \text{AOOH} + \text{A}^+$$  \hspace{1cm} (9)

where HA abbreviates the hyaluronan, in which H stands for a hydrogen atom – the abstraction of which by the AOO• radical leads to the formation of an A• macroradical. In the case when A• is a carbon macroradical, it is especially this reactant, which traps the excess of dioxygen molecule(s) according to the reaction

$$\text{A}^+ + \text{O}_2 \rightarrow \text{AOO}^+$$  \hspace{1cm} (10)

Hence, simply by combining the two reactions 9 and 10, the net reaction

$$\text{HA} + \text{O}_2 \rightarrow \text{AOOH}$$  \hspace{1cm} \text{net reaction}

corroborates the statement that one particular function of (a high-molar-mass) HA is to trap the oxygen excess during the stage of joint re-oxygenation.

**Participation of biogenic transition metals in physiologic catabolism of hyaluronan**

Analogously to the decomposition of lipid hydroperoxides (reactions 6 and 8) caused by a transition metal, similar reactions could be suggested for decomposition of the cumulating AOOH hydroperoxides

$$\text{AOOH} + \text{Fe}^{(II)} \rightarrow \text{AO}^+ + \text{HO}^- + \text{Fe}^{(III)}$$  \hspace{1cm} (11)
$$\text{AOOH} + \text{Fe}^{(III)} \rightarrow \text{AOO}^+ + \text{Fe}^{(II)} + \text{H}^+$$  \hspace{1cm} (12)

As evident, while the reactant/“propagator” entering into reaction 9 is regenerated/recycled by reaction 12, reaction 11 produces an alkoxyl macroradical. The ratio between the generated AOO• to AO• radicals is however governed by the present iron, or more precisely by the ratio of Fe(III) to Fe(II). However, in the case of a sufficiently high level of ascorbate, an efficient reductant of ferric cations, actual...
concentration of Fe(II) ions exceeds that of Fe(III), and thus \( AO^- \) radicals should prevail. These radicals could, similarly to the \( AOO^- \) ones, propagate the radical chain reaction as follows

\[
AO^- + HA \rightarrow AOH + A^\cdot
\]

(13)

however assuming the quenching of \( AO^- \) radicals by ascorbate, the products would now contain "oxidized" hyaluronan macromolecules plus DHA and/or its hydrolyzates.

Higher reactivity of \( AO^- \) radicals with ascorbate compared to that of the \( AOO^- \) radicals, closely correlates with the about 1,000-times higher value of the rate-constant \( k \) in the case of reduction of alkoxyl radicals. For example, the \( k \) values estimated for the reactions between ascorbate and a tocopheryl (T) radical \( TO^- \) or peroxyl-type radical \( TOO^- \), equal \( 1.6 \times 10^9 \) or \( (1–2) \times 10^6 \) \( M^{-1}s^{-1} \), respectively and, hence, the half-life of the alkoxyl radical is much shorter than that of peroxyl – microseconds vs. seconds. Analogously, the redox potential of the pair \( RO^-H^+/ROH \) = 1.6 V exceeds significantly that of \( ROO^-H^+/ROOH \) = 1.0 V.

Yet, concerning the radical reactions of carbohydrate polymers, the strand scission of \( AO^- \) as well as of \( AOO^- \) (intermediate) macroradicals represents a very plausible pathway, by which polymer fragments of shorter molecular size shall be generated (cf. Scheme 2) (see also Refs. Rychlý et al., 2006; Šoltés et al., 2006; Stern et al., 2007).

Along with the above described hyaluronan fragmentation reactions, the radical attack on the \( \beta \)-glucuronate/\( \beta \)-glucuronic acid and \( N \)-acetyl-\( \beta \)-glucosamine moieties can also lead to the "opening" of rings without breaking the polymer chain (Kogan et al., 2007; 2008; Hawkins and Davies, 1996).

**Actors of physiologic catabolism of hyaluronan in the synovial fluid**

The concentration of ascorbate in SF of healthy subjects reaches the values close to those established in blood serum, i.e. 40–140 \( \mu M \) (Wong et al., 1981). The contents of transition metals in both body fluids are listed in Table 3.

As evident from the data listed in Table 3, iron and copper are the two prevailing redox active transition metals in SF. It should be, however, pointed out that the respective levels of ca. 5.2 and 4.3 \( \mu M \) do not represent those, which are (freely) disposable to catalyze the oxidative catabolism of hyaluronan within SF. As has been reported, the availability of iron to stimulate \textit{in vivo} generation of \( \cdot \)OH radicals is very limited, since concentrations of bleomycin-detectable, i.e.

**Table 3. Contents of transition metals in blood serum of healthy human volunteers and in post mortem collected SF from subjects without evidence of connective tissue disease**

| Element   | Mean concentration in blood serum [\( \mu g/100 \text{ mL} \)] \( ^a \) | Mean concentration in synovial fluid [\( \mu g/100 \text{ g} \)] \( ^a \) |
|-----------|-------------------------------------------------|-------------------------------------------------|
| Manganese | 2.4 (0.44) \( ^b \) | 2.4 (0.44) |
| Iron      | 131.7 (23.6) | 29.0 (5.19) |
| Nickel    | 4.1 (0.70) | 1.2 (0.20) |
| Copper    | 97.0 (15.3) | 27.5 (4.33) |
| Zinc      | 115.4 (17.7) | 17.6 (2.69) |
| Molybdenum| 3.4 (0.35) | 1.0 (0.10) |

\( ^a \) Reported by Niedermeier and Griggs (1971).  
\( ^b \) Data in parentheses are the values in \( \mu M \) calculated in assumption that 100 g of SF has a volume of 100 mL.

**Scheme 2.** \( AO^- \) strand scission may be due to \( \beta \)-cleavage of the radical formed at, e.g. \( C(1) \) on the ring of \( \beta \)-glucuronate/\( \beta \)-glucuronic acid.
“free” iron, are rarely larger than 3 μM in human samples (Halliwell and Gutteridge, 1990).

Figure 2 illustrates the viscosity-time profiles of a model situation, where – along with 100 μM ascorbate – a single transition metal is involved (Valachova et al., 2009). As evident, a significant reduction of the solution dynamic viscosity (η), corresponding to the degradation of the high-molar-mass HA sample, clearly indicates a concentration-dependent manner for each metal (cf. left and central panels in Figure 2). As can be seen, the character of the time dependence of η value upon the addition of FeCl₂ (5.0 μM) can be described as a gradual monotonous decline, while the addition of CuCl₂ (5.0 μM) resulted in a literally “dramatic” drop of η value within a short time interval (30 min), after which the decrease of the solution dynamic viscosity continued, yet at a much lower rate. A possible explanation of this dissimilarity lies most probably in different reaction kinetics of the processes leading to generation of oxygen-derived free radicals, yet at a much lower rate. A possible explanation of this dissimilarity lies most probably in different reaction kinetics of the processes leading to generation of oxygen-derived reactive species in the system ascorbate plus Cu(II) and in that comprising ascorbate plus Fe(II), i.e. Weissberger’s and Udenfriend’s oxidative systems.

A similar drop of η value and two-phase reaction kinetics are identifiable upon the addition of a minute (0.1 μM) amount of CuCl₂ (see Figure 2, central panel), however when Cu(II) addition preceded that of ascorbate (right panel), the decline of the solution viscosity was much less marked. A plausible explanation of this observation is that a delay in ascorbate addition (9 min) allowed the Cu(II) ions to reach the “binding sites” within the HA macromolecule. The domain of the coordinate-bound copper formed in such a way seems to be “hindered” and hence less accessible for a direct complexation/interaction with the ascorbate anion, a condition that is required for formation of the Weissberger’s oxidative complex. As postulated, hyaluronate binding sites indicate a (weak) selectivity not only toward copper but also to (isoelectronic) zinc cations (Magnani et al., 1999). On the contrary, manganous and presumably also (isoelectronic) Fe cations form simple hyaluronate salts with α-glucuronate moieties (Pirc et al., 2004). Thus, the four transition metal elements, namely iron, copper, manganese, and zinc, may by their presence/absence create a variety of conditions under which HA oxidative degradation proceeds.

There exists, however, a marked difference between degradation of hyaluronan in vitro/in situ and its in vivo catalysis. Under physiological conditions, the SF viscosity does not indicate any changes since the content of “native” hyaluronan remains constant due to permanent de novo production of megadalton HA macromolecules by (stimulated) synoviocytes. Thus, the self-perpetuating oxidative (non-enzymatic) HA catalysis in SF represents a rather delicate and properly balanced mechanism that presumably plays significant role in regulating the physiological – normoxigen – homeostasis for chondrocytes. At the same time, the polymer fragments formed, which are removed by drainage pathways from the joint, serve most likely as chemical messengers/feedback molecules used for adjustment of the optimum mode of functioning of the synovial membrane and of the HA-producing cells, synoviocytes, localized within. In the other words, during physiologic joint functioning, the hyaluronan in SF plays the role of a “scavenger antioxidant”, whereas the produced polymer fragments can subsequently serve as messengers mediating information on the changes occurring in the homeostasis of the joint.

High “protective/scavenging efficiency” of hyaluronan against the action of e.g. •OH radicals has been earlier pointed out by some authors (Myint et al., 1987; Presti and Scott, 1994). Presti and Scott (1994) described that high-molar-mass hyaluronan (1218 kDa) was much more effective than the lower-molar-mass (668 and 176 kDa) HAs in scavenging •OH radicals generated by a Fenton-type system comprising glucose and glucose oxidase plus Fe²⁺-EDTA chelate.

Pathophysiologic catabolism of hyaluronan in synovial fluid

Despite their exceedingly simple primary structure, the HA macromolecules have extraordinarily wide-ranging and often opposing biological functions. As recently reported
by Stern et al., the unadorned HA polymer functions size-specifically and its fragments constitute a very information-rich system (Stern et al., 2006). While “space-filling” mega-dalton hyaluronans, synthesized by HA synthases (HAS1 and HAS2) (Itano et al., 1999; Weigel and DeAngelis, 2007), are immunosuppressive and anti-angiogenic (McBride and Bard, 1979; Delmage et al., 1986; Feinberg and Beebe, 1983), the intermediate-sized HA-polymer fragments are inflammatory, immunostimulatory, and highly angiogenic (Noble, 2002; West et al., 1985; Jiang et al., 2007).

There are two distinct modes to cleave in vivo the extended native HA chains: the enzymatic, involving participation of the hyaluronidases (e.g. Hyal-2) and the chemical one, involving oxygen-derived reactive species (Stern et al., 2007). Yet, due to the fact that SF is lacking any hyaluronidases, it is reasonable to assume that it is the latter mechanism, which might be involved also during HA catabolism in the inflamed joint. As can be seen from Figure 2, any transition metal, i.e. iron or copper, can play an active role in the oxidative HA catabolism. However, the increase in Cu(II) concentration within the joint (and particularly in SF) could lead to a really very rapid degradation of the native HA macromolecules.

Figure 3 exemplifies the impact of two different oxidative native HA chains comprising Cu(II) ions. As evident, a megadalton HA sample (Mω = 1.2 MDa) degrades in situ to intermediate-sized polymer fragments, which mean molar masses are reduced by one or even two orders of magnitude. Interestingly, the unimodal and relatively narrow molar-mass distribution (MMD) of the megadalton HA sample (Mω/Mn = 1.8) remains retained even upon cleaving the polymer (compare the three MMD curves in Figure 3).

Some differences should be, however, pointed out between the processes taking place in a joint (or locally within SF) and in a well-stirred reaction mixture under in situ conditions. In the bloodstream, the copper atoms are carried by ceruloplasmin, belonging to the so-called “acute phase proteins”, which concentration increases markedly under certain circumstances (Shukla et al., 2006). Higher ceruloplasmin concentration in the blood plasma would mean a larger amount of copper that crosses the synovial membrane. Yet, due to the SF gel-like consistency, the copper ions entering into this specific environment should start their “dramatic” oxidative action in the vicinity of the synovial membrane. How efficiently the chemically generated •OH radicals are “scavenged” within this microenvironment by the locally disposable albumin as well as by the HA polymer fragments of lower molecular size, remains questionable. The oxidative process may escape the control mechanisms and damage/disrupt the synovial membrane. Moreover, the intermediate-sized HA-polymer fragments generated within this microenvironment could participate in the activation of “defender” cells. They may further aggravate the inflammation state of the injured tissue(s) because the HA-polymer fragments can augment in turn inflammatory responses. As reported by Jiang et al., the HA fragments in the e.g. 2×10^5 Da range induce in macrophages the expression of a number of inflammatory mediators, including chemokines, cytokines, growth factors, proteases, and nitric oxide (Jiang et al., 2007). In this way, the oxidants generated by activated defender cells may enlarge the damage within the involved joint tissues such as the synovial membrane.

Moreover, it appears that reactive oxygen species disrupt copper binding to ceruloplasmin, thereby releasing “free” copper ions, which in turn may promote oxidative pathology (Shukla et al., 2006). The damage can be manifested by visually localizable cardinal symptoms of inflammation – color, dolor, rubor, tumor, and functio laesa. Yet, although less distinct, repeated (micro-acute) inflammatory injures may lead to a disastrous outcome – e.g. an autoimmune disease – such as rheumatoid arthritis.

The mutual substitution of (isoelectronic) transition metals, their versatile coordination chemistry and redox properties, along with changes in their compartmentalization and binding to the designated tissue/cell constituents may reflect certain non-specific physiologic responses of the organism to pathologic stimuli (Valko et al., 2005). Rheumatoid arthritis can be classified as one of the diseases of particular interest in this respect. In the synovial fluid from patients with rheumatoid arthritis compared to SF from normal/healthy subjects, the mean concentration of copper is increased by a factor of three and that of iron and zinc by 2 and 3.5, respectively (Niedermeier and Griggs, 1971).

There are several chronic (inflammatory) conditions, in which "abnormalities" in the metabolism of trace/transition metal(s) are at present in the focus of biomedical research. The diseases – such as the autoimmune rheumatoid arthritis, the neurodegenerative Alzheimer’s and Parkinson’s diseases – are classified as socially debilitating. Considering the fact of permanently extending mean lifespan of the human population, these diseases create serious ethical/societal issues, like that of euthanasia.
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