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REVIEW

Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications

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Abstract Hyaluronic acid (hyaluronan, HA) is a linear polysaccharide formed from disaccharide units containing *N*-acetyl-D-glucosamine and glucuronic acid. It has a high molecular mass, usually in the order of millions of Daltons, and interesting viscoelastic properties influenced by its polymeric and polyelectrolyte characteristics. HA is present in almost all biological fluids and tissues. In clinical medicine, it is used as a diagnostic marker for many diseases including cancer, rheumatoid arthritis and liver pathologies, as well as for supplementation of impaired synovial fluid in arthritic patients by means of intra-articular injections. It is also used in certain ophthalmological and otological surgeries and cosmetic regeneration and reconstruction of soft tissue. Herein we present an overview of the occurrence and physiological properties of HA, as well as of the recent advances in production biotechnology and preparation of the HA-based materials for medical application.

Keywords Arthritis · Degradation · Hyaluronan · Reactive oxygen species · Tissue regeneration · Viscosity

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Introduction

Hyaluronan (sodium hyaluronate, hyaluronic acid, HA), a common component of synovial fluid (SF) and extracellular matrix (ECM), is a linear high molar mass, natural polysaccharide composed of alternating $(1 \rightarrow 4)$-β linked D-glucuronic and $(1 \rightarrow 3)$-β linked *N*-acetyl-D-glucosamine residues, Fig. 1. HA belongs to a group of substances known as glycosaminoglycans (GAGs), being structurally the most simple among them, the only one not covalently associated with a core protein, not synthesized in Golgi apparatus, and the only non-sulfated one. The molar mass of HA can reach as high as 10^7 Da. Such high molar mass and its associated unique viscoelastic and rheological properties predispose HA to play important physiological roles in living organisms and make it an attractive biomaterial for various medical applications. Although HA is almost omnipresent (albeit in relatively small amounts) in the human body and in other vertebrates, the highest amounts of HA

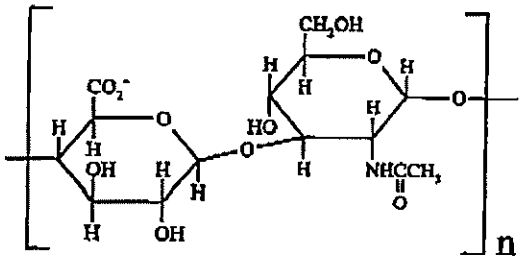

Fig. 1 Structure of the disaccharide repeating unit of hyaluronic acid

are found in the ECM of soft connective tissues (Laurent 1998). Besides vertebrates, HA is also present in the capsules of some bacteria (e.g., strains of *Streptococci*), but is absent in fungi, plants, and insects. The largest content by far of HA is found in rooster combs. Recently, a comprehensive overview of the sources from which HA can be isolated, and the contribution of the potential impurities, has been published (Shiedlin et al. 2004). A brief listing of the occurrence of HA in different animal tissues and its content is provided in Table 1.

Taking into account that recently a very comprehensive volume covering all aspects of hyaluronan chemistry and biology has been published (Garg and Hales 2004), in this review we will concentrate mostly on the biomedical application of HA and the methodologies of preparation of its samples with specified physicochemical and biological properties.

Occurrence of HA in living organisms

Hyaluronic acid occurs primarily in the ECM and pericellular matrix, although recently it has been shown to be present intracellularly (Evanko and Wight 2001). In the human body, the highest content of HA is found in synovial fluid, in umbilical cords, and in vitreous humor of the eye. Almost half of the human body's HA occurs in skin with most of the HA located in the intracellular space, where it may reach 2.5 g/l. Besides serving as a matrix, in which cells are embedded, HA plays a series of other important functions in skin. HA can immobilize water in tissue and thereby change dermal volume and compressibility. It can also influence cell proliferation, differentiation, and tissue repair. Changes in HA observed with ageing, wound healing, and degenerative diseases further emphasize its importance in skin (Juhlin 1997). In the skin, the largest organ of the human body, constituting the primary

Table 1 Occurrence of HA in different animal tissues and its content

Tissue or body fluid	Concentration (μg/ml)	Remarks
Rooster comb	7500	The animal tissue with by far the highest HA content
Human umbilical cord	4100	Contains primarily HA with a relatively high molar mass
Human joint (synovial) fluid	1400–3600	The volume of the synovial fluid increases under inflammatory conditions. This leads to a decreased HA concentration
Bovine nasal cartilage	1200	Often used as a cartilage model in experimental studies.
Human vitreous body	140–340	HA concentration increases upon the maturation of this tissue
Human dermis	200–500	Suggested as a "rejuvenating" agent in cosmetic dermatology
Human epidermis	100	HA concentration is much higher around the cells that synthesize HA
Rabbit brain	65	HA is supposed to reduce the probability of occurrence of brain tumors
Rabbit heart	27	HA is a major constituent in the pathological matrix that occludes the artery in coronary restenosis
Human 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
Human urine	0.1–0.3	Urine is also an important source of hyaluronidase
Human serum	0.01–0.1	HA concentrations increase in serum from elderly people as well as in patients with rheumatoid arthritis and liver cirrhosis

protecting barrier between the underlying tissues and the hostile action of the environment, HA plays a role of a scavenger of free radicals generated by the ultraviolet rays from sunlight. The ultraviolet light inflicts oxidative stress on cells and may damage their genetic material, thus causing degeneration and death.

In cartilage, despite its relatively low content, HA functions as an important structural element of the matrix, forming an aggregation center for aggrecan, a large chondroitin sulfate proteoglycan that retains its macromolecular assembly in the matrix due to specific HA–protein interactions (Prehm 2000). These aggregates have enormous molar mass of up to 100 MDa and are embedded within a collagenous framework (Schiller et al. 2003).

In synovial 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. Under inflammatory conditions of arthritic diseases, such as osteoarthritis or rheumatoid arthritis, high molar mass HA is degraded by reactive oxygen species, which reduces its viscosity and impairs its lubricant and shock absorbing properties leading to deteriorated joint movement and pain (Šoltés et al. 2006).

Although initially it was thought that the major role of HA is to serve as an inert molecular filling of the connective tissue, subsequent identification and study of HA-binding proteins and specific receptors has revealed that HA mediates many other functional activities (Tammi et al. 2002). HA is now recognized to play important roles in embryogenesis, signal transduction and cell motility, and is associated with cancer invasiveness and metastasis (Kogan et al. 2006). Moreover, despite their uniform and simple primary structure, HA polymers have extraordinarily wide-ranging and often opposing biological functions depending on the size of the molecule. Large matrix polymers of HA are space-filling, anti-angiogenic, and immunosuppressive, whereas the intermediate-sized polymers comprising 25–50 disaccharides are inflammatory, immunostimulatory, and highly angiogenic. Smaller oligosaccharides are anti-apoptotic and induce heat shock proteins (Xu et al. 2002). These low molar mass oligosaccharides appear to function as endogenous danger signals. Some of the variably sized fragments trigger different signal transduction pathways. A recently published review provides an insight into the excitingly vast range of size-specific activities of HA polymers (Stern et al. 2006).

Biological sources of the experimentally used HA

As has been described above, HA is an essential functional component of almost all tissues in the vertebrate organism. Thus, various animal tissues—e.g., rooster combs, shark skin, bovine eyeballs—have been used as sources of isolation and production of high molar mass HA (Table 1). Since HA in biological materials is usually present in a complex linked to other biopolymers, several separation procedures have to be applied in order to obtain a pure compound, such as protease digestion, HA ion-pair precipitation (with e.g., cetylpyridinium chloride), membrane ultrafiltration, HA non-solvent precipitation and/or lyophilization (Mendichi and Šoltés 2002; Šoltés and Mendichi 2003). The mean molar mass of the commercially available "extractive" HA preparations obtained from animal tissues is mostly in the range from several hundred thousands Da up to approximately 2.5 MDa. To date, the demand for HA materials approved for applications in human medicine has been satisfied by high molar mass HAs prepared from rooster combs. For example, Healon (Pharmacia & Upjohn, Inc., Peapack, NJ)—used in viscosurgery at eye implant insertion—has a mean HA molar mass of about 2.5 MDa. The current worldwide market for HA is estimated at over $1 billion (Widner et al. 2005; Chong et al. 2005).

Although animal tissues, primarily rooster combs, were involved at the early stages of production of the clinically utilizable materials approved by the Food and Drug Administration (FDA), e.g., in eye surgery (Healon), HA secreted by microorganisms such as certain attenuated strains of *Streptococcus zooepidemicus*, *S. equi*, etc. is currently offered by many companies up to several tons per year, as well. Some of these

"fermentative" HA preparations meet the demand on molar mass in the range of several MDa (Mendichi and Schieroni 2002). However, the risk of mutation of the bacterial strains, possible co-production of various toxins, pyrogens, immunogens, etc., hamper 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., in the eye, knee joint, etc. Yet, even these are also not ideal sources: All HA products obtained from rooster combs carry obligatorily warnings for those who are allergic to avian products. Thus, at present alternative sources for production of HA are being sought.

One of the promising potential candidates is a genetically-modified bacterial strain, *Bacillus subtilis*, carrying the *hasA* gene from *Streptococcus equisimilis* encoding the enzyme HA synthase. Such an engineered strain was able to produce HA with the molar mass in the 1 MDa range. The advantage of using *B. subtilis* is that it is easily cultivatable on a large scale and does not produce exo- or endotoxins, and many products manufactured by this microorganism have received a GRAS (generally recognized as safe) designation. Moreover, *B. subtilis* does not produce hyaluronidase that could degrade the synthesized HA (Widner et al. 2005). At present, microbially produced HA has been approved for treatment of superficial wounds as well as for the use in the cosmetic industry. In a recent review, Stern et al. (2006) provide a list of the major world companies that supply high molar mass HA or HA polymers of defined size for practical application.

Despite the fact that precisely specified molecular size for HA is the most essential parameter, this is often insufficiently specified for marketed HA polymers. Moreover, a frequently neglected fact is that both fermentative and extractive HA samples may contain certain contaminating ingredients. A trace amount of proteins, e.g., in extractive HA samples originates usually from the so-called link proteins. Their presence may detrimentally affect the required non-immunogenicity of HA preparations. The presence of complexed water molecules along with traces of transition metal cations in HA samples can pose a potential risk for the decrease in the high molar mass of HA (even if stored in the solid form) due to their degradation by O_2 (Miyazaki et al. 1998) and subsequent change of their properties. Hygroscopicity of dry HA is another complicating factor when a solution with precisely defined concentration of HA is required. Not only the ubiquitous bacteria or molds, but also the accompanying contaminating substances (proteins, metal cations, etc.) must be critically assessed for their potential to degrade the HA chain.

As pointed out above, the biological properties of the smaller HA fragments may be quite distinct and even opposite of those of the larger precursor molecules. Thus, eliminating a possibility of the presence of lower molecular size fragments in the presumably high molar mass HA samples presents a serious issue. Therefore, Camenisch and McDonald (2000) have emphasized the necessity to control the biological activity of commercial "intact" extractive and fermentative HA preparations of different molar masses, as well as that of the HA fragments prepared by either physicochemical methods or by partial digestion with hyaluronidases. They also proposed to validate the identity/differences of HA samples by a set of certain bioanalytical procedures.

Biomedical applications of HA and its derivatives

The basic areas (modalities) of the clinical applications of HA and its derivatives are classified by Balazs (2004) as follows:

(1) viscosurgery—to protect delicate tissues and provide space during surgical manipulations, as in ophthalmological surgeries,
(2) viscoaugmentation—to fill and augment tissue spaces, as in skin, sphincter muscles, vocal and pharyngeal tissues,
(3) viscoseparation—to separate connective tissue surfaces traumatized by surgical procedures or injury, in order to prevent adhesions and excessive scar formation,
(4) viscosupplementation—to replace or supplement tissue fluids, such as replacement of synovial fluid in painful arthritis, and to relieve pain,

medications have to be injected at least 5 times over 5 weeks (Chong et al. 2005). Recently, Q-Med AB (Uppsala, Sweden) started marketing the product Durolane that is claimed to require only a single injection to reduce pain and to increase mobility for up to 6 months in patients with osteoarthritis of the knee and hip. Administration of HA preparations have been reported to improve symptoms and decrease the use of nonsteroidal anti-inflammatory medications in patients with osteoarthritis. The majority of the evidence suggests that intra-articular administration of HA improves symptoms of osteoarthritis in selected patients and has few side effects (Evanich et al. 2001). Greenberg et al. (2006) indicate that it is unlikely that the beneficial effect of HA treatment could be ascribed only to the restoration of the lubricant and viscoelastic properties of synovial fluid. The authors suggest that it is more plausible that HA therapy has biological effect on the progression of osteoarthritis and propose four mechanisms, by which HA could exert its therapeutic effect:

(1) Restoration of elastic and viscous properties of the synovial fluid;
(2) Biosynthetic stimulatory effect of exogenous HA on cells—injected HA can induce the endogenous synthesis of HA by synovial cells, stimulate chondrocyte proliferation, and inhibit cartilage degradation;
(3) Anti-inflammatory action of HA, since the therapy is associated with decreased inflammatory cell count in synovial fluid, modulation of cytokine expression and reduction of reactive oxygen species content;
(4) Observed analgesic effect of HA administration.

In order to avoid rapid clearance of exogenous HA from the joint, Barbucci et al. (2002) prepared a 50% cross-linked HA, termed Hyal 50% (the number referring to a portion of carboxyl groups involved in cross-linking). The rheological behavior, means of sterilization, and in vitro effect on the chondral defect in rabbit knee was studied. The results obtained demonstrate that the hydrogel injected through the needle still behaved like a gel, although it showed a reduction of the dynamic moduli and the injections resulted in the improved chondrocytes density and matrix appearance.

Otolaryngology

Although HA is ubiquitous in the body, it is most concentrated in developing and specialized tissues such as vocal folds, synovial fluid, umbilical cord, and cartilage. In these tissues, it influences several different functions including tissue viscosity, tissue flow, tissue osmosis, shock absorption, wound healing and space filling. These functions are especially important in vocal folds due to the constant trauma caused by the vibratory actions of phonation. The osmotic, viscoelastic, and space-filling properties of HA are important in voice because they directly affect the thickness and viscosity of the vocal fold (Butler et al. 2001; Chan et al. 2001). Viscoaugmentation of the vocal cord, the repair of injured or scarred vocal cords, and treatment of glottal insufficiency are additional uses of HA derivatives. However, a major drawback to using HA as a lamina propria bioimplant for the treatment of vocal fold disorders is that its residence time within vocal folds is short—its half-life in rabbit vocal folds is only 3–5 days. To overcome this obstacle, the molecular structure of HA should be modified in order to increase the residence time. Various strategies including chemical, enzymatic, and mechanical cross-linking were implemented to prolong HA residence in vocal folds (Ward et al. 2002). Hylan B slurries (a cross-linked HA) injected into vocal cords produce no inflammatory reactions, and the material continues to be present even after one year (Hertegard et al. 2002).

In hearing disorders therapy, films of HA esters, such as HYAFF manufactured by Fidia (Abano Terme, Italy), are used in ear and sinus surgery. These preparations promote wound healing of the tympanic membrane, facilitate re-epithelization, as well as prevent adhesion between layers of mucous tissues.

Dermatology and plastic surgery

Preparations of slightly cross-linked HA are currently commonly used for augmentation, to fill facial wrinkles and depressed scars. Such HA gels

(5) viscoprotection—to protect healthy, wounded, or injured tissue surfaces from dryness or noxious environmental agents, and to promote the healing of such surfaces.

Attesting to the vast array of the physiological functions and properties, HA has found a number of usages in medicine and cosmetics. Already in the 1960s, the product Hyalgan manufactured by Fidia (Abano Terme, Italy) was applied topically for the treatment of burns and skin ulcers.

Ophthalmology

Hyaluronic acid is a major component of the vitreous body of the eye, and is a key macromolecule in ophthalmology. Because of its viscoelastic properties, HA is used in a number of key ophthalmologic surgeries. Preparations of HA protect delicate eye tissues and provide space during surgical manipulations. Its major use, however, is as a substitute or replacement for the vitreous fluid lost during procedures such as cataract surgery or lens implantation. The first product on the market was Healon derived from rooster combs, manufactured initially by Biotrics, Inc. (Arlington, MA) and later by Pharmacia, Sweden, now Pfizer (New York, NY). This product came on the market in 1979 and was soon followed by other products. This preparation was also used as a viscoelastic protector of the corneal endothelium during corneal transplantation. Currently, a number of preparations of varying molecular size HA chains are available, including an HA and chondroitin sulfate combination, termed Viscoat (Alcon Labs, Inc., Fort Worth, TX). Most recently, Maltese et al. (2006), based on the extensive study of the rheological properties of pure materials and their blends, concluded that a new binary combination of sodium hyaluronate and hydroxypropylmethyl cellulose named VISC26 fulfills most optimally the requirements for use as an ophthalmic surgery device.

Orthopedic surgery and rheumatology

The second major application of HA is in viscosupplementation in the joints affected by arthritis in a preparation marketed by Seikagaku (Tokyo, Japan). A normal/healthy joint allows nearly frictionless and pain-free movement. However, when damaged or affected by arthritis, joints become stiff and painful. Of the more than one hundred arthritic disorders, osteoarthritis and rheumatoid arthritis are the most common chronic conditions affecting mostly the elderly population. While osteoarthritis is a degenerative disease of the cartilage and bone resulting in pain and stiffness in the affected joint, rheumatoid arthritis is classified as a systemic inflammatory disease, in which pain of the joint(s) is often accompanied with degenerative changes in additional organs, such as lungs, heart, and blood vessels. It is estimated that over 10% of all people over the age of 55 are affected by osteoarthritis.

Although the etiology and pathogenesis of rheumatoid arthritis are as yet unknown, a progressive degradation of polymeric carbohydrates—mainly HA—in synovial fluid can be observed in the course of the disease. In acute phases, a high number of neutrophils is accumulated in the patient's synovial fluid. These cells alter the oxidative homeostasis and their products, especially reactive oxygen species, can contribute to the destruction of joint structures. Due to chronic inflammation of the joint, the reactive oxygen species alter/destruct the joint structure to such an extent that it is no longer functional. The altered tissues are recognized as "foreign", and subsequently autoimmune reactions promote the disease and make rheumatoid arthritis a systemic ailment affecting the entire body (Šoltés et al. 2006).

Since the end of the 1980s, intra-articular application of HA (viscosupplementation) has been successfully applied in millions of osteoarthritic patients basing on the original concept of Balazs and Denlinger (1989). The molar mass of HA in synovial fluid of a healthy adult person is in the range between 2 and 7 MDa, and the major commercial products Healon (Pfizer, New York, NY) and Synvisc, also known as Hylan G-F 20 (Genzyme, Cambridge, MA) fall into this range, while other products, such as Hyalgan (Fidia, Abano Terme, Italy) and Artz Dispo (Seikagaku, Tokyo, Japan) contain HA of lower molar mass (about 1 MDa). While treatment with higher molar mass preparations requires three injections over 3 weeks, lower molar mass

are more effective in maintaining cosmetic corrections than collagen-based products (Narins et al. 2003). Restylane, produced by the Medicis Corp. (Scottsdale, AZ) is prominent among such HA-based injectable materials (Kanchwala et al. 2005). Unlike collagen-based fillers, HA is extremely elastic, providing the elasticity required by spaces in which it is injected, such as facial wrinkles and depressed scars, vocal cord augmentation, laryngeal and glottal reconstruction, or sphincter muscle support. The HA preparations are also longer lasting. In comparing the applicability of the two commercial products used for soft tissue augmentation—Restylane produced by bacterial fermentation and Hylaform (Hylan B) from rooster combs (Genzyme, Cambridge, MA), Manna et al. (1999) concluded that Hylaform demonstrated better rheological properties behaving as a strong hydrogel, whereas Restylane acted as a weak hydrogel. Moreover, the former product contained four times less protein than the bacterial product, which offered Hylaform a better safety profile.

Shu et al. (2004) described development of novel HA-based cross-linkable hydrogels that did not need surgical implantation, but were injectable and showed improved cytocompatibility with fibroblasts. This demonstrated potential use of such hydrogels for tissue regeneration.

A commercial benzyl ester derivative of HA (HYAFF 11, Fidia, Abano Terme, Italy) and laboratory cross-linked Hylan (Genzyme, Cambridge, MA) were shown to be excellent biomaterials for promotion of adherence of vascular endothelial cells and vascular tissue engineering (Turner et al. 2004; Amarnath et al. 2006).

Surgery and wound healing

High molecular size HA preparations, applied topically, promote healing of fresh skin wounds. They also promote the healing of venous leg ulcers and are useful in the management of chronic wounds (Edmonds and Foster 2006).

A new product, a combination of HA with dexpanthenol (Hylactive, Promedic, Seville, Spain) is used as moisturizing, anti-erythematous, and skin regenerative/protecting topical preparation. Due to its antioxidant properties, HA serves as an anti-inflammatory component in the wound dressing materials (Moseley et al. 2003).

Pharmacology and drug delivery

The carboxylate groups of HA have been used to create a cross-linked hydrogel for DNA entrapment and also for drug delivery. HA can be either conjugated directly to drugs or used to prepare microcapsules for optimized drug delivery (Esposito et al. 2005). HA is also used to improve biocompatibility of chitosan microspheres used as drug delivery capsules (Vasiliu et al. 2005). HA microspheres are also used for the delivery of plasmid DNA and monoclonal antibodies in gene transfer and site-specific targeting (Yun et al. 2004).

Conclusions

Hyaluronic acid is a very attractive subject for biotechnology from several points of view. First, HA can be obtained from various natural sources. Therefore, the issue of formulating an appropriate biotechnological procedure to yield a preparation with the required molecular and biological properties is very important. The anticipated release of HA produced in a heterologous host (genetically modified *B. subtilis* carrying the gene from *S. equisimilis* encoding the HA synthase) signifies the transition of HA manufacturing into modern biotechnology (Widner et al. 2005). The expanding application of HA-derived therapeutics emphasizes the impetus for the development of biotechnological and chemical processes for optimization of the production of HA-based drugs. This offers great promise in various fields of medicine.

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Contribution of Oxidative-Reductive Reactions to High-Molecular-Weight Hyaluronan Catabolism

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Since the content of hyaluronan (HA)-degrading enzymes in synovial fluid (SF), if any, is extremely low, the high rate of HA turnover in SF is to result from a cause different from enzymatic catabolism. An alternative and plausible mechanism is that of oxidative-reductive degradation of HA chains by a combined action of oxygen and transition metal cations maintained in a reduced oxidation state by ascorbate.

Introduction. – Hyaluronan (HA), a glycosaminoglycan widely distributed in vertebrates, is characterized by an extraordinarily high rate of turnover. A 70-kg individual has 15 g of HA, one third of which turns over daily [1][2]. The half-life of HA is a few minutes in blood plasma, several hours in synovial fluid (SF), one to two days in the epidermal compartment of skin, and less than three weeks in cartilage [3–5].

Hyaluronidases cleave the HA chain with moderate specificity. However, since the content of HA-degrading enzymes in SF, if any, is extremely low [6], the high rate of HA turnover in SF should result from a cause different from enzymatic catabolism.

Along with the action of enzymes, HA is known to be degraded under acidic or alkaline conditions, by thermal treatment, ultrasonication, X- or γ-ray irradiation, *etc.* [7]. One relevant group of HA-degrading agents concerns reactive oxygen species. These include OH radicals, peroxynitrite, hypochlorite, *etc.*

The kinetics of HA degradation by the above mentioned enzymes/species was investigated by employing various methods. However, since high-molecular-weight HA solution is characterized by high viscosity, this is the very parameter which seems to be the most appropriate to be studied for monitoring the degradation process. In fact, simple capillary viscometry has been employed in several HA degradation studies. A drawback of this type of viscometry is a high shear-rate under which HA macromolecules flow through the viscometer capillary. Application of rotational viscometry would provide a significant progress in performing more precise measurements. We should, however, point out that although rotational viscometry has already been applied, for e.g. in free-radical HA degradation studies, the material of the sample reservoir as well as that of the rotating spindle – usually stainless-steel – could possibly lead to skewed results.

The aim of this communication is to present results of HA degradation as monitored by Brookfield rotational viscometer equipped with a home-made Teflon cup-spindle pair. The experimental conditions used in this study mimic those of SF in a healthy human joint space.

Results and Discussion. – The HA sample used – *LIFECORE P9710-2* – contained residual transition metal cations [8]. The actual content of Cu ions in the sample solution used was equal to 0.1 μM. The value of the dynamic viscosity of this solution at 12 min equaled 12.1 mPa·s (*Fig.*, curve *b*). The continual increase of the solution viscosity indicates orientation of polymer chains due to spindle rotation – a well-known phenomenon termed 'rheopexy'. The dynamic viscosity *vs.* time profiles recorded for the samples containing transition metal surplus ($CuCl_2$ concentrations tested: 2.5, 10.0, and 40.0 μM) demonstrated a similar nature as that of pure HA solution (compare curves *a* and *b* in *Fig.*).

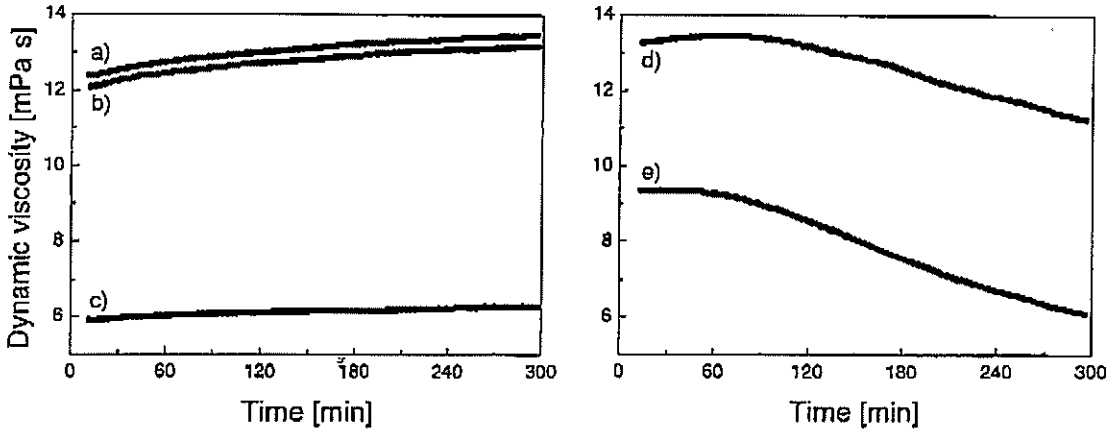

Figure. *Time dependences of dynamic viscosity of hyaluronan solutions.* Samples: *a*) *LIFECORE P9710-2* with addition of 40 μM $CuCl_2$, *b*) *LIFECORE P9710-2* (M_v = 1.215 MDa [9]), *c*) hyaluronan 'CPN' (M_v = 659.4 kDa [9]), *d*) *LIFECORE P9710-2* with addition of 100 μM ascorbic acid, *e*) *LIFECORE P9710-2* with addition of 0.1 μM $CuCl_2$ + 100 μM $CuCl_2$.

On the contrary, upon addition of ascorbic acid, the HA dynamic viscosity demonstrated a biphasic shape: a slight rheopexy until *ca.* the 75th min, thereafter the dynamic viscosity started to decrease continuously, reaching after 5 h a value of 11.3 mPa·s (*Fig.*, curve *d*).

Due to a high reducing capacity of ascorbic acid (100 μM), the copper cations in the HA solution are present in their lower oxidation states (*Reaction 1*). The cuprous ions are then oxidized by air oxygen yielding superoxide anion radicals (*Reaction 2*), which, according to *Reaction 3*, rapidly dismutate spontaneously to form H_2O_2. This is then decomposed in a *Fenton*-type *Reaction 4* to give the hydroxyl radical ($^{\bullet}OH$). The cupric ions (*Reaction 4*) recycle to their lower oxidative state – Cu^+ – according to *Reaction 1* [10].

$$Cu^{2+} + \text{ascorbic acid} \rightarrow Cu^{+} + \text{oxidized ascorbic acid} \quad (1)$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{\cdot -} \quad (2)$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 4H^{+} \rightarrow 2H_2O_2 \quad (3)$$

$$H_2O_2 + Cu^{+} \rightarrow Cu^{2+} + HO^{-} + {}^{\cdot}OH \quad (4)$$

Thus, it can be assumed that during the initial time period (*e.g.*, ≤ 75 min; *cf. Fig.*, curve *d*), an appropriate amount of ·OH radicals is generated by the *Reaction* sequence *1–4*. This statement is supported particularly by the results shown in the *Figure*, curve *e*. The higher the concentration of transition metal ions, the greater the ·OH radical flux.

The ·OH radical extracts H· from the HA polymer chain yielding a macro-radical, which subsequently undergoes fragmentation [11]. Polymer fragments of lower molecular weight naturally demonstrate lower dynamic viscosity, and changes of its values can be very effectively monitored by using the *Brookfield* rotational viscometer.

Physiological and Pathological Implications. – Hyaluronan is continually supplied from the synovial membrane into SF by synoviocytes. The SF in a healthy human knee joint is a highly viscous liquid, in which the HA content is 2–3 mg/ml [12]. The concentration of ascorbate in SF ranges between 50–200 μM [13]; that of copper ions reaches a few μM [14][15]. Thus, the experimental conditions used in our study simulate those of SF in a healthy human joint space.

During the night, when motor activity is minimal, the intra-articular space contains a certain amount of high-molecular-weight HA. Due to a high ascorbate concentration, the transition metals present in SF are in a reduced state. The partial pressure of oxygen is diminished, a status termed 'hypoxia'. In the morning, due to increased motor activity, the SF begins to be supplied by an increased amount of oxygen, a situation termed 're-oxygenation'. This is precisely when excessive oxygen may become 'metabolized' by the reductive action of whatever transition metal present in a healthy knee joint. Due to low contents of both superoxide dismutase and catalase in SF [16], the ·OH radicals become formed by the *Reaction* sequence *1–4*. These are precisely the species that can facilitate the daily catabolism of high-molecular-weight joint HA. The reduced viscosity in the intra-articular space might be the factor that stimulates synoviocytes to produce *de novo* a high-molecular-weight HA, while polymer fragments diffusing outside this space serve as information-carrying species produced by catabolism of joint HA.

It is a well-established fact that the SF of both healthy individuals and patients suffering from rheumatoid arthritis (RA) contains a certain concentration of copper ions [15], and the concentration of total ascorbate in patients with active RA varies markedly [17]. Our finding that the catabolism of HA in the joint SF may be more prominent than previously assumed [1][2] may prove useful for therapeutic considerations in RA.

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Experimental Part

High-molecular-weight HA (M_v 1.215 MDa; *LIFECORE P9710-2* (*Lifecore Biomedical Inc.*, Chaska, MN, USA); 20.0 mg) was dissolved overnight in the dark at r.t. in 0.15M aq. NaCl in two steps. First, 4.0 ml of solvent was added in the morning. The next 3.95-ml portion of the solvent was added after 6 h. On the following morning, 50.0 µl of 16.0 mM ascorbic acid dissolved in 0.15M NaCl was admixed to the formed gel-like soln. at moderate stirring during 30 s. The resulting soln. (8.0 ml) containing HA (2.5 mg/ml) and ascorbic acid (100 µM) was transferred into the *Teflon* cup reservoir of the *Brookfield DV-II+ PRO* rotational viscometer (*Brookfield Engineering Labs.*, Inc., Middleboro, MA, USA). Monitoring of the dynamic viscosity was performed at 25.0°C in 3-min intervals for up to 5 h. For investigating the action of a transition metal surplus, an appropriate amount of $CuCl_2$ was added into the gel-like HA soln. before application of the ascorbic acid. The viscometer spindle rotated at 180 rpm, *i.e.*, at the shear rate equaling 237.6 s^{-1}.

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