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

by **Ladislav Šoltés***[a]), **Monika Stankovská**[a]), **Grigorij Kogan**[b]), **Peter Gemeiner**[b]), and **Robert Stern**[c])

[a]) Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dúbravská cesta 9, SK-84104 Bratislava (fax/phone: +421-2-5477-5928; e-mail: ladislav.soltes@savba.sk)
[b]) Institute of Chemistry, Slovak Academy of Sciences, SK-84538 Bratislava
[c]) University of California, San Francisco, CA-94143-0511, USA

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.

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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.*).

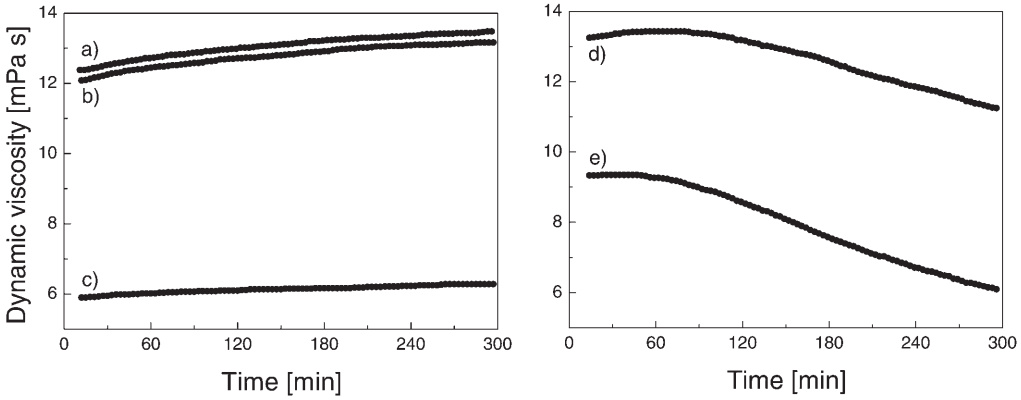

Figure. *Time dependences of dynamic viscosity of hyaluronan solutions.* Samples: *a*) *LIFECORE P9710-2* with addition of 40 μM $CuCl_2$, *b*) *LIFECORE P9710-2* ($M_w = 1.215$ MDa [9]), *c*) hyaluronan 'CPN' ($M_w =$ 659.4 kDa [9]), *d*) *LIFECORE P9710-2* with addition of 100 μM ascorbic acid, *e*)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

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

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

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

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

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_w 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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