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Effects of oxygen-derived free radicals on the molecular weight and the polydispersity of hyaluronan solutions

Barbara M. Praest, Helmut Greiling, Rüdiger Kock *

Institute for Clinical Chemistry and Pathobiochemistry, Medical Faculty, University of Technology Aachen, Pauwelsstr. 30, D-52057 Aachen, Germany

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Abstract

Depolymerization of high-molecular-weight sodium hyaluronan by superoxide anions and hydroxyl free radicals was studied. The changes in number-average and weight-average molecular weight, molecular-weight distribution, polydispersity, and concentration were measured over time by high-performance liquid chromatography on a TSK 6000 PW size-exclusion column coupled to multiangle laser light scattering and refractive index detection. There was no significant change in the elution profile and in the peak height of the refractive index chromatogram for either of the radical species at different reaction times. Furthermore, the weight-average molecular weight of hyaluronan decreased from 1.3×10^6 to 3.0×10^5 g/mol; however, the polydispersity did not significantly increase. These experimental results indicate that superoxide anions and hydroxyl free radicals do not have different modes of action on hyaluronan. Also, both radical species seem to attack hyaluronan randomly from the end of the molecule. © 1997 Elsevier Science Ltd.

Keywords: Depolymerization; Hyaluronan; Light scattering; Molecular weight

1. Introduction

Hyaluronan (HA) is a glucosaminoglycan consisting of alternating units of β-(1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (*N*-acetyl-β-D-glucosamine) and β-(1 → 3)-linked β-D-glucuronic acid. The structure of HA is linear without side-chains. HA is the most ubiquitous glycosaminoglycan of connective tissues and ranges in molecular weight from 4×10^3 to approximately 8×10^6 [1,2]. It has many functions such as regulating oxidative damage [3,4], inhibiting release of proteoglycans from cartilage [5], and modulating the chemotactic, proliferative, and phagocytic response of various inflammatory cells [6–8]. In synovial fluid, high-molecular-weight HA ($6–10 \times 10^6$) shows lubricating and viscoelastic properties [9,10]. The decrease in synovial fluid viscosity was observed during inflammatory joint diseases [11–13], and it has been suggested that

* Corresponding author. Tel.: +49-241-8088681, +49-241-8088670; fax: +49-241-8888512.

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during inflammatory joint diseases oxygen-derived free radicals are responsible for the accelerated depolymerization of HA [14,15]. Sodium HA and HA derivatives have been used as a surgical aid in ophthalmic surgery [16] and in the viscosupplementation therapy of arthritis [5,17–19].

In this paper, the effect of superoxide and hydroxyl free radicals on number-average (M_n) and weight-average (M_w) molecular weight, molecular-weight distribution, polydispersity (M_w/M_n), and concentration of HA were studied to investigate a possible mechanism of HA depolymerization. In particular, the depolymerization of high-molecular-weight HA (sodium salt) was determined by multiangle laser light scattering coupled to a refractive index detector and a size-exclusion column.

2. Experimental

Materials.—High-molecular-weight sodium HA (grade III, from human umbilical cord), xanthine oxidase (grade I, from buttermilk, EC 1.1.3.22), and hypoxanthine were obtained from Sigma Chemical Corp., Deisenhofen, Germany. Dextran standards (molecular weights 148 and 410 kDa) were purchased from PSS, Mainz, Germany. All other chemicals were of the highest purity available from E. Merck, Darmstadt, Germany. Milli-Q HPLC grade water was used after it was filtered through a 0.1 μm filter.

Sample preparation.—HA stock solns (3.4 mg/mL) were prepared by dissolving HA in sterile distilled water. Before use, this soln was dild 1:1 with 50 mM K_3PO_4, pH 7.4. Total concns of HA in soln were also checked after an enzymatic degradation with chondroitin ABC lyase (EC 4.2.2.4) by HPLC.

Time courses of chemically generated hydroxyl free radicals in the depolymerization of HA.—Hydroxyl free radicals were generated by the reaction of $FeSO_4$/EDTA with H_2O_2. HA solns [800 μL of 1.69 mg/mL (in 50 mM K_3PO_4, pH 7.4)] were incubated with 30 μL of H_2O_2 (30% w/v), 100 μL of EDTA (220 μM), 70 μL of K_3PO_4 (50 mM, pH 7.4), and various of fixed concns of Fe^{2+} (7.6, 15, 76, 149, and 764 μM) at 25 °C. Aliquots (100 μL) were collected at different times. The depolymerization of HA was terminated by addition of 100 μL of mannitol (250 mM), and the reaction mixture was analyzed by high-performance size-exclusion–multiangle laser light scattering–differential refractive index detection (HPSEC-MALLS-RI).

Time courses of hypoxanthine – xanthine oxidase generated superoxide free radicals in the depolymerization of HA.—Superoxide free radicals were generated by the reaction of hypoxanthine/O_2/xanthine oxidase in the absence of iron. First 800 μL of HA (1.69 mg/mL) in 50 mM K_3PO_4, pH 7.4, and 200 μL of hypoxanthine (2.3 mM, contained O_2) were mixed and preincubated at 37 °C for 3 min. The reaction was started by addition of various of fixed concns of xanthine oxidase (14.3, 23.5, 28.6, 71.5, and 114 mU/mL). Aliquots (100 μL) were removed at different times; the enzyme was inactivated by boiling. Each mixture was centrifuged and the supernatant was analyzed by HPSEC-MALLS-RI.

The xanthine oxidase activity was assayed by monitoring the absorbance change at 252 nm (for hypoxanthine disappearance).

Analytical methods.—Molecular weight and the molecular-weight distribution of HA were determined by HPSEC-MALLS-RI.

Light scattering was measured at 632.8 nm (5 mW) on a DAWN®DSP-F multiangle laser light scattering (MALLS) (Wyatt Technology, Santa Barbara, CA, USA) equipped with a He–Ne linearly polarized laser and a K5 flow cell ($n = 1.52064$). A Wyatt/Optilab 903 interferometric refractometer (RI) was used as a mass detector. The measuring wavelength of the refractometer was 630 nm; the path length of the RI cell was 10 mm. The operating temperature of both detectors was constantly at 25 °C. The refractive index increment of HA, $dn/dc = 0.155$ mL/g, was determined off-line by flow-injection experiments. The size-exclusion chromatography was performed on a Waters system (600 E pump, 717 autosampler with 200-μL sample loop) equipped with a Toyo Soda guard column TSK PW and a TSK 6000 PW column. The mobile phase was 0.2 M NaCl/0.02 M Na_2HPO_4/0.02 M NaH_2PO_4, pH 7.2; flow rate 0.5 mL/min. The addition of 0.2 M NaCl avoided electrostatic interactions between the column packing material and the samples and also prevented the tendency of HA molecules to aggregate. In particular, the mobile phase was degassed with an on-line degasser (Degasys, DG-1200, uniflow from HPLC Technology Co., UK).

Molecular-weight distributions, M_n, M_w, and M_w/M_n, were established with ASTRA software (v. 4.10 from Wyatt Technology, Santa Barbara, CA, USA).

Pure toluene with a known Rayleigh ratio was used to calibrate the MALLS detector; the calibration

constant of the RI detector was measured experimentally by dextran standards.

3. Results and discussion

Molecular-weight distributions, M_n, M_w, and M_w/M_n, of the HA depolymerization have been characterized by a special analytical method, HPSEC-MALLS-RI. High-molecular-weight HA was depolymerized by hypoxanthine/O_2/xanthine oxidase-induced superoxide free radical generation and by $FeSO_4$/EDTA/H_2O_2-induced hydroxyl free radical production. The decrease of M_w depending on time of HA submitted to treatment with hypoxanthine/O_2/xanthine oxidase (A) and $FeSO_4$/EDTA/O_2 (B) is presented in Fig. 1. The process of depolymerization is accelerated with increasing concentration of radicals. Moreover, the discrepancy between HA depolymerization induced by hypoxanthine/O_2/xanthine oxidase and that induced by $FeSO_4$/EDTA/O_2 is small. However, for the kinetics of xanthine oxidase, high yields of free radicals were observed at the beginning of the reaction, so that the HA depolymerization was more effective. The depolymerization did not proceed further after 12 min and was shown to be independent of radical concentrations and radical species.

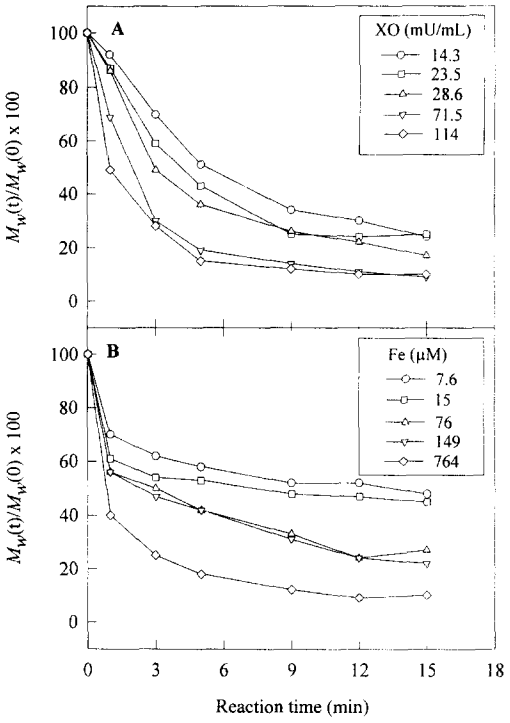

Fig. 1. Effect of different (A) xanthine oxidase activities (14.3, 23.5, 28.6, 71.5, and 114 mU/mL) and (B) Fe^{2+} concentrations (7.6, 15, 76, 149, and 764 μM) on M_w of HA. The HA depolymerization was measured by HPSEC-MALLS-DRI.

Fig. 2(A) and Fig. 3(A) show the difference in elution time and especially in the peak height of the 90° light scattering corresponding to the decrease of the molecular weight of HA during the incubation time. The HA depolymerization was performed with superoxide free radicals (A) as well as with hydroxyl free radicals (B). The concentrations of Fe^{2+} and XO in Figs. 2 and 3 were chosen as an example for the degradation process; all other concentrations of Fe^{2+} and XO have a similar effect on HA. In contrast to the 90° scattering signal, no change in elution profile and height of the peak of the concentration signal

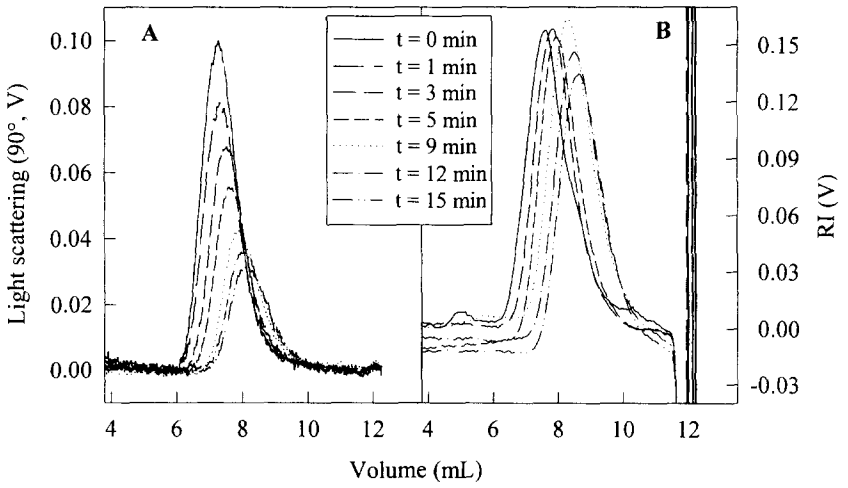

Fig. 2. Elution profile of HA in the presence of superoxide free radicals generated by the hypoxanthine/O_2/xanthine oxidase system. HA was incubated with hypoxanthine, O_2, and xanthine oxidase (14.3 mU/mL), and the depolymerization was measured by HPSEC-MALLS-RI at various times. (A) 90° light scattering chromatogram. (B) Concentration (RI) signal.

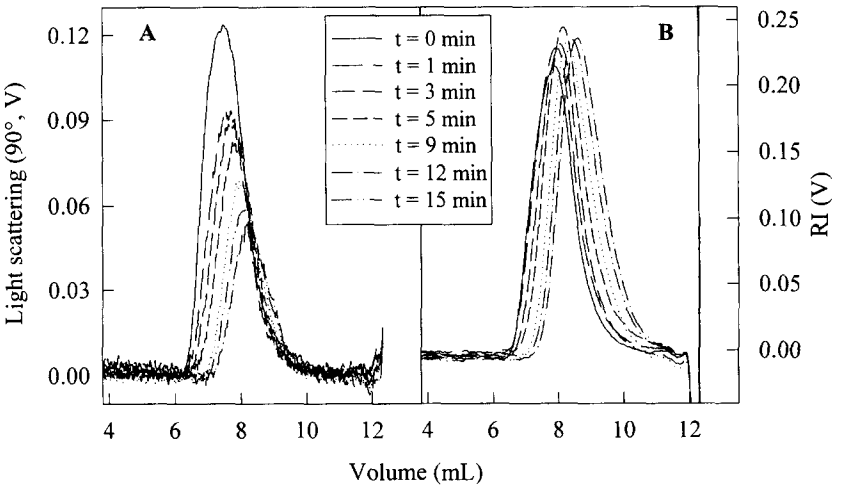

Fig. 3. Elution profile of HA in the presence of hydroxyl free radicals generated by the $FeSO_4/EDTA/H_2O_2$ system. HA was incubated with Fe^{2+} (76 μM), EDTA, and H_2O_2, and the depolymerization was measured by HPSEC-MALLS-RI at various times. (A) 90° light scattering chromatogram. (B) Concentration (RI) signal.

(RI) was observed [Fig. 2(B) and Fig. 3(B)]. Both types of free radicals seem to attack HA randomly from the end of the molecule so that the change in molecular weight is continuous for a given concentration. Strand breaking in the middle did not occur; in this case M_w/M_n should have increased more significantly (Table 1). Saari investigated the effect of radicals on HA by a simple SEC combined with a ^{125}I–HA binding protein assay [20]. He reported that only hydroxyl free radicals reduced the molecular weight of HA in synovial fluid; superoxide free radicals and hydrogen peroxide caused only a decrease of the HA peak without changes in the retention time. In this study large amounts of protein were present in the measurements. UV detection of HA in the presence of high-molecular-weight protein may give misleading results, because UV detection is more dependent on the composition of the analyte than is refractive index detection.

Differential molecular-weight distributions of HA after treatment with hypoxanthine/O_2/xanthine oxidase (14.3 mU/mL) for 1 min (A) and 15 min (B) are shown in Fig. 4. These results indicate a significant reduction in molecular weight after 15 min reaction time of HA. The initial distribution is broadened by the depolymerization process, while the shape is almost unchanged.

The depolymerization effect of superoxide free radicals and hydroxyl free radicals is accompanied by a significant decrease in molecular weight (Fig. 1), within a small increase in M_w/M_n of HA (Table 1). Low changes of M_w/M_n with increasing yields of radicals were observed. For the highest concentration of superoxide free radicals, M_w changed from ini-

Table 1
Depolymerization of HA for various times by superoxide and hydroxyl free radicals. The M_w/M_n were measured by HPSEC-MALLS-RI

Reaction time (min)	M_w/M_n									
	(14.3) [a]	(23.5) [a]	(28.6) [a]	(71.5) [a]	(114) [a]	(7.6) [b]	(15) [b]	(76) [b]	(149) [b]	(764) [b]
0	1.32	1.18	1.18	1.17	1.17	1.14	1.14	1.12	1.13	1.12
1	1.18	1.17	1.15	1.21	1.36	1.12	1.12	1.11	1.11	1.16
3	1.23	1.17	1.25	1.21	1.27	1.11	1.10	1.12	1.14	1.12
5	1.25	1.17	1.23	1.26	1.35	1.10	1.10	1.17	1.14	1.28
9	1.30	1.39	1.29	1.41	1.29	1.13	1.17	1.20	1.24	1.30
12	1.34	1.36	1.27	1.34	1.23	1.13	1.16	1.24	1.27	1.37
15	1.44	1.34	1.43	1.44	1.31	1.12	1.15	1.24	1.25	1.36

[a] Xanthine oxidase activity in mU/mL.
[b] Fe^{2+} concentration in μM.

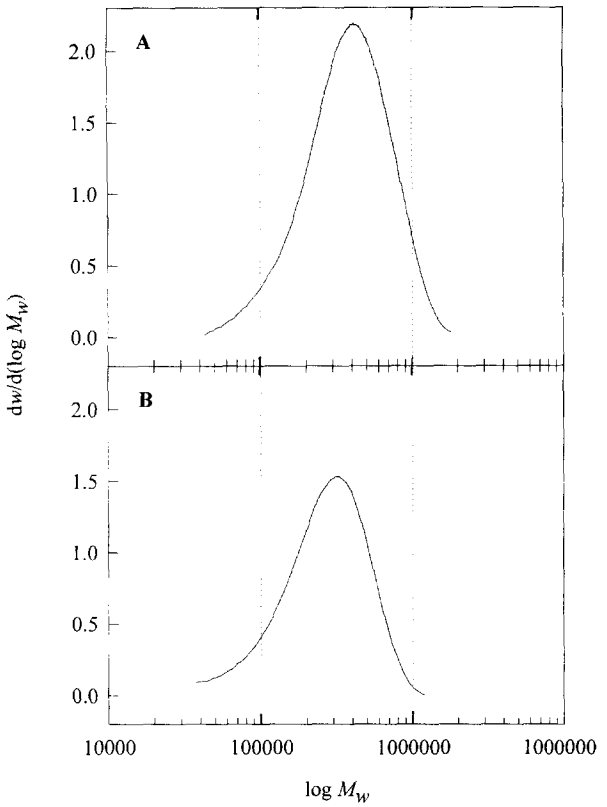

Fig. 4. Differential molecular-weight distribution curves of HA depolymerization by superoxide free radicals generated by hypoxanthine, O_2, and xanthine oxidase (14.3 mU/mL). (A) Depolymerization of HA for 1 min. (B) Depolymerization of HA for 15 min.

tially 1300 to 300 kg/mol after 15 min depolymerization, whereas M_w/M_n changed from 1.17 to 1.31.

Nevertheless, it seems that HA reacts with both superoxide free radicals and hydroxyl free radicals in the same manner. In vivo superoxide free radicals react with various components and generate other oxygen-derived free radicals including singlet oxygen and hydroxyl free radicals [15]. High-molecular-weight HA is not susceptible to depolymerization by lysosomal carbohydrases. However, after partial depolymerization of HA by free radicals, lysosomal enzymes cleave HA. Although several investigators have studied the HA depolymerization, including studies on the effect of free radicals on HA by viscosity measurements [15,21] and molecular-weight measurements [3,4,22], their results were limited by the sensitivity of their analytical methods. In the present study a promising method for the determination of molecular weight and molecular-weight distribution of HA has been described. The instrumental configuration as described has several advantages. MALLS allows not only the accurate determination of the molecular weight of large polymers but also facilitates the quantification as well as the analysis of M_w/M_n simultaneously. This method may be useful in the development on HA derivatives. HA derivatives with optimized resistance against free radical-induced depolymerization are needed as more effective agents for the medical treatment of arthritis [19].

In addition, we have investigated in vitro a possible mechanism of HA depolymerization. Our results indicate that both types of free radicals attack HA randomly and that HA is depolymerized into smaller molecules of similar size and low-molecular-weight products.

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