Suppression of Ischemic Edema in Mice by Manganese-Hyaluronate Conjugate

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ABSTRACT—Manganese-hyaluronate conjugate (Mn-HA) was synthesized from a diethylenetriaminepentaacetic acid derivative of hyaluronic acid and manganese ion. The conjugate markedly scavenged superoxide anion in vitro and exhibited much higher anti-inflammatory activity than superoxide dismutase in suppressing paw edema in mice when intravenously injected 30 min before the initiation of ischemia.

Keywords: Manganese-hyaluronate conjugate, Ischemic edema, Reactive oxygen species

Reactive oxygen species including superoxide anion (O_2^-), hydroxyl radical ($OH\cdot$) and hydrogen peroxide (H_2O_2) have been implicated as mediators of a wide variety of diseases such as radiation injury, hyperoxemia and inflammation, especially arthritis (1). In the case of ischemia-reperfusion, these oxygen intermediates are suggested to be generated by xanthine oxidase in endothelial cells as well as by NADPH oxidase and myeloperoxidase in accumulated phagocytes, and they play a major role in the reperfusion injury of tissues (2).

To remove these harmful oxygen species, various antioxidants and radical scavengers have been widely investigated. Superoxide dismutase (SOD), a unique metal enzyme containing copper and zinc (Cu/Zn) or manganese (Mn) at the catalytic site, has an activity of scavenging superoxide anion by dismutation into oxygen and hydrogen peroxide. Because of this ability, SOD has been experimentally or clinically applied for the treatment of inflammatory diseases (3, 4). In addition, various copper complexes synthesized as SOD model compounds show the superoxide anion-scavenging activity (5). However, these compounds as well as Cu/Zn-SOD could have a very short half-time in blood circulation and may potentially generate extremely reactive hydroxyl radical from hydrogen peroxide by a Haber-Weiss reaction (6). Although Mn-SOD does not form the harmful

byproduct, mammalian Mn-SOD may be less stable than Cu/Zn-SOD and the bacterial one may cause severe immune responses.

To overcome these disadvantages, we prepared a Mn complex coupled with hyaluronic acid (HA), a biodegradable and non-immunogenic biopolymer of typical glycosaminoglycan with a repeating disaccharide structure. HA is susceptible to degradation by reactive oxygen species and may protect tissues from oxidative damage in patients with inflammatory diseases (7). The present study deals with the oxygen radical-scavenging activity of manganese-hyaluronate conjugate (Mn-HA) and its suppressive effect on ischemic edema.

Manganese-hyaluronate conjugate (Mn-HA) was prepared as shown in Fig. 1. HA with molecular weight of 1,000,000 from rooster comb (Seikagaku Co., Tokyo) has approximately 2,500 repeating dimeric units of *N*-acetyl-D-glucosamine and D-glucuronic acid. The carboxyl groups of HA were coupled with ethylenediamine dihydrochloride by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; Dojindo, Kumamoto). The amine derivative of HA (NH_2 -HA) was then coupled with diethylenetriamine-*N,N,N',N',N'*-pentaacetic dianhydride (DTPA anhydride, Dojindo) to prepare the DTPA derivative of HA (DTPA-HA). Mn-HA was obtained by adding manganese(II) chloride to the DTPA-HA solution. In each step, excess reagents were removed by dialysis against 0.15 M NaCl. The amount of HA was

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calculated on the basis of uronic acid determined by the carbazole-sulfate method (8).

The molar ratios of ethylenediamine and DTPA coupled to the carboxyl groups of HA were determined to be 6.4 mol% and 6.0 mol%, respectively, by the ninhydrin method. The content of Mn in the Mn-HA was 5.5 mol% as determined by an inductively coupled plasma (ICP) atomic emission spectrometer (SPS7000A; Seiko Instruments, Inc., Chiba), and the value closely agreed with that of DTPA content. Further dialysis of the conjugate against 0.15 M NaCl could not decrease its Mn content. On the other hand, Mn ion was released from the mixture of MnCl₂ and HA during an equilibrium dialysis against 0.15 M NaCl. Therefore, Mn in the conjugate forms a

Fig. 1. Preparation of manganese-hyaluronate conjugate (Mn-HA). To HA (1 g, 2.5 mmol as COOH group) dissolved in 200 ml of H₂O was added ethylenediamine (5 mmol) and EDC (10 mmol). To NH₂-HA was then added DTPA anhydride (1.5 mmol) in 200 ml of 0.1 M HEPES-HCl containing 0.15 M NaCl (pH 7.0), followed by the addition of MnCl₂ (1.5 mmol). In each step, sufficient dialysis against 0.15 M NaCl was carried out.

stable chelate complex with DTPA, which may be due to the high complex stability constant between DTPA and Mn²⁺ (log K = 16) (9).

To estimate the stability of the conjugate in blood circulation, 1 mg of Mn-HA dissolved in 0.4 ml of saline was injected into a tail vein of male ddY mice (10-weeks-old; Kiwa Laboratory Animals, Tokyo). At given times, the Mn concentrations in serum were determined by the ICP method (data not shown). Forty minutes after the injection, approximately 10% of the initial concentration of Mn remained in the serum. On the contrary, the concentration fell to 10% within 2 min after the injection of free Mn ion or a mixture of Mn and HA.

Figure 2a shows the ESR spectra of superoxide anion in the presence of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Dojindo). The intensity of the control signals was clearly diminished by adding Mn-HA in a dose-dependent manner, but was not by HA only. The signals were also diminished by adding MnCl₂ or Mn-SOD from *Bacillus stearothermophilus* (Seikagaku Co.). The 50% inhibitory concentration (IC₅₀) of Mn-HA determined by the cytochrome *c* method (10) was 6×10^{-6} M based on the Mn concentration. The suppression of cytochrome *c* reduction could not be observed by adding HA up to 1.7 mg/ml. The values of MnCl₂ and Mn-SOD were 1×10^{-6} M and 2×10^{-8} M, respectively. The superoxide scavenging activity of Mn-HA is similar to that of free Mn ion. In the case of hydroxyl radical, the intensity of the ESR signals (Fig. 2b) was partially diminished with Mn-HA, while a clear decrease in the intensity could not be observed with HA, MnCl₂ or Mn-SOD.

The anti-inflammatory activity of Mn-HA was determined using an experimental ischemia model (11). Mn-HA was intravenously injected to male ddY mice (8-weeks-old). After 30 min, a suture was tied around the right hind leg of each mouse. One end of the suture was fixed while the other end was tensed with a weight of 300 g. After 40 min of ischemia, the suture was removed and blood flow was reperfed for 20 min. The thickness of the instep of the ischemic foot was measured with slide calipers, which was normalized as a percentage of increase by comparing it with that measured before the ischemia.

As a result of ischemic paw edema, the thickness of the mouse paw was increased by $30.8 \pm 3.6\%$ in comparison with that before ischemia (Fig. 3). The administration of Mn-HA markedly suppressed the edema; the increase in the foot-pad thickness was only $10.7 \pm 5.2\%$. On the contrary, the thickness was increased by $21.2 \pm 4.8\%$, $28.3 \pm 2.8\%$ and $30.1 \pm 4.2\%$ when mice were treated with HA, free Mn ion, and a mixture of Mn and HA, respectively, in the same amount as Mn-HA on a molar basis. Furthermore, the foot-pad thickness was also increased

Fig. 2. ESR spectra of superoxide anion (a) and hydroxyl radical (b) in the presence of Mn-HA and DMPO. The Mn concentration of each sample is indicated in parentheses. Superoxide anion was generated by the hypoxanthine-xanthine oxidase system (10) containing 30 mM DMPO, and hydroxyl radical was generated from ferrous sulfate (0.1 mM), hydrogen peroxide (1.5 mM) and DMPO (30 mM) in 0.1 M phosphate buffer, pH 7.5. ESR was carried out with a Jeol FR-80 spectrometer (Tokyo) under the following conditions: modulation frequency (50 or 200 kHz), modulation amplitude (0.079 mT), scanning field (336.1 ± 5 mT), receiver gain (5×100), response time (0.1 sec), sweep time (1 min) and microwave power (4 mW) at room temperature. The xanthine oxidase activity monitored by urate formation (15) was not inhibited by the conjugate.

($25.4 \pm 1.8\%$) by a injection of Mn-SOD at the same SOD activity as Mn-HA.

Ischemia-reperfusion paw edema, a model of superoxide anion-related inflammatory diseases, is reported to be suppressed by SOD injection just before the ischemia (11). The suppression of edema by Mn-HA suggested its effective scavenging of harmful radicals in vivo as demonstrated in our in vitro experiments. As the suppressive effect on the edema was only observed with Mn-

Fig. 3. Suppression of ischemia-reperfusion paw edema by Mn-HA. Male ddY mice were treated with saline ($n=7$) or a sample: Mn-HA (50 μg , $n=7$), HA (50 μg , $n=5$), MnCl₂·4H₂O (1.6 μg , $n=5$), a mixture of HA and MnCl₂·4H₂O (50 and 1.6 μg , respectively, $n=4$), and Mn-SOD (1 U, $n=6$). Each bar indicates a mean $\pm$ S.D. * $P < 0.01$ vs control group by Student's *t*-test.

HA but not with a mixture of Mn and HA or Mn-SOD, the advantage may be mainly due to the longer clearance time of Mn-HA in blood circulation. Although the radical scavenging activity of HA was hardly detected in vitro, ischemic paw edema was slightly suppressed by HA only. As is expected, HA was endowed with superoxide anion scavenging activity by the conjugation with Mn. The suppression of paw edema by Mn-HA was significantly effective in comparison with that by HA only. Moreover, Mn-HA seems to have a hydroxyl radical scavenging activity.

HA, bound to proteoglycan in amorphous intercellular matrix, is reported to act as an anti-inflammatory molecule (12) by inhibiting the adherence of immune complexes to neutrophils (13) and by protecting tissues from the attachment of inflammatory mediators (14) in inflamed joints. Mn-HA with an enhanced radical scavenging activity may exhibit more suppressive activity by a local injection to the inflammatory regions.

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