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Anti-inflammatory activity of superoxide dismutase conjugated with sodium hyaluronate

Katsukiyo Sakurai[1], Kyousuke Miyazaki[2], Yoh Kodera[1], Hiroyuki Nishimura[1], Masao Shingu[3] and Yuji Inada[1]*

[1]*Toin Human Science and Technology Centre, Department of Materials Science and Technology, Toin University of Yokohama, 1614 Kurogane-cho, Aoba-ku, Yokohama 225, Japan*
[2]*The Tokyo Research Institute, Seikagaku Corporation, 3-1253 Tateno, Higashiyamato, Tokyo 207, Japan*
[3]*Department of Internal Medicine, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumihara, Beppu 874, Japan*

Superoxide dismutase (SOD) from bovine erythrocytes was conjugated with sodium hyaluronate (HA) with a mean molecular weight of 10^6 to have greater anti-inflammatory activity *in vivo.* Amino groups of SOD were coupled with carboxyl groups in the hyaluronate molecule using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The HA-SOD conjugate was composed of 1.5 mol of SOD molecule per 1 mol of hyaluronate on the average, and retained 70% of the activity of unmodified SOD. The conjugate was essentially non-immunogenic in mice, and exhibited much higher anti-inflammatory activities than HA or SOD in models of inflammatory diseases such as ischemic oedema of the foot-pad in mice, carrageenin-induced pleurisy and adjuvant arthritis in rats.

***Keywords*: superoxide dismutase, sodium hyaluronate, glycoconjugate, ischemic oedema, carrageenin-induced pleurisy, adjuvant arthritis**

Introduction

Superoxide dismutase (SOD) catalyses the dismutation of highly reactive superoxide anion to molecular oxygen and hydrogen peroxide. Catalase catalyses the conversion of hydrogen peroxide into molecular oxygen and water. These two enzymes have been tested as potential therapeutic agents for adverse inflammatory reactions mediated by superoxide anion, such as allergic reactions [1], ischemic myocardial damage [2], Crohn's disease [3] and virus infection [4]. However, the clinical applications of these enzymes have been limited because they are rapidly cleared from the circulation and induce an immune reaction when injected *in vivo*. Covalent attachment of polyethylene glycol has been studied as an effective technique to solve the problems of therapeutic enzymes [5, 6]. SOD chemically modified with polyethylene glycol (PEG) has a prolonged half-life in the circulation of rodents. It does not react with anti-SOD antibodies and, on intramuscular or intravenous injection, elicits antibodies neither to itself nor to unmodified SOD [7]. PEG-SOD in combination with PEG-catalase has been successfully administered to experimental models of hyperoxia [8, 9], ischemia-reperfusion [10–12] and carrageenin-induced pleurisy [13] in rodents.

Sodium hyaluronate (HA) has been known as a unique anti-inflammatory polymer with the capacity to scavenge hydroxyl radical [14–16], that is highly reactive and thought to be involved in the tissue injury associated with inflammatory reaction. HA consists of repeating dimeric units of *N*-acetyl-D-glucosamine and D-glucuronic acid with alternating $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 4$ linkages. HA with molecular weights ranging from 0.5 to 1×10^6 has been clinically applied to the treatments of osteoarthritis of the knee and periarthritis of the shoulder as a biodegradable and non-immunogenic biopolymer with anti-inflammatory activities [17]. In the present studies, we prepared HA-SOD conjugates with the hope that the coupling of SOD with superoxide-dismuting activity and HA with hydroxyl radical-scavenging capacity may yield a unique anti-inflammatory agent with improved biocompatibilities.

Materials and methods

Materials

Female Swiss-Webster mice, male ddY mice, and male Sprague-Dawley rats were purchased from SLC Inc., Shizuoka, Japan. Lewis rats were purchased from Charles River Inc., Atsugi, Japan.

* To whom correspondence should be addressed. Tel: + 81-45-974-5060; Fax: + 81-45-972-5972.

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Sodium hyaluronate from rooster comb (molecular weight 10^6) was obtained from Seikagaku Co., Tokyo, Japan. Xanthine, cytochrome *c* from horse heart, Cu/Zn-SOD from bovine erythrocytes (molecular weight 31 200 [18]; specific activity 3200 U mg^{-1} protein) and Mn-SOD from *Escherichia coli* (specific activity 3300 U mg^{-1} protein) were purchased from Sigma Chemical Co., St Louis, MO. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) was purchased from Dojindo Laboratories, Kumamoto, Japan. Xanthine oxidase was purchased from Boehringer Mannheim GmbH, Mannheim, Germany. All other chemicals were of analytical grade commercially available.

Conjugation of SOD with sodium hyaluronate

Six grams of sodium hyaluronate (15 mmol as COOH) was dissolved in 600 ml of distilled water, and its pH adjusted to 4.8 by adding 0.1 M hydrochloric acid. To this solution was added stepwise 1.5 mmol of EDCI at 4 °C, and the pH was maintained at 4.8 by titration with 0.1 M hydrochloric acid.

To this reaction mixture containing an *O*-acyl-isourea derivative of HA [19] was added bovine Cu/Zn-SOD (288 mg) or *E. coli* Mn-SOD (288 mg) solution, and the mixture was maintained at 4 °C for 20 h. The reaction mixture was ultrafiltered against 0.5 M NaCl to remove the unreacted SOD using an omega series PVS membrane (EYELA, Tokyo, Japan; fractionating molecular weight of 3×10^5), and subsequently dialysed against distilled water and lyophilized. HA-SOD was obtained as a white powder (5.88 g).

Analytical methods

The amount of HA and SOD in the conjugate was determined as described by Bitter and Müir [20], and Lowry [21], respectively. The free amino groups in the conjugate were determined by the TNBS method [22] Metal contents of the conjugate were determined with a Hitachi Z-8100 atomic absorption spectrometer (Tokyo, Japan). The molecular weight of the conjugate was determined by high performance liquid chromatograph equipped with G6000PW columns (7.5 mm ID × 30 cm × 2, Tosoh Co., Tokyo, Japan) using 0.2 M NaCl as an eluent at a flow rate of 0.5 ml min^{-1}.

The enzymic activity of SOD was measured as follows [23]. The reaction mixture (3 ml) contained cytochrome *c* (10 μM), xanthine (50 μM), ethylenediaminetetraacetic acid (0.1 mM), xanthine oxidase (about 10 nM) and a sample of SOD in 50 mM potassium phosphate buffer (pH 7.8). The reduction of cytochrome *c* due to the generation of superoxide anion was determined as an absorbance increase at 550 nm. One unit of SOD activity is defined as the amount of SOD required for 50% inhibition of the rate of cytochrome *c* reduction.

Immunogenicity

Four female mice (Swiss-Webster, 6 weeks old) were immunized by 12 weekly intraperitoneal injections of either SOD or HA-SOD (0.1 mg of protein in 0.05 M phosphate buffered saline, pH 7.3, PBS). Levels of IgG-class anti-SOD or anti-HA-SOD antibodies at week 12 were determined by ELISA [24].

Ischemic paw oedema

Oedema of the foot-pad was induced by ischemia (40 min) and recirculation (20 min) in 8-week-old male ddY mice [25]. Thirty minutes before the initiation of ischemia (Experiment 1), each group of five mice was pretreated with a sample solution (200 μl) containing either SOD (10 000 U kg^{-1}), HA (27 mg kg^{-1}), SOD plus HA (10 000 U kg^{-1} and 27 mg kg^{-1}) or HA-SOD (500 or 2000 U kg^{-1}). Alternatively, each group of mice was pretreated similarly just before the initiation of the ischemia (Experiment 2). Twenty minutes after recirculation, the thickness of the foot-pad of the animal was measured with slide calipers.

Carrageenin-induced pleurisy

Pleurisy in Sprague-Dawley rats (male, 6 weeks old) was induced by the intrapleural injection of carrageenin according to the method of Oh-ishi [26]. In Experiment 1, each group of eight rats received intrapleural injection (200 μl) of either HA-SOD (500 U kg^{-1}), SOD (10 000 U kg^{-1}), indomethacin (10 mg kg^{-1}) or PBS 30 min before the intrapleural injection of carrageenin (10 mg kg^{-1}). In Experiment 2, each group of mice received an intravenous injection of either HA-SOD (4000 U kg^{-1}), SOD (4000 U kg^{-1}), indomethacin (10 mg kg^{-1}) or PBS immediately before the intrapleural injection of carrageenin (1 mg kg^{-1}).

A volume of pleural fluid was measured 4 h after the carrageenin injection. Protein concentration in the pleural fluid was measured by the method of Lowry [21] and number of leukocytes in the pleural fluid was measured by an EKDS hemocytometer (Irika Kogyo, Ltd., Tokyo, Japan) after staining with Türk solution.

Adjuvant arthritis

Adjuvant arthritis in rats was induced according to Shingu [27]. Lewis inbred rats (male, 6 weeks old, 200 ± 15 g) were injected with complete adjuvant (0.1 ml) containing *Mycobacterium tuberculosis* H37/Ra (Difco, Detroit, MI) in the tail at day 0. A group of 6 rats was injected weekly with 0.2 ml of the solution containing either HA-SOD (90 U kg^{-1}), SOD (5000 U kg^{-1}), HA (10 mg kg^{-1}), or saline into the periarticular soft tissue of the right ankle three times a week from day 3 to day 84. The swelling of the

foot joint was measured using a water plethysmometer three times a week and a weekly mean foot volume was calculated in each group.

Results

Properties of HA-SOD conjugates

Physicochemical properties of HA-SOD prepared by conjugating SOD from bovine erythrocytes or that from *Escherichia coli* with *O*-acyl-isourea derivative of HA are summarized in Table 1. No trace of unconjugated SOD was detected in the preparations by cellulose acetate membrane electrophoresis and by gel filtration chromatography (data not shown). Unmodified bovine SOD has a molecular weight of 31 200 and has two atoms each of Cu and Zn per molecule. HA-SOD (bovine) conjugate, in which four out of 24 amino groups in the molecule were modified, retained 70% of the activity of the unmodified enzyme. Atomic absorption analysis revealed that 1.7 and 1.8 mol mol^{-1} protein of Cu and Zn, respectively, were retained in the conjugate. The HA-SOD conjugate was composed of 1.6 mol of SOD molecule per 1 mol of hyaluronate on the average. On gel filtration chromatography, HA-SOD (bovine) showed nearly the same elution profile as unconjugated HA indicating the mean molecular weight of 1×10^6. HA-SOD from *E. coli*, which has Mn atom instead of Cu or Zn, was also coupled with HA. The conjugate exhibited nearly the same activity and physicochemical properties as that of bovine SOD.

Immunological properties of HA-SOD

Since bovine SOD was not as immunogenic as *E. coli* SOD in Swiss-Webster mice, effects of HA-conjugation on immunoreactivity and immunogenicity of SOD were tested only for SOD from *E. coli*. As shown in Table 2, immunization with *E. coli* SOD induced high titres of anti-SOD antibodies in Swiss-Webster mice. The immunoreactivity of SOD was shown to be greatly reduced by conjugating with HA. Furthermore, immunization of mice with HA-SOD induced only marginal level of antibodies towards SOD or HA-SOD.

Suppression of ischemic paw oedema by HA-SOD

Ischemic paw oedema was induced in ddY mice and the swelling of foot-pads was measured 20 min after the recirculation. Anti-inflammatory potency of the HA-SOD conjugates was measured as its activity to suppress the swelling. By injecting unmodified SOD (10 000 U kg^{-1}) 30 min before the initiation of ischemia, only the low level of suppression (24%) was observed (Figure 1, Exp. 1a). Injection of HA alone and HA plus SOD induced 22 and 16% suppression, respectively (Exp. 1b and c). HA-SOD showed much higher suppressive effect than unmodified SOD. As little as 500 U kg^{-1} of HA-SOD could induce 48% suppression (Exp. 1d). By injecting 2000 U kg^{-1} of HA-SOD, 67% of the swelling was suppressed (Exp. 1e). Unmodified SOD could not exhibit effective suppression presumably because it was rapidly cleared from the circulation. Injection of 10 000 U kg^{-1} of unmodified SOD immediately after the initiation of ischemia was significantly more suppressive (35%, Exp. 2a) than that 30 min before ischemia (24%, Exp. 1a). Injection of HA-SOD (500 U kg^{-1}) just before ischemia (Exp. 2b) was nearly as effective as that obtained by HA-SOD treatment 30 min before ischemia (Exp. 2b).

Suppression of carrageenin-induced pleurisy by HA-SOD

It is proposed that carrageenin-induced pleurisy is a model of inflammatory diseases mediated by reactive oxygen species [28]. Rats were pretreated intrapleurally either with PBS, HA-SOD or SOD 30 min before the carrageenin injection, and suppressive effects of each agent was scored (Figure 2, Exp. 1). The pleurisy indexes as measured by the volume of pleural fluid, total protein and number of leukocytes in the fluid were all significantly suppressed by

Table 1. Chemical properties of HA-SOD.

Properties	*HA-SOD (bovine)*	*HA-SOD* (E. coli)	*SOD (bovine)*
HA content (w/w)%	92.5	92.6	0
Cu(mol mol^{-1} protein)	1.7	0.0	2.0
Zn(mol mol^{-1} protein)	1.8	0.0	2.0
SOD content (w/w)%	4.66	4.58	100
Mean MW	1×10^6	1×10^6	31 200
Free NH_2 (mol mol^{-1} protein)	20.0	20.0	24.0
Enzymatic activity			
(U mg^{-1} protein)	2200	2000	3100
(% to SOD)	70	67	100

Table 2. ELISA antibody titers from Swiss-Webster female mice immunized with SOD (*E. coli*) or HA-SOD (*E. coli*).

Antiserum against[a]	*Route of injection*	*Tested against antigen*	*Antibody titer*
SOD(*E. coli*)	i.p.	SOD	1 : 150 000
		HA-SOD	1 : 2500
HA-SOD(*E. coli*)	i.p.	SOD	1 : 90
		HA-SOD	1 : 150

[a] Swiss-Webster mice were intraperitoneally injected with SOD from *E. coli* or HA-SOD 12 times weekly.

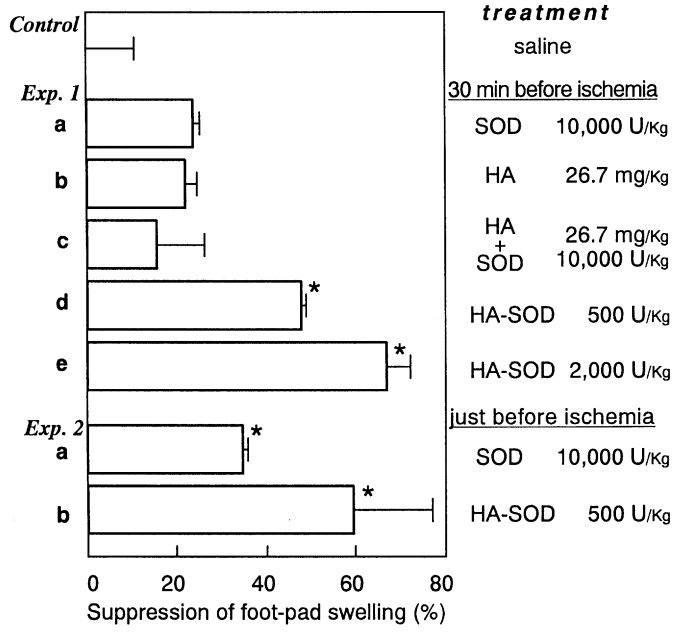

Figure 1. Suppressive effect of HA-SOD on paw oedema induced by post-ischemic reperfusion. The thickness of the foot-pad increased from 2.2 ± 0.0 mm to 3.1 ± 0.1 mm. The sample was intravenously injected 30 min before the initiation of ischemia (Exp. 1) or just before the ischemia (Exp. 2). HA-SOD conjugate (500 U and 2000 U) contains approximately 4 mg and 18 mg of HA, respectively. Each bar indicates mean ± SD. *The suppression of swelling was statistically significant ($p < 0.01$, *t*-test).

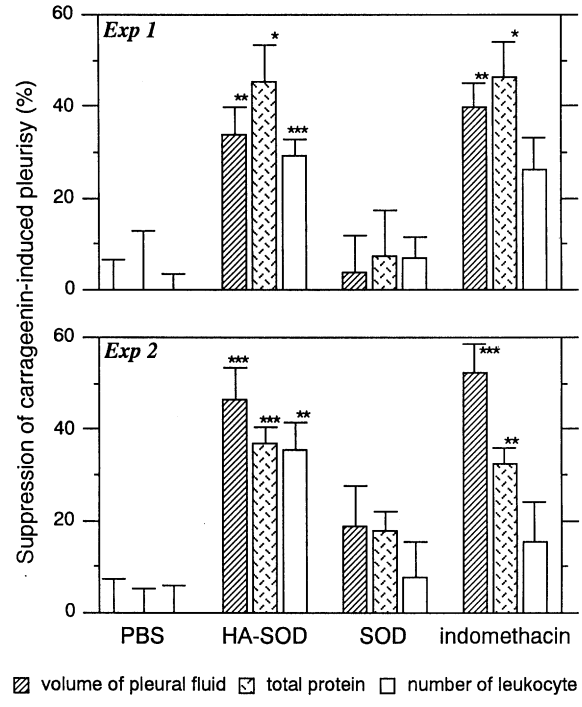

Figure 2. Suppression of pleural exudate accumulation and leukocyte migration by HA-SOD during rat pleurisy. Experiment 1: PBS, HA-SOD (500 U kg^{-1}), SOD (10 000 U kg^{-1}) or indomethacin (10 mg kg^{-1}) was injected intrapleurally 30 min before the intrapleural injection of 10 mg of carrageenin. In PBS-treated rats, the volume of pleural fluid, amount of protein and number of leukocytes was 0.83 ± 0.15 ml, 22 ± 8 mg and $5.5 \pm 0.5 \times 10^7$ cells, respectively, 4 h after the carrageenin injection. Experiment 2: PBS, HA-SOD (4000 U kg^{-1}), SOD (4000 U kg^{-1}) or indomethacin (10 mg kg^{-1}) was injected intravenously just before the intrapleural injection of 1 mg of carrageenin. The volume of pleural fluid, amount of protein and number of leukocytes were 0.16 ± 0.03 ml, 7 ± 1 mg and $1.3 \pm 0.2 \times 10^7$ cells, respectively. Each bar indicates mean ± SE. *$p < 0.10$, **$p < 0.05$, ***$p < 0.01$ (*t*-test).

pleural administration of HA-SOD (500 U kg^{-1}). Administration of SOD (10 000 U kg^{-1}) alone could not induce significant suppression. The levels of suppression induced by HA-SOD (500 U kg^{-1}) was comparable to those induced by a high dose in indomethacin (10 mg kg^{-1}). Similar results were also observed when rats were pretreated intravenously (Exp. 2).

Suppression of adjuvant arthritis by HA-SOD

Arthritis was induced in Lewis rats by a single injection of *Mycobacterium tuberculosis* H37/Ra emulsified in paraffin oil. The polyarthritis appeared in about 90% of rats at week 3. The foot-pad volume reached at maximum levels of 3.0–3.3 ml between weeks 4 and 12 (curve A in Figure 3). In normal rats that had not been injected with adjuvant, their foot-pad volumes were between 1.6–1.8 ml (curve E). Repetitive treatments with high dose of SOD (5000 U kg^{-1}) or HA (10 mg kg^{-1}) alone did not suppress the swelling of foot-pads (curves C and D, respectively). The anti-inflammatory activity of the mixture of SOD (5000 U kg^{-1}) and HA (10 mg kg^{-1}) was also as low as that of SOD or HA alone (data not shown). Contrary to this, HA-SOD induced significant suppression. In rats that received injections of HA-SOD (90 U kg^{-1}) three times a week, swelling of foot joints was suppressed to the levels of 2.1–2.5 ml (curve B).

Discussion

SOD from bovine erythrocytes in which 20% of the amino groups were conjugated with HA retained 70% of the original superoxide anion-scavenging activity as measured by the cytochrome *c* method. Therefore the activity of HA-SOD is comparable to those reported for PEG-SODs (90–50%) in which 20–90% of the amino groups were coupled with a PEG derivative (molecular weight 5000) [7–13].

HA-SOD conjugate exhibited a much higher therapeutic activity than unconjugated SOD in experimental models of inflammatory diseases. In contrast, SOD or HA alone,

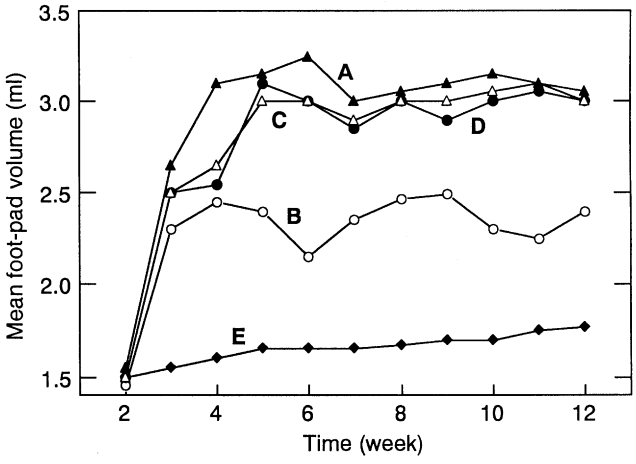

Figure 3. Anti-inflammatory effects of HA-SOD on adjuvant arthritis. Adjuvant arthritis was induced in Lewis rats by a single injection of *M. tuberculosis* H37/Ra emulsified in paraffin oil. The test sample was administered three times a week and a mean foot volume was calculated in each group weekly. Saline: Curve A (▲), HA-SOD 90 U kg^{-1}: curve B (○), HA 10 mg kg^{-1}: curve C (△), SOD 5000 U kg^{-1}: curve D (●), normal: curve E (◆).

or the mixture of SOD and HA was not as effective as HA-SOD conjugates. In artificially induced paw ischemia in mice and carrageenin-induced pleurisy in rats, superoxide anion radical has been shown to be involved in the development of inflammatory reaction [10–13]. Hydroxyl radical may also be generated and cause the tissue injury [29, 30]. It is possible that combined scavenging abilities of superoxide anion radical by SOD and of hydroxyl radical by HA [14–16], both contributed to the greater antiinflammatory potentials of HA-SOD. As hydroxyl radical is also known to be generated as a by-product in the Cu/Zn-SOD-catalysed reaction [31, 32], HA-SOD conjugates with the capacity to scavenge these reactive oxygen radicals should serve as safer anti-inflammatory agents than SOD. The higher anti-inflammatory activity of HA-SOD may also be attributable to its prolonged life-time in the circulation. Further experiments remain to be conducted to clarify the advantage of using the conjugate of SOD and a unique biopolymer, HA.

In the present studies, we demonstrated that HA-SOD was also effective in suppressing adjuvant arthritis in rats, a model of chronic rheumatoid arthritis (RA) in humans. To our knowledge, PEG-SOD has not been tested in models of chronic diseases such as adjuvant arthritis. As HA-SOD did not induce an immune response to exogenous SOD, HA-SOD can probably be safely injected into patients with RA without immunological side effects. Further preclinical studies remain to be investigated to verify the high pharmacological potentials of HA-SOD.

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