Food and Chemical Toxicology 58 (2013) 401–407

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Immunostimulatory and antiangiogenic activities of low molecular weight hyaluronic acid

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ARTICLE INFO

Article history:
Received 25 February 2013
Accepted 18 May 2013
Available online 29 May 2013

Keywords:
Hyaluronic acid
Low molecular weight of hyaluronic acid
Immunostimulatory activity
Antiangiogenic activity

ABSTRACT

The immunostimulatory activities of two low molecular weight hyaluronic acids (LMWHA-1 and LMWHA-2 with MW of 1.45×10^5 and 4.52×10^4 Da, respectively) and HA (MW, 1.05×10^6 Da) were evaluated by using *in vitro* cell models and *in vivo* animal models, and their effects on angiogenesis were measured *in vivo* by using the chick embryo chorioallantoic membrane (CAM) assay. The results demonstrated that LMWHA-1, LMWHA-2 and HA could promote the splenocyte proliferation, increase the activity of acid phosphatase in peritoneal macrophages and strengthen peritoneal macrophages to devour neutral red *in vitro* in a dose-dependent manner. Furthermore, LMWHA-1 and LMWHA-2 exhibited much stronger immunostimulatory activity than HA. For assay *in vivo*, LMWHA-1 and LMWHA-2 significantly increased the indices of spleen and thymus, the activity of lysozyme in serum and the swelling rate of earlap in delayed-type hypersensitivity in a dose-dependent manner. In the CAM model, the results showed that LMWHA-1, LMWHA-2 and HA suppressed angiogenesis in chicken embryos. Moreover, LMWHA-1 exhibited higher antiangiogenesis activity than LMWHA-2 and HA. All these results suggested that LMWHA might be a potential natural immunomodulator and a potential candidate compound for antiangiogenic.

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1. Introduction

Cyclophosphamide (CY) is an anticancer and immunosuppressant drug that induces the production of reactive oxygen species (ROS). The excessive production of ROS plays multiple important roles in tissue damage and loss of function in a number of immune tissues and organs. For example, ROS can induce cell death by injuring the DNA of normal cells, resulting in the damage of the immune system (Diaz-Montero et al., 2012; Ma et al., 2009). It has been reported that polysaccharides can attenuate this oxidative damage of a tissue indirectly by enhancing natural defenses of cell and/or directly by raising the immunostimulatory activity (Chen et al., 2012; Wang et al., 2011). Accordingly, many natural polysaccharides with potential and potent capability of immunostimulatory activity and antiangiogenic activity have been isolated and characterized (Chen et al., 2010; Luo et al., 2009; Qiao et al., 2010; Yang et al., 2009). Furthermore, some polysaccharides have been used as immunomodulatory agents that often act by inducing lymphocyte proliferation and cytokine production and exhibiting protective effects toward the hematopoietic function of bone marrow and immune organs (Nie and Xie, 2011; Wang et al., 2011). Therefore, more attentions have gradually been paid to the polysaccharides characterized as immunomodulator and antiangiogenic natural products.

Hyaluronic acid (HA), also known as hyaluronan, is a linear glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA), namely, [→4)-β-D-GlcA-(1 → 3)-β-D-GlcNAc-(1→]. Low molecular weight HA (LMWHA), defined as HA with less than 500 kDa, is the degradation product of high molecular weight HA. Increased evidence has been gathered that LMWHA, comparing with the native HA, exhibits some functional differences such as in immunostimulatory and antiangiogenic activities (Alaniz et al., 2011; Knoflach et al., 1999; Stern et al., 2006, 2007). HA functions are well known to be size-dependent (Noble, 2002). However, there is little information about the activities both *in vitro* and *in vivo* of LMWHA and the relation between immunostimulatory or antiangiogenic activity and the molecular weight of LMWHA is unknown. Therefore, evaluation of the immunostimulatory and antiangiogenic activities of LMWHA will be important for the elucidation of function and utilization of the polymers.

Recently, we reported the preparation of two LMWHAs (LMWHA-1 and LMWHA-2, molecular weights of 1.05×10^5 and 4.5×10^4 Da, respectively) degraded from HA by sulfate radical ($SO_4^{\cdot-}$) and their antioxidant activities (Ke et al., 2011). But there is little information about the immunostimulatory and

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http://dx.doi.org/10.1016/j.fct.2013.05.032

antiangiogenic activities of LMWHA degraded from HA by sulfate radical ($SO_4^{\cdot-}$). In this paper, therefore, we report in detail their immunostimulatory activities by using CY-induced immunosuppressed mice as *in vivo* model and *in vitro* cell models and their antiangiogenic activities *in vivo* by using the chick embryo chorioallantoic membrane (CAM) assay.

2. Materials and methods

2.1. Reagents and materials

Male Kunming mice (8-weeks-old), grade of specific pathogen free with body weight (BW) of 20 ± 2 g, were purchased from the Experiment Animal Center, Academy of Military Medical Sciences (Beijing, China). Chick embryos were obtained from Xingdian Poultry Plant (Nanjing, China). Concanavalin A (ConA), 3-(4,5-dimethylthiaz-ol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and D-glucuronic acid sodium salt monohydrate were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). CY was purchased from Jiangsu Hengrui Medcine Co. (Nanjing, China). 5-Fluorouracil was purchased from Nanjing Duly Biotechnology Co., Ltd. (Nanjing, China). Assay kit for lysozyme was the product of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from Shanghai Sangon Biological Engineering Technology & Services Co. (Shanghai, China). High molecular weight HA (MW, 1.05×10^6 Da) was obtained from Zhenjiang Dong Yuan Biotechnology Co., Ltd. (Zhenjiang, China). All other reagents were of analytical grade.

2.2. Preparation and analysis of LMWHA

High molecular weight HA was partially degraded by $SO_4^{\cdot-}$ in the ferrous ion-persulfate anion oxidative system to low molecular weight fragments according to the reported method (Ke et al., 2011). Briefly, one volume solution of ferrous ion (Fe^{2+}, 0.2 mmol/L), persulfate anion ($S_2O_8^{2-}$, 0.4 mmol/L) and HA (0.2 mg/mL) in a ratio of 1:2:1 (v/v/v) were mixed with 1 volume of phosphate buffered saline (PBS, pH 5.0), and the mixture was kept at 50 °C for 30 or 60 min. The reaction solution was transferred to 4 volume of ethanol, resulting in the precipitation of HA. The LMWHA was recovered by centrifugation at 1700*g* for 10 min and drying at ambient condition. Then, LMWHA was routinely estimated by the carbazole assay (Bitter and Muir, 1962). Molecular weight of LMWHA was determined by HPLC equipped with a refractive index detector and a TSKgel G3000SW×l column (7.5 × 300 mm, Tosoh Corp., Tokyo, Japan) according to our previously reported method (Ke et al., 2011), and shodex P-82 standards (Showa Denko K.K, Tokyo, Japan) in a range of 0.59–78.8 × 10^4 Da were used as the standards for molecular weight measurement.

2.3. Determination of immunostimulatory activity of LMWHA in vitro

2.3.1. Assay of splenocyte proliferation

The assay of splenocyte proliferation was done according to the MTT-based colorimetric method with minor modifications (Dai et al., 2009; Yang et al., 2006). Briefly, male Kunming mice were killed by cervical dislocation (All the procedures involving animals were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals) and the spleens were removed aseptically. The suspension of single spleen cells was prepared by homogenization in 5.0 mL RPMI-1640 medium. The suspension was centrifuged to afford cell pellet. The erythrocytes in cell pellet were lysed with Tris-NH_4Cl lysing buffer (0.15 M NH_4Cl and 20 mM Tris) for 3 min. The lysed solution was then centrifuged, washed twice with RPMI-1640 medium and adjusted to a density of 1×10^7 cells/mL in the RPMI-1640 medium supplemented with FBS (10%), penicillin (100 unit/mL) and streptomycin (100 μg/mL). The spleen cell suspension was pipetted into 96-well flat-bottom plate (50 μL/well). Then, Group I (normal control group) was treated with RPMI-1640 medium (50 μL/well), and Group II (lower dose, 25 μg/mL), Group III (low dose, 50 μg/mL), Group IV (medium dose, 100 μg/mL) and Group V (high dose, 200 μg/mL) were treated with LMWHA (50 μL/well, dissolved in PBS buffer). Group VI (positive control group) was treated with ConA (50 μL/well, final concentration 2.5 μg/mL). After incubated for 72 h at 37 °C in a humidified 5% CO_2 incubator, 10 μL of MTT solution (5.0 mg/mL) was added to each well and the plate was further incubated for 4 h. Then, 100 μL of 10% SDS in 0.01 N HCl was added to per well and the plate was kept overnight for the dissolution of formazan crystals. The absorbance (Abs) at 570 nm was measured by an ELISA plate reader (TECAN Infinite F200, Switzerland).

2.3.2. Assay of acid phosphatase activity in peritoneal macrophages

The activity of acid phosphatase in peritoneal macrophages was determined according to the reported method with slight modifications (Liu et al., 2008). Briefly, sterile 3% thioglycollate medium (50 mL/kg body weight) was injected intraperitoneally into male Kunming mice as a stimulant to elicit peritoneal macrophages. Three days later, peritoneal exudates cells were harvested by a lavage of the peritoneal cavity with 5 mL of ice-cold RPMI-1640 medium. The resulting cell suspension was centrifuged (1700 rpm, 5 min), washed twice with RPMI-1640 medium and adjusted to a density of 2×10^6 cell/mL in the RPMI-1640 medium supplemented with FBS (10%), penicillin (100 unit/mL) and streptomycin (100 μg/mL). The cell suspension was added into a 96-well flat-bottom plate (100 μL/well) and the cells were allowed to adhere to the bottom of the plate at 37 °C in a humidified 5% CO_2 incubator for 3 h. The non-adherent cells were removed by washing three times with RPMI-1640 medium. Then, fresh medium (50 μL/well, control group) or test sample (50 μL/well, LMWHA at a final concentration 25, 50, 100 and 200 μg/mL as lower dose, low dose, medium dose and high dose, respectively) was added, and the plate was incubated at 37 °C for 24 h. The culture medium was removed by rapid inversion and flicking of the plate. The macrophage monolayer in each well was solubilized by addition of 1% Triton X-100 (25 μL). Thereafter, freshly prepared *p*-nitrophenyl phosphate (150 μL, 1 mg/mL) in 0.1 M citrate buffer (pH 5.0) was added as a substrate for acid phosphatase, and the plate was incubated at 37 °C for 1 h. The reaction was stopped by addition of 50 μL of 3.0 M NaOH solution, and Abs of the culture well was measured at 405 nm by using an ELISA plate reader. The stimulating index of acid phosphatase activity was calculated by the following equation:

$$\text{Acid phosphatase activity} = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}})/\text{Abs}_{\text{control}}$$

2.3.3. Assay of phagocytosis of peritoneal macrophages

Phagocytosis of peritoneal macrophages was measured according to the reported method with modification (Xu et al., 2006). In brief, the peritoneal macrophages were prepared, seeded in a 96-well flat-bottom plate and allowed to adhere as done as previously described. Non-adherent cells were removed by washing three times with RPMI-1640 medium. Then, fresh medium (50 μL/well, control group) or test sample (50 μL/well, LMWHA at a final concentration 25, 50, 100 and 200 μg/mL as lower dose, low dose, medium dose and high dose, respectively) was added to each well and the plate was incubated at 37 °C in a 5% CO_2 incubator for 48 h. Then, neutral red solution (1%, 100 μL/well) was added to the cell plate. After further incubation for 60 min, the cells were washed three times with RPMI-1640 medium to remove excess neutral red. A mixture of glacial acetic acid (1%) and ethanol (1:1) was added to each well (100 μL/well), and the cell plate was incubated again at 37 °C for 15 h. Finally, the Abs of each well was measured at 540 nm by an ELISA plate reader.

2.4. Determination of immunostimulatory activity of LMWHA in vivo

2.4.1. Animal selection and experimental design

Female Kunming mice were housed in an air-conditioned animal room with the conditions of 21 ± 1 °C and 50%–60% relative humidity in a 12 h light/dark cycles, and were free to access to food and water. After adapting to their environment for 1 week, a total of 72 mice were randomly divided into twelve groups (6 for each), including normal control group (NCG), CY model control group (MCG), positive control group (PCG), HA groups (20, 60 and 120 mg/kg body weight), LMWHA-1 groups (20, 60 and 120 mg/kg body weight) and LMWHA-2 groups (20, 60 and 120 mg/kg body weight). Mice in NCG were given gastric gavage of 0.9% NaCl (15 mL/kg body weight) once daily for seven days and hypodermic injection of 0.9% NaCl (15 mL/kg body weight) on days 1, 3 and 5. Mice in MCG were fed with physiological saline solution (15 mL/kg body weight on days 1–7) via gastric gavage per day and CY injection (75 mg/kg body weight on day 1, 3, 5). Mice in PCG were given Shengxuetiaoyuan Tang (SXTY Tang, 15 mL/kg body weight on day 1–7) by gastric gavage each day and CY injection (75 mg/kg body weight on days 1, 3 and 5). The mice in dose-dependent HA and LMWHA groups were given HA, LMWHA-1 and LMWHA-2 at dose of 20, 60 and 120 mg/kg body weight on days 1–7 through gastric gavage and CY injection (75 mg/kg body weight on days 1, 3 and 5) respectively. At the third day of treatment by gastric gavage, mice hair on the abdominal part (1.5 × 1.5 cm) was shaved and 50 μL of DNFB (1%, a mixture of acetone and vegetable oil in 1:1) was smeared on the shaved abdomen. At the seventh day of treatment by gastric gavage, 20 μL of 1% 2,4-dinitrofluorobenzene (DNFB) was painted on both sides of right earlap, while the unpainted left earlap was used as control (Kuang et al., 2011).

2.4.2. Biochemical assay

After overnight fasting following the last drug administration, the mice were weighed and sacrificed. Blood samples were collected immediately in centrifuge tubes. After 1 h, the blood samples were centrifuged at 4000 rpm for 10 min to afford the serums. The serum samples were stored at −20 °C until use. The concentration and activity of lysozyme in serum were determined by using the commercial reagent kit according to the instruction manual. The enzyme activity was expressed as unit per milliliter of serum (U/mL) and concentration of enzyme was expressed as microgram per milliliter of serum (μg/mL).

2.4.3. Analysis of delayed-type hypersensitivity response

The delayed-type hypersensitivity (DTH) response to DNFB was evaluated by measuring weight difference of right and left ear with an analytical balance (Kuang et al., 2011). Briefly, the earlaps of both sides were cut off and hole-punched to afford circle earlaps (diameter 0.6 cm), and the circle earlaps were weighed. Indices of organs and swelling rate of earlap were calculated by using the following formula:

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