Pharmacological Reports 68 (2016) 1126–1132

Contents lists available at ScienceDirect

Pharmacological Reports

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

Original article

Hyaluronan oligosaccharides promote diabetic wound healing by increasing angiogenesis

Yi Wang[a,b], Guanying Han[c,*], Bin Guo[c], Jianhua Huang[c,*]

[a] Graduated School of Liaoning Medical University, Jinzhou, China
[b] School of Pharmaceutical Science, Liaoning Medical University, Jinzhou, China
[c] First Affiliated Hospital of Liaoning Medical University, Jinzhou, China

ARTICLE INFO

Article history:
Received 26 April 2016
Received in revised form 21 June 2016
Accepted 14 July 2016
Available online 16 July 2016

Keywords:
Hyaluronan oligosaccharides
Diabetic wound healing
Angiogenesis

ABSTRACT

Background: Hyaluronan (also known as hyaluronic acid) oligosaccharides (O-HA) can promote angiogenesis and wound healing; however, there are few reports on whether O-HA also plays a role in healing wounds of diabetic patients.
Methods: In this study, we prepared a special ointment containing a mixture of hyaluronan fragments from 2 to 10 disaccharide units and investigated its effects on healing the wounds of diabetic rats.
Results: We found that O-HA significantly increases proliferation, migration, and tube formation of endothelial cells under high glucose conditions, and topical administration of O-HA ointment promotes wound healing by increasing angiogenesis in the wounded area of the skin. The underlying mechanisms are that O-HA increases the phosphorylation of Src and ERK, and expression of TGF beta1, thereby increasing angiogenesis.
Conclusions: This suggests that topical application of O-HA could be a useful method by which to treat diabetic wounds in clinical practice.

Introduction

Skin is the first barrier that protects the human body against microorganisms and physical and chemical damage. When there is damage to the skin, a series of biological and molecular events occur to help repair the damage, such as hemostasis, inflammation, proliferation, vascularization, and production and remodeling of the extracellular matrix (ECM) [1]; however, in diabetic patients, wound healing is often impaired [2]. Insufficient blood supply to the wound area of a diabetic patient is one of the most important factors that impairs wound healing [3,4]. It is estimated that 15% of diabetic patients have foot ulcerations, that result in prolonged hospitalization and even amputation [5], because current interventions and therapies on the diabetic wound are unsatisfactory. Looking for the new ways to increase angiogenesis is crucial for promoting diabetic wound healing in clinical practice.

Hyaluronan (also known as hyaluronic acid, HA) is a naturally occurring nonsulfated, linear glycosaminoglycan consisting of repeating units of (β,1–4)-D-glucuronic acid-(β,1–3)-*N*-acetyl-D-glucosamine [6]. HA is found in its native state as a high molecular–mass polymer (>10^6 kDa) in the ECM of nearly all animal tissues and in significant amounts in the skin. HA plays an important role in maintaining tissue homeostasis, including angiogenesis. Native high molecular–weight HA is anti-angiogenic[7], whereas products of degradation that are a specific size (e.g., 3–10 disaccharide units; O-HA) are pro-angiogenic[8]. Thus, HA/O-HA might play important roles in diabetic wound healing. Although there are many *in vitro* studies related to O-HA and angiogenesis, few studies on the role O-HA plays *in vivo*, especially in healing wounds in diabetic animal models, have been reported.

In the present study, we prepared a special slow-releasing ointment that contained a mixture of HA fragments from 2 to 10 disaccharides, investigated the effects of O-HA on endothelial cells in a high glucose environment, and further studied the role that O-HA plays and its mechanisms in wound healing in a diabetic rat model.

Materials and methods

Preparation of HA oligosaccharide ointment

HA oligosaccharides were prepared as described previously [9]. Briefly, native high molecular–weight HA (Sigma-Aldrich Corporation, St. Louis MO, USA) was digested by hyaluronidase, and

* Corresponding authors.
E-mail addresses: hgy19800223@163.com (G. Han), hjhuadr@163.com (J. Huang).

http://dx.doi.org/10.1016/j.pharep.2016.07.001
1734-1140X/

oligomers were fractionated by size exclusion chromatography using a Bio-Gel P-10 column (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Fragments from 2 to 10 disoligosaccharide units (4–20mers) were pooled. An endotoxin activity check was performed using a *Limulus* amebocyte lysate assay. HA oligosaccharide–containing ointment was prepared by emulsification. Briefly, the oil phase was made by mixing 2.4 g vaseline, 2.4 g stearic acid, 1.6 g glycerol monostearate, and 2.5 g liquid paraffin wax. The mixture was melted at 75 °C. The water phase was made by adding 2.4 g glycerin sodium, 0.2 g dodecyl sulfate, and 0.02 g methylparaben to 9.6 mL purified water and heating to 85 °C to dissolve the agents. After cooling the water phase to 35–40 °C, 0.03 g HA oligosaccharides was added and completely dissolved. The solution was slowly poured into the oil phase and stirred to a uniform paste. The final concentration of HA oligosaccharide was 0.15%.

Cell proliferation assay

Human umbilical vein endothelial cells (HUVECs) were plated at a density of 1.0×10^4 cells/well in complete medium containing 10% fetal bovine serum (FBS) in 96-well plates. After attachment of HUVECs, the medium was removed and replaced with complete medium containing 33 mmol/L glucose either with or without 10 μg/mL O-HA. The cells were then cultured for 72 h, after which 20 μL methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent were added to each well, and the plates incubated for 4.0 h at 37 °C. The supernatants were discarded and 150 μL dimethyl sulfoxide were added to each well. The 96-well plate was shaken in oscillators at room temperature for 10 min. The wells without cells were used as the zero point of absorbance. The absorbance was measured using a microplate reader at 570 nm.

Tube formation assay

Two hundred microliters Matrigel were polymerized in the wells of a 24-well plate at 37 °C for 30 min. HUVECs (1.0×10^5) were dispensed into each well in 1000 μL complete medium containing 33 mM glucose, with or without 10 μg/mL O-HA. Cord morphogenesis of HUVECs was assessed by phase-contrast microscopy. Tubule structure was photographed with a microscope 8.0 h after cell seeding. Vascular cross points were counted in five randomly selected fields under the microscope in a blinded manner.

In vitro assay of wound recovery

HUVECs were plated at a density of 4.0×10^5 cells/well in complete medium containing 10% FBS in six-well plates. After 90% confluence of cultured cells, the cell layer was rinsed with phosphate buffered saline (PBS), wounded using a mechanical wounder, rinsed again in PBS to remove loose and dislodged cells, and placed into a fresh medium containing 30 mM glucose either in the presence or absence of 10 μg/mL O-HA. Cells were cultured again for 24 h. Movement of cells into the denuded area was quantified using a Seescan computerized image analysis system (Seescan Ltd., Cambridge, UK).

Animals

Male Sprague-Dawley (SD) rats weighing 200–250 g were provided by the Experimental Animal Center of our university. All experimental procedures were carried out in accordance with the recommended guidelines for the care and use of laboratory animals issued by the Chinese Council on Animal Research.

Diabetes was induced in the male SD rats (n = 20) by a one-time tail-vein injection of streptozotocin (STZ, Sigma-Aldrich, St Louis, MO, USA) dissolved in 100 mmol/L sodium citrate buffer (pH = 4.5) at a dose of 60 mg/kg body weight. For the preparation of citrate buffer, 3 g Na3C6H5O7.2H2O and 0.4 g $C_6H_8O_7 \cdot H_2O$ were mixed and dissolved in 100 mL double distilled water, the final concentration is 100 mmol/L, pH is about 4.5. For this study, a diabetic model was defined as an experimental model characterized by a non-fasting blood glucose measurement of 16.7 mmol/L or higher, detected by three consecutive measurements. A total of 16 rats complied with the standard.

After 7 days of streptozotocin administration, the diabetic rats were randomly and equally divided into two groups as follows: control (ointment) and treatment (ointment + O-HA). After anesthesia using an intramuscular injection of 40 mg/kg ketamine and 4.0 mg/kg xylazine, the hair was shaved, and a 2.0-cm full-thickness wound was made on the dorsal surface of the right flank. The ointment with or without O-HA was smeared on the surface of the wound twice a day during the whole experimental process.

Measurement of blood flow

Immediately after the wound was made and every 7.0 d during the wound-healing period, the blood flow around the wound area was measured using laser Doppler (Perimed, PSI, Perimed).

Histological examination

After 14 days, the skins around the wound were harvested, skin specimens were fixed in 10% neutral buffered formalin; after fixation, the tissues were embedded in paraffin. Five micrometer thick sections were mounted on glass slides, de-waxed, rehydrated to distilled water and stained with haematoxylin and eosin. All slides were examined by a pathologist without knowledge of the previous treatment, and the granulation tissue and re-epithelialization were evaluated.

Capillary density measurement

After 14 days, the skins around the wound were harvested, snap frozen in liquid nitrogen, and 10-μm cryosections were prepared. Endothelial cells were stained with mouse monoclonal anti-CD31 primary antibody (Catalogue#555027, BD Pharmingen, SD, CA, USA) at a dilution of 1:200, followed by biotinylated anti-mouse IgG secondary antibody, and an avidin-HRP conjugate for color reaction (DAB paraffin IHC staining module, Ventana Medical Systems, Inc., Tucson, AZ, USA). Hemotoxilin was used for counter staining. The sections were analyzed by microscopy with five high-power fields randomly selected for each section. CD31-positive cells were counted in a blinded manner. The number of CD 31-positive cells in each field was used as an index of capillary density.

Western blot

The tissues from the wound area were washed twice with cold PBS and resuspended in cold lysis buffer containing 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.0 mmol/L ethylenediaminetetraacetic acid, 0.5% Triton X-100, and protease inhibitors (Roche). Similar quantities of total protein (20 μg) were separated by electrophoresis using sodium dodecyl sulfate polyacrylamide gel, transferred onto polyvinylidene fluoride membranes, and blocked overnight in blocking solution at 4.0 °C. To detect p-Src and p-ERK, and TGF beta1, the membranes were incubated for 1.0 h with a rabbit polyclonal antibodies raised against p-Src (Catalogue#44-662G, Invitrogen Corporation, Carlsbad, CA, USA, dilution 1:1000), a mouse monoclonal antibody against p-ERK (Catalogue#4696, Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000), and a rabbit polyclonal antibody against TGF-beta1(Catalogue#ab92486, Abcam, dilution 1:1000) respectively, followed by