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"The Anti-angiogenic Effects of Hyaluronic Acid Oligosaccharides with Different Molecular Weights"

Liyun Dai, Danyu Zhang, Junliang Zhang and Yue Wu *

Bloomage Biotechnology Corp., Ltd., Jinan, Shandong 250101, China

1. Introduction

When the skin is exposed to external stimuli such as (UV/hypoxia/low temperature), it may develop a "red face", which may be caused by abnormal response of skin vascular such as telangiectasia or angiogenesis, leading to facial erythema. Angiogenesis is rarely seen under normal physiological conditions [1], and occurs during wound healing to repair damaged vasculature and accelerate the healing process [2].

Angiogenesis is a process in which new blood vessels develop from existing capillaries and eventually create a complete, regular, and mature vascular network. This process includes degradation of the basement membrane and activation, proliferation, and migration of the endothelial cells (ECs), which is regulated by various pro-angiogenic and anti-angiogenic factors.[4]

Various biomolecules that promote or inhibit angiogenesis constitute a complex and dynamic angiogenic system, including growth factors (such as vascular endothelial growth factor, fibroblast growth factor, transforming growth factor, hepatocyte growth factor), adhesion factors (integrin, cadherin), proteases (such as matrix metalloproteinase), extracellular matrix proteins (fibronectin, collagen), transcription factors (hypoxia-inducible factor, nuclear factor), signaling molecule mechanistic target of rapamycin (mTOR), protein kinase B (AKT), p38 mitogen-activated protein kinases (p38 MAPK), nitric oxide (NO), angiopoietin, thrombospondin-1, angiostatin, endostatin, and interleukin (IL) [3]. A recent study demonstrated that complement protein C1q, like VEGF, is involved in wound healing vascular changes and promoted tube formation, independently of complement activation [4]. Hyaluronic acid (HA) is a common glycosaminoglycan in skin extracellular matrix, negatively charged polysaccharide of repeating disaccharide units of D-glucuronic acid (β-1, 3) and N-acetyl-β-D-glucosamine. It was proposed that the HA of different molecular sizes can display different and sometimes opposing biological actions. For instance, High MW HA (>2x10^6 Da) represses mitogenic signalling and has anti-inflammatory properties, whereas low MW HA (1x10^5Da-1x10^6Da) promotes inflammation, angiogenesis and tissue remodeling in the wound healing process. However, Hyaluronic Acid Oligosaccharides（HAOs, 10000Da）exhibit opposite effects in different cell, and the researchers analyzed that HA interactions with its receptors initiate various signaling pathways regulating cellular responses to a variety of signals.[5] Therefore, this article will discuss the anti-angiogenic effects of HAO with different molecular weights.

2. Materials and Methods

2.1 Cell Culture

The human endothelial EA.hy926 cells (EA.hy926, purchased from SUNNCELL, Wuhan, China) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 g/mL of streptomycin(all from Gibco, Carlsbad, CA, USA). The human umbilical vein endothelial cells (HUVECs, purchased from ETHEPHON, Shanghai, China)were cultured in endothelial cell medium (ECM) supplemented with 5% fetal bovine serum, 1% endothelial cell growth factor, 100 U/mL of penicillin, and 100 g/mL of streptomycin (all from ScienCell, San Diego, CA, USA). Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2 Cell Proliferation Assay

Cells were seeded into 96-well plates (Corning Coster, Corning, NY, USA) with a density of 1.5×10^4 cells/well and were incubated at 37°C and 5% CO_2 for 24 h. Every plate was incubated with different treatments for 24 h. Subsequently, MTT (0.5 mg/mL, 100 µL/well) (Sigma–Aldrich Co., St. Louis, MO, USA) was added and incubated with the cells for 3 h. Then, the MTT working solution was discarded; DMSO (100 µL/well) was added, and the absorbance at 550 nm was measured using a Multiskan Sky^{TM} microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.3 Tube formation assay

Differentiation of HUVECs was examined by tube formation on Matrigel. Growth factor-reduced Matrigel was thawed on ice overnight, spread evenly over each well (50 µL/well) of 96-well plates and polymerized for 1 h at 37 °C. HUVECs (1×10^4 cells/well) were plated onto the Matrigel layer and cultured in ECM supplemented with 1 % FBS and different growth factors. After 16 h of incubation at 37 °C, tube formation was observed and captured with a phase contrast microscopy. Total tubular length per well was determined by computerassisted image analysis using Image Pro Plus software.

2.4 Wound healing assay

HUVECs were cultured on 6-well plates (5×10^5 cells/well) in basal medium. After confluent, HUVECs were starved with free-serum medium for 24 h. Then, HUVECs monolayer was scratched horizontally with a yellow pipette tip to obtain a monolayer culture with a space without cells. Cellular debris as removed by gently washing twice with PBS and then cultured in DMEM supplemented with 1 % FBS and different growth factors. Three randomly selected fields along the scraped line were photographed using a phase contrast inverted microscope. After incubation at 37 °C for 16 h, images were taken and cell migration distances were evaluated.

2.5 *In vivo* chick embryo chorioallantoic membrane (CAM) assay

Fertilized White Leghorn chicken eggs (purchased from Boehringer Ingelheim, Beijing, China) were incubated at 37°C at constant humidity. On day 4, 2–3 mL of egg albumin was removed with a hypodermic needle, allowing the CAM and yolk sac to drop away from the shell membrane.On day 7, a 2.5-cm diameter window was made with a razor and tweezers, the mixed cellulose filter membrane discs were cut to a size of 6 mm in diameter and placed on top of a growing CAM, drop the solution containing the C1q and Hyaluronic Acid Oligosaccharides (5µL) onto the disc for 3 days. For every test group, 10 eggs were utilized.The angiogenic response was evaluated 96 hours after the implants by means of a stereomicro-scope connected to an image analyser system (Olympus Italia, Italy).

2.6 Western Blotting Assay

HUVECs were cultured in a 100-mm dish at a concentration of 2.5 × 105 cells/mL for 24 h were treated with the specific test compound at 37 °C in a 5% CO_2 incubator for 48h. Cells were washed with phosphate-buffered saline (PBS) three times and lysed with 2% sodium dodecyl sulfate (SDS) and 1% sodium deoxycholate (0.5 mL). Supernatants were collected as whole-cell lysates, and protein concentrations were determined using the BCA protein assay reagent (Thermo Fisher Scientific, Rockford, MA, USA). Each sample was adjusted to the same protein concentration and heated to 95 °C for 5 min. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–20% gel (TEFCO, Hachioji, Japan), and then transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Cork, Ireland). The membrane was blocked with 5% skim milk in tris-buffered saline (TBS) at room temperature for 30 min, then incubated with human anti-Erk1/2, anti-phosphor- Erk1/2, anti-β-actin antibody (diluted 1:1000; Cell Signaling Technology, Boston, USA) with TBS at 4°C overnight. The membrane was washed 3 times with tris buffered saline with tween 20 (TBST) for 30 min and then incubated with a goat anti-rabbit IgG H&L (HRP) (diluted 1:10000; Abcam, Cambridge, UK) for 1 hour at room temperature. The membrane was washed three times with TBST for 30 min, and a ECL western blotting substrate (Thermo Fisher Scientific, Pittsburgh, PA, USA) was added; the membrane was then incubated for 5 min at room temperature. Finally, immunoreactive bands were visualized using a Tanon Chemi Dog 5200T (Tanon, Shanghai, China).

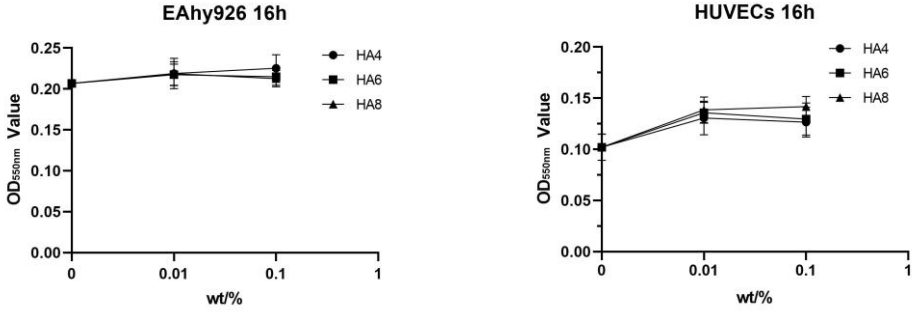
Enzyme‑linked immunosorbent assay (ELISA)

HUVECs culture supernatants were harvested and IL-6,IL-8,VCAM-1,ICAM-1 (MULTI SCIENCES, Hangzhou, China) ELISA kits were used to detect the respective cytokines following the manufacturers' instructions.

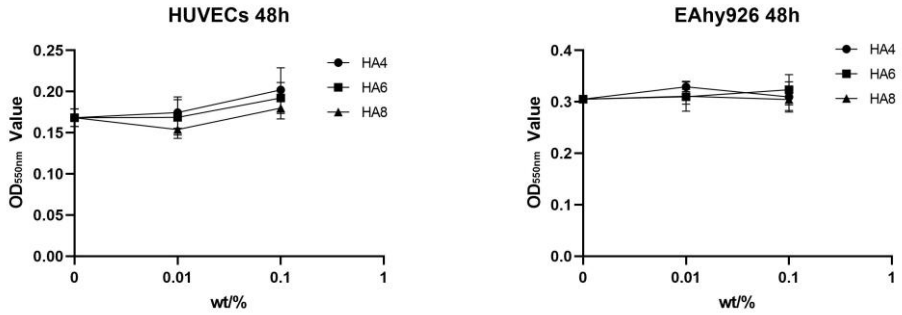
3. Results

3.1 The Effect of Hyaluronic Acid Oligosaccharides on the Proliferation of EAhy926 and HUVECs

To study the effect of hyaluronic acid oligosaccharides on cell growth, the proliferation of EAhy926 and HUVECs was detected through MTT assay. As shown in Figure 1, hyaluronic acid oligosaccharides did not significantly affect the proliferation of EAhy926 and HUVECs, after incubation for 16 hours and 48 hours respectively.



(**a**) (**b**)

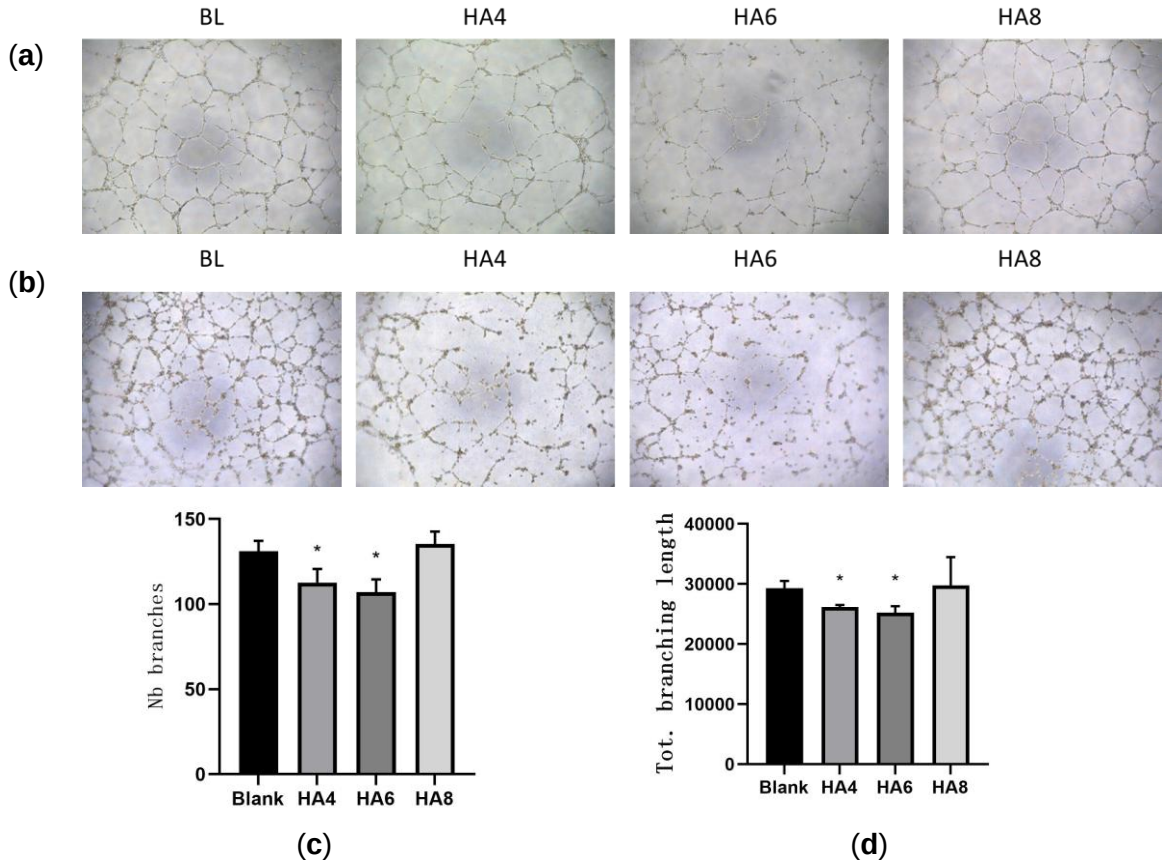


(**c**) (**d**)

Figure 1. The Effect of Hyaluronic Acid Oligosaccharides on the Proliferation of EAhy926 and HUVECs: (a)EAhy926 were incubated with hyaluronic acid oligosaccharides for 16 hour; (b)HUVECs were incubated with hyaluronic acid oligosaccharides for 16 hour; (c)EAhy926 were incuated with hyaluronic acid oligosaccharides for 48 hour; (d)HUVECs were incubated with hyaluronic acid oligosaccharides for 48 hour.

3.2 The Effect of Hyaluronic Acid Oligosaccharides on the tube formation of EAhy926 and HUVECs

EAhy926 and HUVECs completely reorganize when cultured with complete medium in three-dimensional matrigel and finally form a network of tubular structures with multiple cell–cell contacts (Figure 2). The anti-angiogenic effect was observed when HA4 and HA6 were added, and HA8 was opposite.



(**c**) (**d**)

Figure 2. The effect of hyaluronic acid oligosaccharides on the tube formation of EAhy926 and HUVECs: (a)HUVECs tube formation in vitro induced by hyaluronic acid oligosaccharides;

(b)EAhy926 tube formation in vitro induced by hyaluronic acid oligosaccharides; (c)The effect of hyaluronic acid oligosaccharides on the number of tube branches and total of branching length(d) of HUVECs.

3.3 The Effect of Hyaluronic Acid Oligosaccharides on the migration of EAhy926

Endothelial cells play an important role in vessel formation. These processes involve the directed migration of cells into the damaged or injured area and the stimulation of growth. In a confluent monolayer of HUVECs the ability of cells adjacent to a scratch to close this ‘‘wound’’ was evaluated microscopically in response to Hyaluronic Acid Oligosaccharides stimuli. As shown in Fig. 3, addition of 0.1% HA4,HA6 to the culture medium of a confluent HUVECs layer immediately after ‘‘wounding’’ resulted in a invasion of adjacent cells into the scratch after approximately 16 hours.

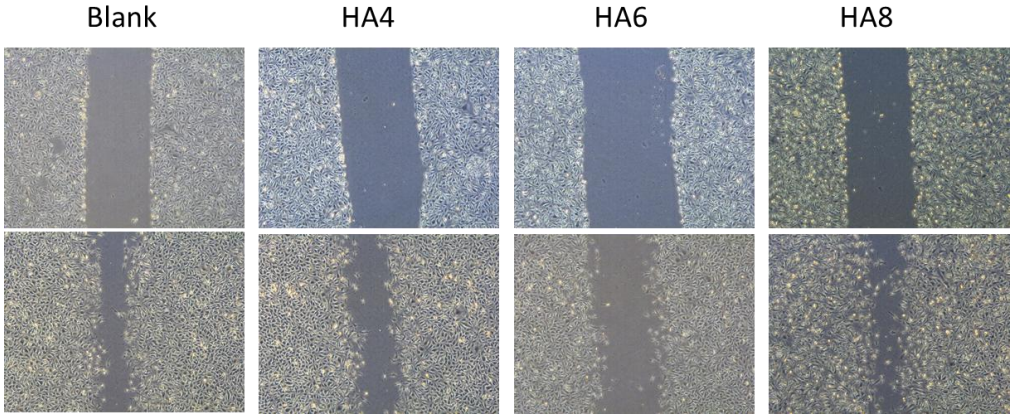
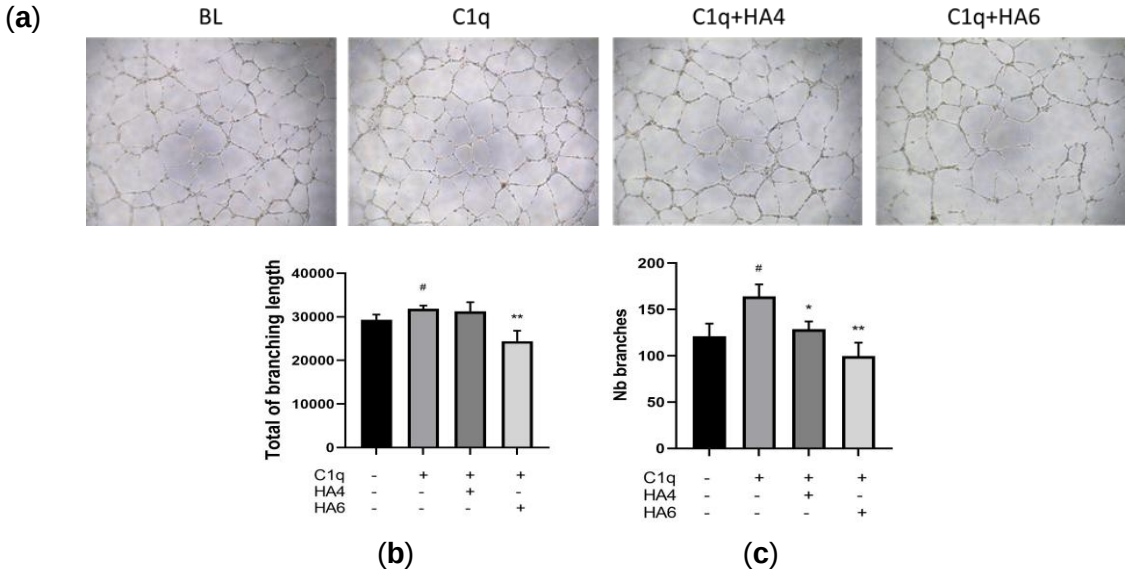


Figure 3. The Effect of Hyaluronic Acid Oligosaccharides on the migration of EAhy926

3.4 The Effect of HA4 and HA6 on C1q-induced tube formation and migration of HUVECs

To study the effect of HA4 and HA6 reduced C1q-induced on tube formation, HUVECs were cultured with complete medium in three-dimensional matrigel and finally form a network of tubular structures. As shown in Figure 4(a), C1q significantly promotes angiogenesis, while HA4 and HA6 attenuate this effect. Similarly, C1q promotes cell migration, while HA4 and HA6 exhibit an inhibitory trend.



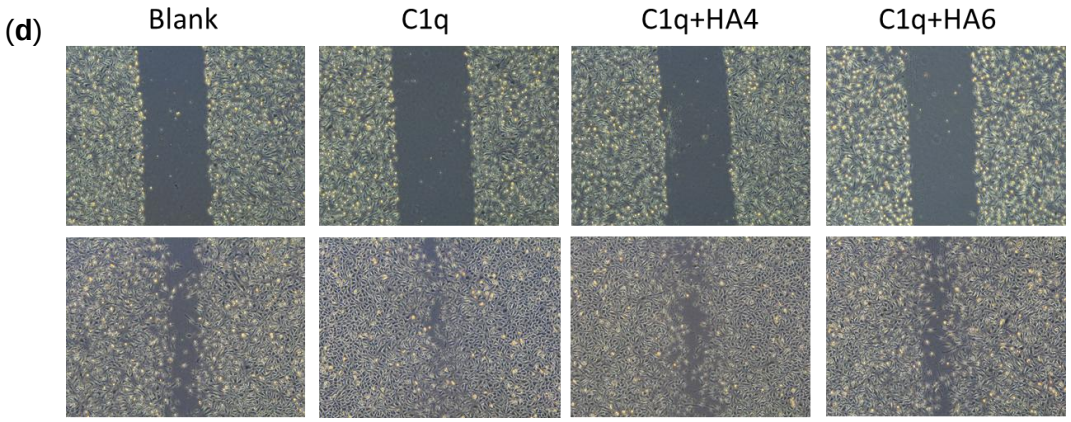


Figure 4. The effect of HA4 and HA6 on the tube formation and migration of HUVECs: (a)HUVECs tube formation in vitro induced by C1q,HA4 and HA6; (b)EAhy926 tube formation in vitro induced by hyaluronic acid oligosaccharides; (b)The effect of hyaluronic acid oligosaccharides on the number of tube branches and total of branching length(c) of HUVECs. (d) The Effect of HA4 and HA6 on the migration of HUVECs

3.5 The Effect of HA4 and HA6 on C1q-induced angiogenesis in CAM model

To determine whether HA4 and HA6 have anti-angiogenic activity, CAM assay was first carried out. After 10 days of incubation, C1q elicited on angiogenic response, which is visible with the microscope as a spoke-wheel-like pattern of blood vessels. However, around the control discs containing C1q+HA4 and C1q+HA6, no growth of new blood vessels was observed (Fig. 5).

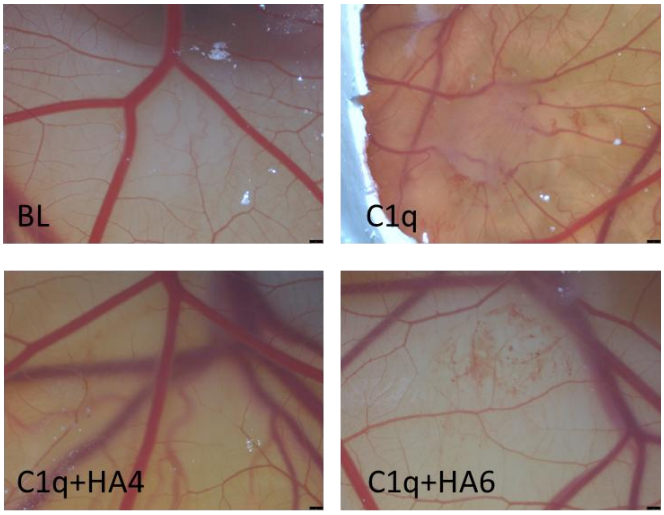


Figure 5. CAM assay. On day 7, discs contained HA4(0.1%), HA6(0.1%),C1q(10μg/mL) were applied to the chicken CAM, and their effects on vascular development were evaluated 4 days later.

3.6 The Effect of HA4 and HA6 on C1q-induced activation of the ERK1/2 pathway

Activation of the ERK1/2 pathway is closely associated with angiogenesis. It was reported that ERK pathways are also the downstream effectors of KDR,which is highly expressed on the surface of HUVECs.To validate this hypothesis,phosphorylation of ERK were detected at 24 h

after treatment, The C1q induced phosphorylation of ERK1/2, which was significantly inhibited by HA6 but unaffected by HA4.

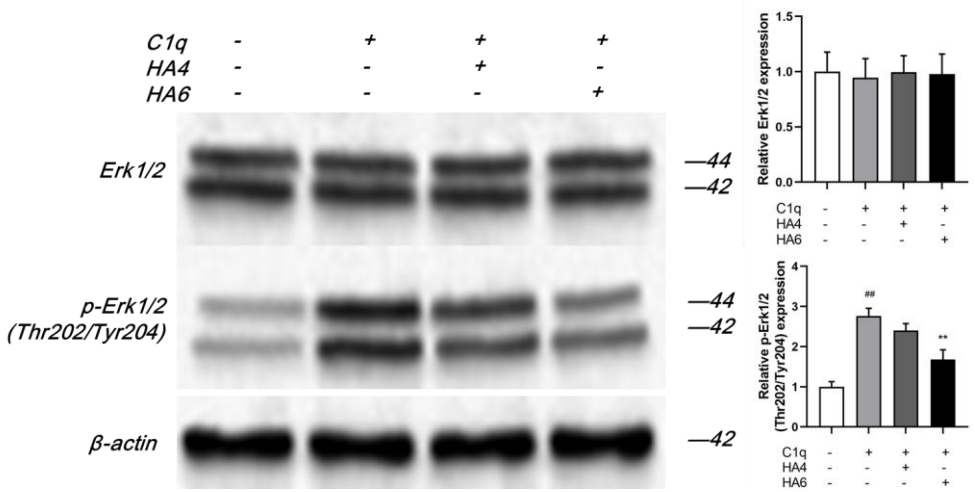


Figure 6. The Effect of HA4 and HA6 on C1q-induced activation of the ERK1/2 pathway

4. Discussion

Hyaluronic acid (HA), a naturally occurring polysaccharide, has demonstrated broad application potential in vascular medicine due to its excellent biocompatibility, biodegradability, and angiogenesis-modulating properties. Studies reveal that HA fragments of different molecular weights influence vascular functions through distinct mechanisms: low molecular weight HA (LMW-HA, <10 kDa) typically promotes angiogenesis by activating endothelial TLR-2/4 or CD44 receptors, making it suitable for ischemic disease therapy, whereas high molecular weight HA (HMW-HA, >500 kDa) exerts anti-angiogenic effects by suppressing MMP-9 and VEGF signaling pathways, showing therapeutic value in targeted tumor treatment [5]. The complement component C1q, as the initiator of the classical complement pathway, exhibits dual roles in vascular homeostasis regulation and pathological processes. Research demonstrates that C1q can interact with vascular endothelial cell surface receptors (such as gC1qR and cC1qR) to suppress TNF-α-induced vascular inflammatory responses and maintain endothelial barrier integrity[6]. Recent studies have revealed that C1q can regulate the proliferation, migration, and inflammatory responses of vascular endothelial cells (ECs) and smooth muscle cells (VSMCs) through activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway[6].

In vascular endothelial cells, C1q binding to the gC1q receptor (gC1qR) triggers ERK1/2 phosphorylation, subsequently upregulating anti-apoptotic proteins (e.g., Bcl-2) and enhancing endothelial cell survival [7]. Furthermore, during vascular injury repair, C1q activates ERK1/2 signaling in VSMCs to stimulate their migration and proliferation - a process potentially involved in vascular remodeling [8].

In this study, the anti-angiogenic property of HA Disaccharide/Tetrasaccharide/ Hexasaccharide/Octasaccharide (HA2/4/6/8) and hydrolyzed hyaluronic acid (Hyd-HA) on EAhy926 and

HUVEC cells was compared at non-toxic concentration. HA2/4/6 showed inhibitory effect on angiogenesis. The effects of HA2/4/6/8 and Hyd-HA on proliferation and migration were further compared, and they had no promoting effect. Researchers hypothesized that HA binding protein 1(HABP1) was involved in the angiogenesis process. Therefore, it was further found that HA2/4/6 significantly inhibited C1q-induced angiogenesis, phosphorylation of extracellular signal-related kinase (ERK).

5. Conclusion

In conclusion, we provide direct evidence that HA4 and HA6 inhibit angiogenesis, meanwhile HA6 significantly inhibits C1q-induced angiogenesis through the ERK-mediated pathway. HA4 and HA6 might serve as a potential target for antiangiogenic therapy.

6.Reference

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[7] C1q enhances microglial clearance of apoptotic neurons and neuronal blebs, and modulates subsequent inflammatory cytokine production.
[8] The role of complement component C1q in angiogenesis.