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Enzymatic fragments of hyaluronan inhibit adipocyte differentiation in 3T3-L1 pre-adipocytes

Byong-Gon Park [a, 1], Chang Won Lee [b, 1], Joo Woong Park [c], Yuan Cui [d], Yoon-Sun Park [e], Woon-Seob Shin [e, *]

[a] Department of Physiology, Catholic Kwandong University College of Medicine, Gangneung 210-701, Republic of Korea
[b] Department of Biotechnology, The Catholic University of Korea, Bucheon, Gyeonggi-do 420-743, Republic of Korea
[c] Biostream Technologies, Yongin-si, Gyeonggi-do 446-930, Republic of Korea
[d] Department of Anesthesiology, Affiliated Hospital of Yanbian University, Yanji 119, China
[e] Department of Microbiology, Catholic Kwandong University College of Medicine, Gangneung 210-701, Republic of Korea

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ABSTRACT

Hyaluronan has diverse biological activities depending on its molecular size. High molecular weight hyaluronan (2000 kDa) is a major component of extracellular matrix, and has been used in wounding healing, extracellular matrix regeneration, and in the treatment of osteoarthritis. Hyaluronan fragments can stimulate inflammation or induce loss of extracellular matrix. Hyaluronan is expressed during adipocyte differentiation, and down regulation of hyaluronan synthesis can reduce adipogenic differentiation. However, the direct effects of hyaluronan fragments on adipocyte differentiation have not been elucidated. Therefore, we prepared hyaluronan fragments by enzymatic digestion, and examined the inhibitory effects of these hyaluronan fragments on the accumulation of lipid droplets and on adipogenic gene mRNA expression in differentiating 3T3-L1 pre-adipocytes. Medium sized hyaluronan fragments (50 kDa) decreased lipid droplet accumulation in a dose-dependent manner. However, high molecular weight hyaluronan did not inhibit lipid droplet accumulation when used at a concentration of 600 μg/ml. Two or 4 day treatments with medium molecular weight of hyaluronan resulted in similar inhibitory levels of lipid accumulation as did treatment for 8 days. Medium sized hyaluronan inhibited the differentiation of 3T3-L1 pre-adipocytes during the early stages of adipogenesis. When 3T3-L1 cells were treated with 180 μg/ml of medium sized hyaluronan, the mRNAs for the master adipogenic transcription factors PPAR-γ and C/EBP-α were inhibited. Additionally, medium molecular weight hyaluronan suppressed mRNA expression of PPAR-γ target genes, including aP2 and FAS. This study is the first to report that medium molecular weight hyaluronan fragments can inhibit adipocyte differentiation.

1. Introduction

Hyaluronan is a major component of the extracellular matrix (ECM) in multiple tissues. It is a negatively charged, non-sulfated, glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetyl-glucosamine, with a molecular weight usually approaching a few million Daltons [1]. Despite its simple structure, hyaluronan has numerous functions, depending on its molecular weight [2]. High molecular weight hyaluronan (HMW-HA) has been used in diverse clinical applications, including ECM regeneration, wound healing, and in the treatment of osteoarthritis [3]. Researchers have observed that the production of matrix metalloproteinase-1 (MMP-1) was significantly suppressed by HMW-HA [4]. In contrast, others have found that hyaluronan oligosaccharides increased MMP-13 expression which subsequently induced the loss of ECM proteoglycan and collagen in human articular cartilage [5,6].

It was reported that hyaluronan oligosaccharides and HMW-HA modulate inflammation through the regulation of peroxisome proliferator activated receptor-gamma (PPAR-γ) in chondrocytes [7]. Calvo et al. [8] reported that hyaluronan exists in the extracellular media of differentiated 3T3-L1 pre-adipocyte

* Corresponding author.
E-mail addresses: bgpark@cku.ac.kr (B.-G. Park), idis1985@naver.com (C.W. Lee), pjwrnds@empas.com (J.W. Park), cuiyuan1222@163.com (Y. Cui), pyskth@cku.ac.kr (Y.-S. Park), shinws@cku.ac.kr (W.-S. Shin).
[1] These authors contributed equally to this work.

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cultures, and subsequently, a few papers regarding the relationship between hyaluronan and adipogenesis have been published. Interestingly, Allingham et al. demonstrated that the expression of genes for the biosynthesis and degradation of hyaluronan was positively involved in the differentiation of adipocytes [9]. Also, hyaluronan down regulation has been reported to have inhibitory effects on adipogenesis in 3T3-L1 cells in vitro, and on fatty liver development in high fat diet-feeding C57BL/6J mice in vivo [10]. Although many effects of hyaluronan have been reported, a direct inhibitory effect of hyaluronan on adipogenesis has not been demonstrated.

Obesity, or excessive fat deposition, results from an energy imbalance between energy intake and expenditure, and is a major cause of metabolic disease, especially in developed countries [11,12]. Obesity is one of the main factors responsible for the increased incidences of cardiovascular disease, Type 2 diabetes and several types of cancer [13,14]. Therefore, obesity is an increasing issue in healthcare, and mechanisms to control obesity have been actively studied worldwide [15,16]. Although several drugs have been developed to treat obesity, no ideal compounds or drugs have been developed yet [17]. Therefore, a novel material for the treatment of obesity is needed. In this study, we investigated the inhibitory effect of different molecular weights of hyaluronan on adipogenesis in 3T3-L1 pre-adipocytes.

2. Materials & methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Logan, UT, USA). Dimethylfumarate (DMF), insulin, 3-isobutyl-1-metylxanthine (IBMX), dexamethasone, and Oil Red O dye were purchased from Sigma–Aldrich (St. Louis, MO, USA). The HMW-HA (2000 kDa) sample (fermented with *Streptococcus zooepidemics*) of high purity was obtained from Biostream Technologies, (Yongin, Korea). Pullulan polysaccharide calibration kits were purchased from Agilent Technologies Inc. (CA, USA). Hyaluronidase was purified from a culture of *Vibrio splindidus* BST398 (KCTC-11899).

2.2. Preparing MMW-HA and LMW-HA

To obtain low molecular weight hyaluronan (LMW-HA) and medium molecular weight hyaluronan (MMW-HA), HMW-HA was hydrolyzed using hyaluronidase isolated from *V. splindidus* BST398 (KCTC-11899). HMW-HA (1 g) was dissolved in 100 ml of 0.1 M tris buffer (pH 7.0), and incubated with hyaluronidase for 4 h at 30 °C. After enzymatic hydrolysis, the MMW-HA fragments were filtered using an ultrafilter (Sartorius slice, NMWCO 10 kDa, Sartorius Co., Germany). To obtain LMW-HA, the ultrafiltrate from the 10 kDa ultrafilter was hydrolyzed for an additional 4 h at 30 °C and filtered using a 1 kDa ultrafilter (Sartorius slice NMWCO 1 kDa, Sartorius Co.). Each ultrafiltrate of MMW-HA and LMW-HA was dried using a freeze dryer. The white powder obtained was analyzed using an HPLC system (Waters 2695 Separations Module, Waters, USA) with a Shodex OHpak column (SB-805HQ, 8.0 × 300 mm, Showa Denko Co., Japan) under conditions of a flow rate of 0.7 ml/min in a mobile phase of 10 mM sodium phosphate (pH 7.0), monitored using a refractive index detector (Waters 2414 RIDetecter, Waters, USA).

2.3. MTT assay

The 3T3-L1 pre-adipocytes were seeded at a density of 1×10^4 cells per well in 96-well plates and incubated in culture medium. The cells were then treated with different molecular weights of hyaluronan. After 8 days, the cells were incubated in the dark with an MTT solution for 4 h at 37 °C. The supernatants were aspirated, DMSO was added to each well, and the plates were agitated to dissolve the formazan crystal product. Absorbance was then measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage of viable cells was calculated by defining the cell viability without treatment as 100%.

2.4. Cell culture and differentiation

Murine 3T3-L1 pre-adipocytes cells (ATCC, Manassas, VA, USA) were cultured in high-glucose DMEM supplemented with 10% bovine serum (Gibco by Life Technologies Co., Auckland, NZ), penicillin (100 μg/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. Two days after reaching confluence, 3T3-L1 pre-adipocytes were induced to differentiate using differentiation medium (MDI) supplemented with 1 μg/ml insulin, 1 μM dexamethasone, 0.5 mM IBMX, and 10% FBS (Gibco by Life Technologies Co., NY, USA). After 2 days, the medium was replaced with medium containing 10% FBS supplemented with 1 μg/ml insulin, and changed every 2 days thereafter with medium containing 10% FBS.

2.5. Oil Red O staining

After 8 days, differentiated 3T3-L1 pre-adipocytes were fixed with 10% formalin in phosphate buffer saline (PBS) for 1 h and washed with 60% isopropyl alcohol. After drying at room temperature, cells were stained with filtered Oil Red O solution for 30 min. Stained cells were washed four times with distilled water. The phenotypic changes of adipogenic differentiation were observed using an inverted phase-contrast microscope (Olympus IX70, Tokyo, Japan). To quantify the amount of Oil Red O stained lipids, stained cells were eluted with 100% isopropyl alcohol for 10 min and the absorbance of the extracts was measured at 500 nm in a microplate reader.

2.6. Real-time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and an aliquot (3 μg) of total RNA was reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK), according to the manufacturer's instructions. First strand cDNAs were amplified by PCR using gene-specific primers. Real-time PCR was carried out using SYBR Green (SYBR Green Master Mix, Applied Biosystems) with the Rotor-Gene system (Corbett Research Co., Mortlake, Australia). The detector was programmed with the following PCR conditions: 40 cycles of 10 s denaturation at 95 °C, 30 s annealing at 53 °C, and 20 s amplification at 72 °C. All reactions were run in triplicate and were normalized to the housekeeping gene GAPDH. Primers for adipogenic genes used in this study were as follows: mouse GAPDH: forward, 5′-TTGTTGCCATCAACGACCCC-3′ and reverse, 5′-GCC GTTGAATTTGCCGTGAG-3′; mouse PPAR-γ: forward, 5′-CAGTGGG-GAGAGAGGACAGA-3′ and reverse, 5′-AGTTCGGGAACAAGACGTTG-3′; mouse C/EBP-α: forward, 5′-TTGTTTGGCTTTATCTCGGC-3′ and reverse, 5′-CCAAGAAGTCGGTGGACAAG-3′; mouse SREBP-c: forward, 5′-TGGTTGTTGATGAGCTGGAG-3′ and reverse, 5′-GGCTC TGGAACAGACACTGG-3′; mouse aP2: forward, 5′-CACTTTCC TTGTGGCAAAGC-3′ and reverse, 5′-AATGTGTGATGCCTTTGTGG-3′; mouse FAS: forward, 5′-GTTGGCCCAGAACTCCTGTA-3′ and reverse, 5′-GTCGTCTGCCTCCAGAGC-3′.