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Comparative Study

ROLE OF HYALURONIC ACID IN MESENCHYMAL STEM CELL DIFFERENTIATION: A CELLULAR INVESTIGATION ON INFLAMMATION BIOLOGICAL NETWORK

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ABSTRACT

Hyaluronic acid (HA) is the major structural component of the extracellular matrix, involved in signaling pathways, inflammation, wound repair, and morphogenesis.

HA is considered an important biomaterial for tissue engineering, drug delivery systems and various medical and pharmaceutical applications based on its characteristics, such as its good biocompatibility, biodegradability, and viscoelastic properties. The physiological properties of HA largely depend on its molecular weight and ability to interact with specific cell receptors.

In this study, we evaluated the correlation between the molecular weight and physiological function of HA by measuring the expression of some inflammatory mediators, such as cytokines, chemokines, and interleukins in mesenchymal stem cells.

Gene expression of genes belonging to the "Inflammatory Cytokines and Receptors" pathway was measured by Real-Time PCR after 24h of treatment with high, medium, and low molecular weight HA solution.

The results confirm the anti-inflammatory activity of high molecular weight HA and the pro-inflammatory and immuno-stimulating activity of medium and low molecular weight HA.

KEYWORDS: *Hyaluronic acid, inflammation, mesenchymal stem cells, real-time PCR*

INTRODUCTION

Hyaluronic acid (HA) is a linear polymer belonging to the family of unbranched polysaccharides called glycosaminoglycans (GAGs), composed of repeated disaccharide units of β-1, 3-N-acetyl-D-glucosamine, and β-1, 4

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acid-glucuronic (1). It is a natural extracellular matrix component found in various body fluids, organs, and tissues. It is found at high levels in the umbilical cord, synovial fluid, skin dermis, epidermis, vitreous of the eye, and blood (2). It is synthesized by fibroblasts, keratinocytes, and endothelial cells in the skin region to regulate various biological processes, including signaling, inflammation, wound repair, morphogenesis, and others (3-5).

The molecular size of HA varies from 0.8 kDa to 3000 kDa and is determined by the number of repeated disaccharide units. The biological functions of HA depend on molecular weight. HA with a molecular weight greater than 1000 kDa exerts antiangiogenic, immunosuppressive, and anti-inflammatory effects (6). High molecular weight HA is also highly viscoelastic and viscous and can protect cartilage by acting as a lubricant in the synovial fluid. Conversely, medium and low molecular weight HA possesses pro-inflammatory, pro-angiogenic, and immunostimulating properties (1, 3).

The physiological responses mediated by HA usually correlate with the immune functions that cause inflammation of the tissues and are based on the different molecular sizes related to the different receptors used. Low molecular weight HA binds to toll-like receptor (TLR) 4, an innate bacterial lipopolysaccharide (LPS) receptor, while CD44 glycoprotein is the major high molecular weight HA binding receptor.

HA degradation is mainly mediated by members of a family of enzymes called hyaluronidases (HYAL1 and HYAL2) via the CD44 receptor in macrophages (7, 8). HYAL1 and HYAL2 are the main types of hyaluronidase and are distinguished by their action at the protein level. HYAL2 is responsible for the cleavage of high molecular weight HA, which is mainly bound to the CD44 receptor, where HYAL1 degrades HA in the lysosomes to generate HA oligosaccharides (9).

One of the best-known functions of HA is hydration, thanks to its good ability to trap water (2). Based on its characteristics, such as its good biocompatibility, biodegradability, and viscoelastic properties, HA is considered an important biomaterial for tissue engineering, drug delivery systems, and various medical and pharmaceutical applications (10, 11). HA is also known to reduce the appearance of wrinkles and accelerate wound healing. In addition to these functions, HA-based formulations have shown remarkable efficacy in treating a wide range of inflammatory skin diseases (12-14). In this study, we tested the effects of three different weight hyaluronic acids (high, medium, and low molecular weight) on a population of mesenchymal stem cells isolated from dental pulp.

Mesenchymal stem cells are an excellent model for testing biomaterials' effects or substances' cellular compatibility thanks to their multilineage differentiation potential, high proliferation activity, and self-renewal (15). Moreover, thanks to the ease of availability and isolation, the dental pulp is an excellent source of mesenchymal stem cells (16).

MATERIALS AND METHODS

Dental pulp stem cells isolation

Dental germ pulp was extracted from the third molars of healthy subjects. Pulp was digested for 1 h at 37 °C in a solution containing 3 mg/ml type I collagenase, 4 mg/ml dispase, in 4 ml phosphate-buffered saline (PBS) solution supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 500 µg/ml clarithromycin. The solution was then filtered with 70 µm Falcon strainers (Sigma Aldrich). Filtered cells were cultivated in α-MEM culture medium (Sigma Aldrich) supplemented with 20% FCS, 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and placed in 25 cm^2 flasks. Flasks were incubated at 37°C, and 5% $CO_{2,}$ and the medium changed twice a week.

Flow cytometric analyses

The purity of dental pulp stem cells (DPSC) cultures was determined by analysis of different antigens after staining with a fluorochrome (FITC- or PE-) conjugated mAbs anti-human CD14-FITC, CD14-PE, CD34-FITC, CD45-FITC, CD90-PE, CD105-PE (Immunotech) and analyzed by FACScan. The nonspecific mouse IgG was used as isotype control (Immunotech). In order to avoid nonspecific fluorescence from dead cells, live cells were gated tightly using forward, and side scatter.

Cell viability test

PrestoBlue™ Reagent Protocol (Invitrogen) was used to evaluate the viability of cells treated with high molecular weight hyaluronic acid (HMW-HA), medium molecular weight hyaluronic acid (MMW-HA), and low molecular weight hyaluronic acid (LMW-HA) solutions at different concentration. A stock solution of 10 g/mL of each molecular weight HA was prepared. Further dilutions were made with the culture medium to the desired concentrations before use.

Serial dilutions of each different molecular weight HA solution (1000 mg/mL, 100 mg/mL, 10 mg/mL, 1 mg/mL) were added (three wells for each concentration). The cell culture medium alone was used as a negative control. Cells were

seeded into 96-well plates at a density of 10^4 cells per well containing 100 µl of cell culture medium. After 24h of incubation, cell viability was measured using PrestoBlue™ reagent protocol. The percentage of viable cells was determined by comparing the average absorbance in drug-treated with average absorbance in control wells exposed to vehicle alone. The results were presented as the mean ± standard deviation of three measures.

Cell treatment

Cells were seeded at a 1.0 x 10^5 cells/ml density into 9 cm^2 (3 ml) wells and subjected to serum starvation for 16 hours at 37°C. After serum starvation, cells were treated with the following solutions: a) 10 mg/mL of HMW-HA; b) 10 mg/mL of MMW-HA; c) 10 mg/mL of LMW-HA. All solutions were obtained in DMEM supplemented with 2% FBS, antibiotics, and amino acids. For each treatment, three biological replicates were performed.

The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24 hours. Cell medium alone was used as a negative control. After the end of the exposure time, cells were trypsinized and processed for RNA extraction.

RNA isolation, reverse transcription and quantitative real-time RT-PCR

According to the manufacturer's instructions, total RNA was isolated from cell lines using GenElute mammalian total RNA purification miniprep kit (Sigma-Aldrich). Pure RNA was quantified at NanoDrop 2000 spectrophotometer (Thermo Scientific).

Table I. *Selected genes used in Real-Time PCR grouped by functional pathway.*

Phatway	Gene symbol	Gene name
Chemokine	CCL1	C-C motif chemokine ligand 1
	CCL2	C-C motif chemokine ligand 2
	CCL2D	C-C motif chemokine ligand 2 D
	CCL5	C-C motif chemokine ligand 5
	CCL8	C-C motif chemokine ligand 8
	CXCL5	C-X-C motif chemokine ligand 5
	CXCL10	C-X-C motif chemokine ligand 10
Chemokine receptor	CCR1	C-C motif chemokine receptor 1
	CCR2	C-C motif chemokine receptor 2
	CCR5	C-C motif chemokine receptor 5
	CCR6	C-C motif chemokine receptor 6
	CCR10	C-C motif chemokine receptor 10
	CXCR5	C-X-C motif chemokine receptor 5
Interleukin	IL1A	interleukin 1 alpha
	IL1B	interleukin 1 beta
	IL2	interleukin 2
	IL3	interleukin 3
	IL5	interleukin 5
	IL6	interleukin 6
	IL7	interleukin 7
	IL8	interleukin 8
Interleukin receptor	ILR1	interleukin 1 receptor type 1
	IL1RN	interleukin 1 receptor antagonist
	IL6R	interleukin 6 receptor
	IL10RB	interleukin 10 receptor subunit beta
Cytokine	BMP2	bone morphogenetic protein 2
	SPP1	secreted phosphoprotein 1
	TNFSF10	TNF superfamily member 10
	TNFSF11	TNF superfamily member 11
	VEGFA	vascular endothelial growth factor A
Cytokine receptor	TNFRSF	tumor necrosis factor receptor superfamily
Reference gene	RPL13	ribosomal protein L13

cDNA synthesis was performed starting from 500 ng of total RNA, using PrimeScript RT Master Mix (Takara Bio Inc.). The reaction was incubated at 37 °C for 15 min and inactivated by heating at 70 °C for 10 sec. cDNA was amplified by Real-Time Quantitative PCR using the ABI PRISM 7500 (Applied Biosystems).

All PCR reactions were performed in a 20 µl volume. Each reaction contained 10 µL of 2x qPCRBIO SYGreen Mix Lo-ROX (Pcrbiosystems), 400 nM concentration of each primer, and cDNA. Custom primers belonging to the "Inflammatory Cytokines and Receptors" pathway were purchased from Sigma Aldrich. The selected genes grouped by functional pathway are listed in Table I.

All experiments were performed, including non-template controls, to exclude reagent contamination. PCR was performed, including two analytical replicates. The amplification profile was initiated by 10 minutes of incubation at 95°C, followed by two-step amplification of 15 seconds at 95 °C and 60 seconds at 60 °C for 40 cycles. As a final step, a melt curve dissociation analysis was performed.

Statistical analysis

The gene expression levels were normalized to the expression of the reference gene (RPL13) and were expressed as fold changes relative to the expression of the untreated cells. Quantification was done with the delta/delta Ct calculation method (17).

RESULTS

DPSC was treated with three different dilutions of hyaluronic acid stock solutions (high, medium, and low molecular weight) to establish the correct concentration to be used in the treatment of cells cultured *in vitro*. After 24 hours of treatment, cell viability was measured using the PrestoBlue™ assay establishing that the optimal concentration of the treatment that did not significantly affect cell viability was 10 mg/ml for all three types of hyaluronic acid.

Gene expression of genes belonging to the "Inflammatory Cytokines and Receptors" pathway was measured by Real-Time PCR in DPSC treated with high, medium, and low molecular weight hyaluronic acid solution 10 mg/ml for 24 h hours. Table II shows significant gene expression levels after 24 hours of treatment with high molecular weight hyaluronic acid (HMW-HA) compared to untreated cells.

Table II. *Significant gene expression levels after 24h treatment with HMW-HA, as compared with untreated cells.*

Gene	Fold change	SD (+/-)	Gene function
CCL1	3.24	1.19	Chemokine
CCL2	0.41	0.05	Chemokine
CCL2D	0.40	0.08	Chemokine
CCL8	0.14	0.00	Chemokine
CXCL5	0.13	0.04	Chemokine
CXCL10	0.30	0.01	Chemokine
CCR2	0.14	0.00	Chemokine receptor
CCR5	0.17	0.00	Chemokine receptor
CCR6	0.09	0.02	Chemokine receptor
CXCR5	0.23	0.03	Chemokine receptor
IL2	0.15	0.03	Interleukin
IL3	0.24	0.03	Interleukin
IL5	0.04	0.01	Interleukin
TNFRSF	0.22	0.04	Cytokine receptor
TNFSF11	0.26	0.01	Cytokine

All the significantly deregulated genes, except the chemokine CCL1, were down-regulated in treated cells. Genes were "Chemokine" (CCL2, CCL2D, CCL8, CXCL5, CXCL10), "Chemokine receptor" (CCR2, CCR5, CCR6, CXCR5), "Interleukin" (IL2, IL3, IL5), "Cytokine" (TNFSF11) and "Cytokine receptor" (TNFRSF). Fig. 1 represents the gene expression profile of treated stem cells compared with control (untreated cells).

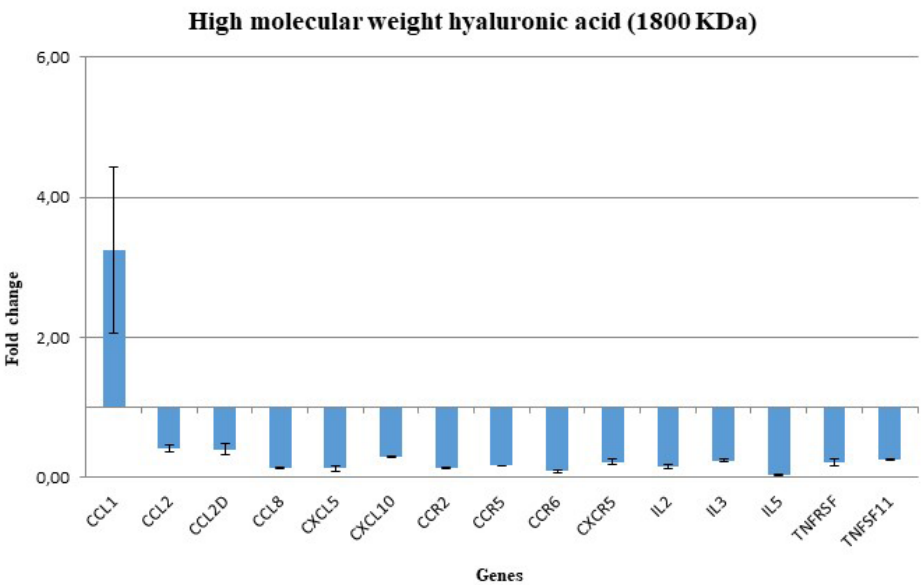

Fig. 1. *Gene expression profile of human DPSC treated with HMW-HA 10 mg/ml.*

Table III reported the significant gene expression levels after 24h treatment with medium molecular weight hyaluronic acid (MMW-HA) compared to untreated cells. The treatment induces the down-regulation of genes belonging to "Chemokine" (CCL1, CCL2, CCL2D, CCL8, CXCL5, CXCL10), "Chemokine receptor" (CCR1, CCR2, CCR4, CCR5, CCR6, CXCR5), "Interleukin" (IL2 and IL5), "Interleukin receptor" (IL1RN), "Cytokine" (SPP1, TNFSF11) and "Cytokine receptor" (TNFRSF). Only three genes, the chemokine CCL5 and the interleukins IL1A and IL6 were significantly up-regulated, as shown in Fig. 2.

Table IV reported the significant gene expression levels after 24h treatment with low molecular weight hyaluronic acid (LMW-HA) compared to untreated cells. Significantly up-regulated genes were chemokine CCL1 and chemokine receptor CCR10, interleukins IL1A, IL5 and interleukin receptor IL6R, cytokines SPP1 and TNFSF11. Conversely, among the down-regulated genes after treatment, there were chemokines CCL2 and CXCL10, chemokine receptors CCR1 and CCR2, and interleukin receptor ILR1. Fig. 3 shows the expression profile of genes up-and down-regulated in stem cells treated with low molecular weight hyaluronic acid.

Table III. *Significant gene expression levels after 24h treatment with MMW-HA, as compared with untreated cells.*

Gene	Fold change	SD (+/-)	Gene function
CCL1	0.09	0.01	Chemokine
CCL2	0.21	0.01	Chemokine
CCL2D	0.49	0.02	Chemokine
CCL5	2.92	0.33	Chemokine
CCL8	0.15	0.01	Chemokine
CXCL5	0.22	0.02	Chemokine
CXCL10	0.10	0.01	Chemokine
CCR1	0.19	0.02	Chemokine receptor
CCR2	0.06	0.00	Chemokine receptor
CCR4	0.10	0.03	Chemokine receptor
CCR5	0.32	0.01	Chemokine receptor
CCR6	0.19	0.02	Chemokine receptor
CXCR5	0.34	0.05	Chemokine receptor
IL1A	2.57	0.01	Interleukin
IL2	0.21	0.06	Interleukin
IL5	0.18	0.01	Interleukin
IL6	3.70	0.14	Interleukin
IL1RN	0.48	0.04	Interleukin receptor
SPP1	0.43	0.01	Cytokine
TNFRSF	0.33	0.01	Cytokine receptor
TNFSF11	0.17	0.17	Cytokine

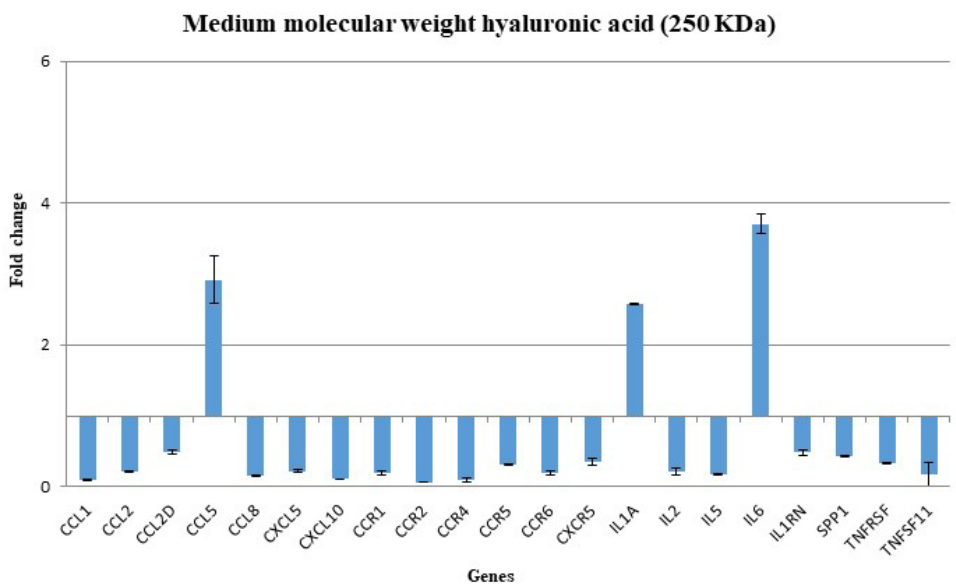

Fig. 2. *Gene expression profile of human DPSC treated with MMW-HA 10 mg/ml.*

DISCUSSION

HA is a linear glycosaminoglycan composed of alternating units of β-1,4-bonded D-glucuronic acid and β-1-3-bonded N-acetyl-D-glucosamine-linked and synthesized by fibroblasts, keratinocytes and endothelial cells (18). It is the major structural component of the extracellular matrix, involved in signaling pathways, inflammation, wound repair, and morphogenesis (3). Numerous studies have highlighted how the physiological properties of hyaluronic acid largely depend on its molecular weight and ability to interact with specific cell receptors (3, 19). High molecular weight hyaluronic acid interacts with the CD44 receptor and has antiangiogenic, immunosuppressive, and anti-inflammatory effects (6). Low molecular weight HA, in contrast, binds to the toll-like rector (TLR) 4, an innate bacterial lipopolysaccharide (LPS) receptor, and possesses pro-inflammatory, pro-angiogenic, and immunostimulating properties (20, 21).

Table IV. *Significant gene expression levels after 24h treatment with LMW-HA, as compared with untreated cells.*

Gene	Fold change	SD (+/-)	Gene function
CCL1	2.35	0.55	Chemokine
CCL2	0.21	0.04	Chemokine
CXCL10	0.17	0.04	Chemokine
CCR1	0.42	0.08	Chemokine receptor
CCR2	0.41	0.04	Chemokine receptor
CCR10	2.29	0.17	Chemokine receptor
IL1A	2.39	0.40	Interleukin
IL5	2.24	0.15	Interleukin
ILR1	0.48	0.13	Interleukin receptor
IL6R	2.03	0.36	Interleukin receptor
SPP1	2.97	0.27	Cytokine
TNFSF11	2.91	0.50	Cytokine

In this study, we evaluated the correlation between HA's molecular weight and physiological function by measuring the expression of some inflammatory mediators, such as cytokines, chemokines, and interleukins, in mesenchymal stem cells extracted from the dental pulp after 24 hours of treatment with HA.

Gene expression levels of genes belonging to the "Inflammatory Cytokines and Receptors" pathway were measured by Real-Time PCR. All the genes (except the cytokine CCL1) expressing pro-inflammatory mediators were significantly down-regulated by treatment of cells with HMW-HA, confirming this polymer's anti-inflammatory and restorative activity at the high molecular weight.

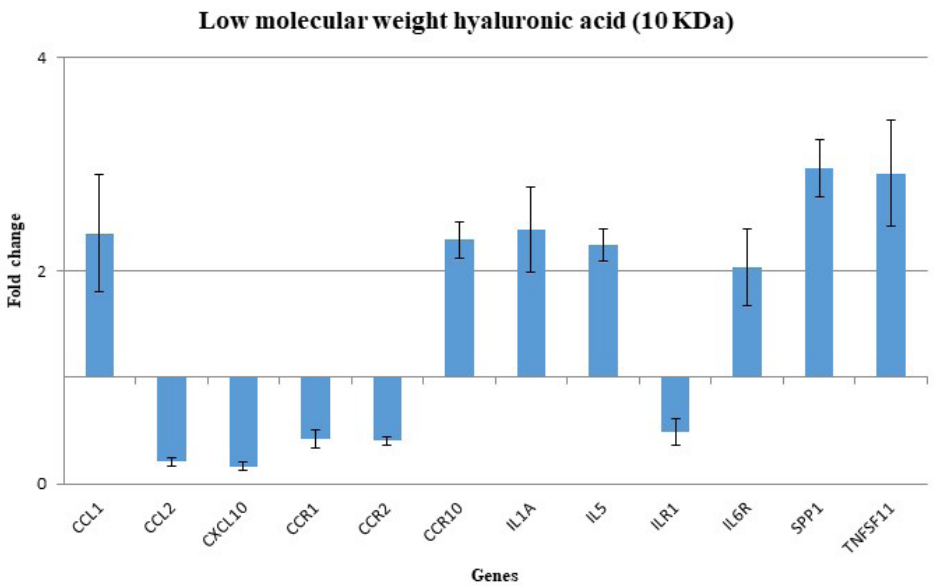

Fig. 3. *Gene expression profile of human DPSC treated with LMW-HA 10 mg/ml.*

Instead, treatment with medium and low molecular weight HA seems to confirm the pro-inflammatory and immunostimulating properties exerted by low and medium molecular weight HA. Stem cells treated with MMW-HA showed significant over-expression of pro-inflammatory interleukins such as IL1A and IL6; this trend becomes more evident in the treatment with LMW-HA, where in addition to the overexpression of interleukins IL1A, IL5 and IL6, up-regulation of cytokines SPP1 and TNSF11, were also observed.

Both medium and low molecular weight HA treatments induce significant over-expression of IL6. LMW-HA binds to toll-like receptor (TLR) 4, activating nuclear factor kappa B (NF-κB) via a myeloid differentiation factor (MyD) 88 dependent pathway leading to nuclear translocation of NF-κB to induce cytokine expression pro-inflammatory, such as interleukin 6 (IL-6) (22). The results confirm the anti-inflammatory activity of high molecular weight HA and the pro-inflammatory and immuno-stimulating activity of medium and low molecular weight HA.

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