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The effect of hyaluronic acid on human fibroblasts: an in vitro study

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ABSTRACT

The linear glycosaminoglycan hyaluronic acid is a component of many body organs and tissues, such as the extracellular matrix of connective tissue, skin, synovial fluid, and embryonic mesenchymal tissue, naturally occurring with a high molecular weight. Hyaluronic acid can also be detected in the soft periodontal tissues (gingiva and periodontal ligament).

Hyaluronic acid biocompatibility, biodegradability, non-toxicity, non-immunogenic and non-inflammatory properties make it applicable to bioengineering and biomedicine fields. Its molecular weight influences the biological effects of hyaluronic acid.

High-molecular weight hyaluronic acid causes the suppression of immune response, avoiding the exacerbations of inflammation; on the other hand, low-molecular-weight hyaluronic acid takes part in tissue damage signaling and immune cell mobilization. Thanks to these properties, hyaluronic acid can be considered a promoter of soft tissue and bone healing.

This study evaluates the effect of hyaluronic acid with different molecular weights on fibroblasts.

Keywords: *hyaluronic acid, inflammation, fibroblasts*

INTRODUCTION

The linear glycosaminoglycan hyaluronic acid (HA) is composed of repeating units of N-acetylglucosamine and a linear chain of D-glucuronic acid, with β-1,3 and β-1,4 glycosidic bonds linking the monosaccharide units (1-4). HA is naturally occurring with a high molecular weight (up to $2x10^4$ kDa) and is a component of extracellular matrix of connective tissue, skin, synovial fluid, and embryonic mesenchymal tissue (5, 6). Regarding the oral cavity, HA can be detected in the soft periodontal tissues (gingiva and periodontal ligament) and hard tissues such as bone; moreover, it represents a key element of crevicular fluid and saliva (7, 8). This natural linear polysaccharide is synthesized by three transmembrane enzymes 1, 2, and 3 (HAS1, 2, and 3) on the cytoplasmic surface of the plasma membrane, while the hyaluronidase enzyme family

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plays a major role in its catabolism (9). The HAS synthesized HA with different molecular weights since their expression during morphogenesis, and pathological conditions are different: HAS1 and HAS2 are responsible for the production of high-molecular sized molecules (> 2 x 10^6), while low-molecular-weight molecules are generated by HAS3 (1 x 10^5 – 1 x 10^6) (10-13). HA biocompatibility, biodegradability, non-toxicity, non-immunogenic and non-inflammatory properties make it applicable to bioengineering and biomedicine fields (14). Its molecular weight influences the biological effects of HA: HA with a high-molecular size (> 500 kDa) acts as an anti-angiogenic, anti-inflammatory, and immunologic depressant molecule, and it can enhance tissue integrity and cell quiescence (9, 15). Molecular weight from 6 to 20 kDa presents angiogenic, immunostimulatory, and phlogotic properties; embryonic development, ovulation, and wound healing involve HA with molecular weight ranging from 20 to 200 kDa (9).

Data reported in the literature demonstrates that HA can contribute to the oral soft tissue wound healing process: it first acts as a high molecular weight molecule whose anti-angiogenic and immunosuppressive properties facilitate the accumulation of polymorphonuclear leukocytes in the wound site. In the next step, the inflammatory stage, high molecular weight HA is fragmented by hyaluronidase or reactive oxygen species activity, inducing angiogenesis, stimulating the production of pro-inflammatory cytokines by fibroblasts, cementoblasts, and osteoblasts and thus promoting the inflammatory response (16-18). It has been demonstrated that high-molecular-weight HA causes the suppression of immune response, avoiding the exacerbations of inflammation, while on the other hand, low-molecular-weight HA takes part in tissue damage signaling and immune cell mobilization (19). Thanks to these properties, HA can be considered a valid promoter of soft tissues and bone healing (20).

The aim of our research was to study the effect of HA with different molecular weights on human fibroblasts, assessing the role of this natural linear polysaccharide in gingival inflammatory conditions.

MATERIALS AND METHODS

Primary Human Fibroblast cells culture

Primary gingival fibroblasts obtained from a 60-year-old woman were purchased from ATCC® Cell Lines (LGC Standards, Middlesex, UK). Cryopreserved cells at the second passage were cultured in 75 cm^2 culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 50% fetal calf serum, antibiotics (Penicillin 100U/ml and Streptomycin 100 micrograms/ml-Sigma Aldrich, Inc., St Louis, Mo, USA).

Cells were incubated in a humified atmosphere of 5% CO_2 at 37°C. The medium was changed the next day and twice a week. After 15 days, the pieces of tissue were removed from the culture flask. Cells were harvested ted after an additional 24h of incubation.

Cell viability test

PrestoBlue™ Reagent Protocol (Invitrogen) was used to evaluate the viability of cells treated with high, low, and medium molecular weight hyaluronic acid (HA) solutions at different concentrations. A 10 g/mL stock solution 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 104 cells per well containing 100 µl of cell culture medium.

After 24h of incubation, cell viability was measured using PrestoBlue™ reagent protocol (Invitrogen). The percentage of viable cells was determined by comparing the average absorbance in drug-treated wells with the average absorbance in control wells exposed to vehicles 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 solution: a) 10 mg/mL of high molecular weight HA; b) 10 mg/mL of medium molecular weight HA; c) 10 mg/mL of low molecular weight 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 h.

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

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

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 A minute 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.

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

Pathway	Gene symbol	Gene name
Chemokine	CCL2	C-C motif chemokine ligand 2
	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
Housekeeping gene	RPL13	ribosomal protein L13

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 (21).

RESULTS

To establish the right concentration of hyaluronic acid (high, medium, and low molecular weight) to be used in treating fibroblasts cultured in vitro, serial dilutions of the stock solutions were made. After treating the cells for 24 hours with these solutions, cell viability was measured using the PrestoBlue™ assay, and it was established 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 investigated in fibroblast treated high, medium, and low molecular weight hyaluronic acid solution 10 mg/ml for 24 h. Table II shows significant gene expression levels after 24h treatment with high molecular weight hyaluronic acid (HMW-HA) compared to untreated cells.

The treatment of fibroblasts with HMW-HA modulates the expression of various genes belonging to the "Inflammatory Cytokines and Receptors" pathway. Among these, the up-regulated genes are the chemokines CCL2D and CCL8 and

the receptor for the chemokines CXCR5. In addition, the treatment induces a significant up-regulation of interleukins IL1B, IL2, and IL5 and the over-expression of a cytokine that belongs to the tumor necrosis factor (TNF) ligand family, TNFSF10.

Among the genes down-regulated by HMW-HA are the CCR1 chemokine receptor, interleukin 1 A (IL1A) and its ILR1 receptor, and the two cytokines BMP2 and SPP1. Fig. 1 represents the gene expression profile of treated fibroblast with HMW-HA compared with control (untreated cells).

Table III reports the significant gene expression levels after a 24h treatment with medium molecular weight hyaluronic acid (MMW-HA) compared to untreated cells. Significant up-regulated genes were chemokine receptors CCR2 and CCR10, the interleukin 2 (IL2), and the cytokine belonging to the tumor necrosis factor (TNF) ligand family, TNFSF11. Conversely, MMW-HA induces down-regulation of genes like the chemokine receptor CCR6, the interleukin 6 (IL6), the receptor IL6R, and the cytokine SPP1.

Fig. 2 shows the expression profile of genes up-and down-regulated in treated with medium molecular weight hyaluronic acid.

Table IV reported the significant gene expression levels after a 24h treatment with low molecular weight hyaluronic acid (LMW-HA) compared with untreated cells. The treatment induces the down-regulation of genes such as chemokine CCL5 and chemokine receptors CCR1, CCR5, CCR6, and CCR10. Other down-expressed genes were Interleukin IL1A, the interleukin receptor IL6R and the cytokine SPP1.

Only the interleukin IL2 and the cytokine belonging to the tumor necrosis factor (TNF) ligand family, TNFSF11, were up-regulated by the treatment. Fig. 3 shows the fibroblast gene expression modulation after the LMW-HA treatment.

DISCUSSION

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 (22, 23).

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

Gene	Fold change	SD +/-)	Gene function
CCL2D	2,30	0,34	Chemokine
CCL8	3,79	0,11	Chemokine
CCR1	0,28	0,04	Chemokine receptor
CXCR5	2,49	0,87	Chemokine receptor
IL1A	0,34	0,00	Interleukin
IL1B	2,83	0,03	Interleukin
IL2	7,35	0,15	Interleukin
IL5	2,94	0,01	Interleukin
ILR1	0,41	0,02	Interleukin receptor
BMP2	0,06	0,01	Cytokine
SPP1	0,31	0,01	Cytokine
TNFSF10	5,12	1,19	Cytokine

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

Gene	Fold change	SD (+/-)	Gene function
CCR2	3,56	0,14	Chemokine receptor
CCR6	0,28	0,02	Chemokine receptor
CCR10	3,06	0,68	Chemokine receptor
IL2	11,12	3,07	Interleukin
IL3	0,31	0,04	Interleukin
IL6R	0,25	0,02	Interleukin receptor
SPP1	0,49	0,03	Cytokine
TNFSF11	4,71	0,09	Cytokine

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

Gene	Fold change	SD (+/-)	Gene function
CCL5	0,42	0,0312	Chemokine
CCR5	0,09	0,0009	Chemokine receptor
CCR1	0,47	0,1241	Chemokine receptor
CCR10	0,50	0,0385	Chemokine receptor
CCR6	0,26	0,0115	Chemokine receptor
IL2	6,84	0,0931	Interleukin
IL6R	0,30	0,0070	Interleukin receptor
SPP1	0,50	0,1163	Cytokine
TNFSF11	2,27	0,0178	Cytokine

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 (24). In this study, the effects of HA with different molecular weights on human fibroblasts were evaluated, assessing the role of this natural linear polysaccharide in gingival inflammatory conditions.

The main receptor for HA is CD44, a glycoprotein expressed on the T cell's surface. The binding of exogenous HA to CD44 plays multiple roles in T cell biology, including their autocrine proliferation (25) and the regulation of T cell trafficking to inflamed sites (26). Mahaffey et al. showed that exogenous HA enhances IL2 expression, which binds to its IL2R receptor inducing T cell proliferation (27).

In this study, the treatment of fibroblasts with hyaluronic acid at all three molecular weights (high, medium, and low) induces the overexpression of IL2, suggesting that IL2 is an important mediator involved in the proliferation of T lymphocytes following the binding of HA to its CD44 receptor. SPP1 encodes for osteopontin, a protein that plays a fundamental role in the immune response and tissue repair associated with inflammatory diseases. Its expression increases in immune cells, epithelial cells, endothelial cells, and fibroblasts of inflamed tissues.

Mori et al. (28) showed that inflammation-triggered osteopontin expression inhibits the repair rate and contributes to wound fibrosis. In our study, hyaluronic acid determines the under-expression of SPP1. Therefore, hyaluronic acid would increase tissue repair by improving the rate and quality of healing.

In our study, high molecular weight hyaluronic acid exerts an anti-inflammatory effect on treated fibroblasts, as demonstrated by the significant under-expression of IL1 and its IL1R receptor. IL-1 is a pro-inflammatory cytokine whose role in regulating the mechanisms leading to the amplification of inflammation has been widely demonstrated. Its role is primarily expressed in regulating genes that amplify or support inflammation, such as metalloproteinases, prostaglandins, adhesion molecules on leukocytes, and chemokines necessary to recruit circulating leukocytes. Its inflammatory activity has also been extensively demonstrated in vivo in mice, in which it has been observed that the deletion of the gene leads to a reduction of the inflammatory response, exposing them to many types of infections (29).

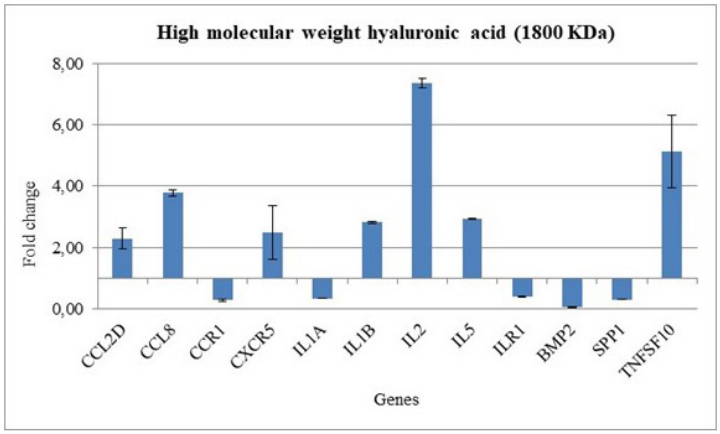

Fig. 1. *Gene expression profile of fibroblast treated with HMW-HA 10 mg/ml.*

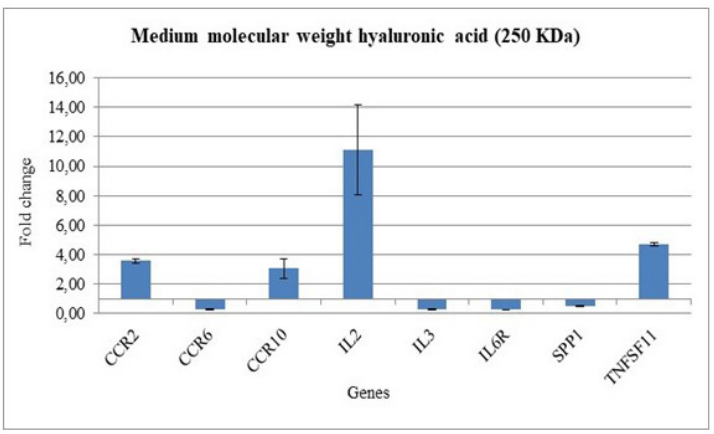

Fig. 2. *Gene expression profile of fibroblast treated with MMW-HA 10 mg/ml.*

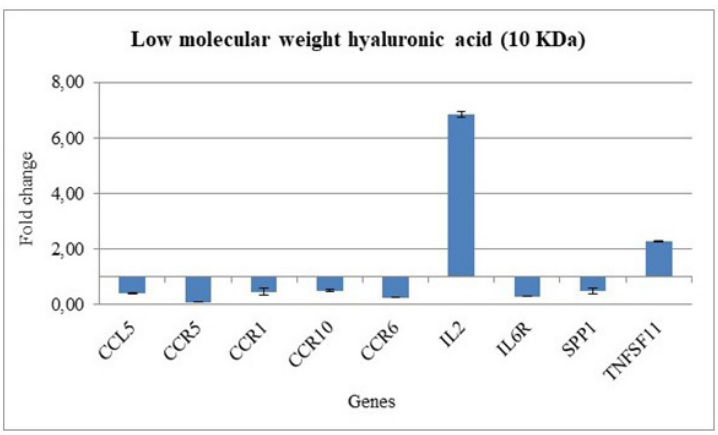

Fig. 3. *Gene expression profile of fibroblast treated with LMW-HA 10 mg/ml.*

In general, the results obtained show the tendency of hyaluronic acid to reduce inflammation and tissue regeneration,

with a more marked aptitude for high molecular weight hyaluronic acid.

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