Evaluation Study

INTERACTION BETWEEN HUMAN FIBROBLASTS AND HYALURONIC ACID: OUTPUT IN THE EXTRACELLULAR MATRIX

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ABSTRACT

Hyaluronic acid is the major constituent of the extracellular matrix. It is important in cell signaling and proliferation, extracellular matrix structural organization, tissue reparation, angiogenesis, and inflammatory and immune response. Nevertheless, it was demonstrated that hyaluronic acid's biological functions and properties are strictly dependent on its molecular weight, showing opposite effects between high-molecular-weight and low molecular weight. Here, we tested the effect of hyaluronic acid at the different molecular weights (low, medium, and high) on the extracellular matrix deposition and remodeling in fibroblasts treated for 24 hours, measuring the gene expression levels of genes belonging to "Extracellular Matrix and Adhesion Molecules" pathway. The most significant effects in cell proliferation seem to occur with the administration of high and medium molecular-weight hyaluronic acid, which induces the expression of genes such as HAS1, COL4A1, and COL9A1. These results demonstrated that hyaluronic acid activates fibroblasts by stimulating the deposition of the extracellular matrix and its remodeling.

KEYWORDS: *hyaluronic acid, fibroblasts, extracellular matrix, gene expression*

INTRODUCTION

Hyaluronic acid (HA), a non-sulfated glycosaminoglycan, is a polymer of disaccharides composed of D-glucuronic acid and N-acetyl-D-glucosamine. HA is present at the extracellular matrix (ECM) level and plays a key role during wound healing phases and in any regulatory process ECM. It is important in cell signaling and proliferation, ECM structural organization, tissue reparation, angiogenesis, and inflammatory and immune response (1-4). Nevertheless, it was demonstrated that HA's biological functions and properties are strictly dependent on its molecular weight, also showing opposite effects between high-molecular-weight (HMW) (i.e., higher than 1000 KDa) and low-molecular-weight (LMW) (i.e., lower than 1000 KDa) (2-7). HA is a major constituent of ECM in the human body; it is constantly synthesized as HMW-HA and is degraded very fast by hyaluronidases (8). Moreover, it plays an important role in supporting cells during wound healing (9, 10), recognizing specific surface receptors during the healing process (11), and favoring collagen deposition and angiogenesis (9, 10). HA is known to activate fibroblasts, and it is involved during the proliferation, migration, and tissue maturation phases of the healing process (12). However, HA is rapidly metabolized,

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and its half-life is less than a day. HA is also actively degraded within 24 h by the hyaluronidase enzymes or reactive oxygen species (12).

The aim of our research was to study the effect of HA with different molecular weights on human gingival fibroblasts, assessing the role of this natural linear polysaccharide in extracellular matrix deposition and remodeling. For this purpose, we treated human fibroblasts with hyaluronic acid at three different molecular weights, high, medium, and low, for 24 hours. Then we measured the expression levels of genes involved in the "Extracellular Matrix and Adhesion Molecules" pathway by real-time PCR.

MATERIALS AND METHODS

Primary gingival fibroblasts purchased from ATCC® Cell Lines were cultured in flasks containing medium and antibiotics and incubated in a humified atmosphere. PrestoBlue™ Reagent Protocol (Invitrogen) was used to evaluate the viability of cells.

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. For each treatment, three biological replicates were performed. Cell medium alone was used as a negative control. After the end of the exposure time, cells were trypsinized and processed for RNA extraction. Primers from the "Extracellular Matrix and Adhesion Molecules" pathway were purchased from Sigma Aldrich. The selected genes grouped by functional pathway are listed in Table I. Standard cDNA synthesis was performed, and cDNA was amplified by Real-Time Quantitative PCR using the ABI PRISM 7500 (Applied Biosystems). For statistical analysis, the delta/delta Ct calculation method was used. (13).

Table I. *Selected genes grouped by functional pathway.*

Pathway	Gene symbol	Gene name
Collagens & Extracellular Matrix Structural constituent	COL1A2	collagen type I alpha 2 chain
	COL2A1	collagen type II alpha 1 chain
	COL3A1	collagen type III alpha 1 chain
	COL4A1	collagen type IV alpha 1 chain
	COL5A1	collagen type V alpha 1 chain
	COL6A1	collagen type VI alpha 1 chain
	COL7A1	collagen type VII alpha 1 chain
	COL8A1	collagen type VIII alpha 1 chain
	COL9A1	collagen type IX alpha 1 chain
	COL10A1	collagen type X alpha 1 chain
	COL11A1	collagen type XI alpha 1 chain
Cell Adhesion Molecule	CCTNA1	catenin alpha 1
	CTNNB	catenin beta 1
	CTNND2	catenin delta 2
	VCAN	versican
Transmembrane Receptor	HAS1	hyaluronan synthase 1
	ILF3	interleukin enhancer binding factor 3
	ITGA1	integrin subunit alpha 1
	ITGA2	integrin subunit alpha 2
	ITGA3	integrin subunit alpha 3
	ITGA4	integrin subunit alpha 4
	ITGA5	integrin subunit alpha 5
	ITGA6	integrin subunit alpha 6
	ITGA7	integrin subunit alpha 7
	ITGA8	integrin subunit alpha 8
	ITGB1	integrin subunit beta 1
	ITGB2	integrin subunit beta 2
	ITGB4	integrin subunit beta 4
	ITGB5	integrin subunit beta 5
	LAMA1	laminin subunit alpha 1
	LAMA2	laminin subunit alpha 2
	LAMA3	laminin subunit alpha 3
	LAMB1	laminin subunit beta 1
	LAMB2	laminin subunit beta 2
	LAMB3	laminin subunit beta 3
Extracellular Matrix Protease	MMP2	matrix metallopeptidase 2
	MMP7	matrix metallopeptidase 7

	MMP8	matrix metallopeptidase 8
	MMP9	matrix metallopeptidase 9
	MMP10	matrix metallopeptidase 10
	MMP11	matrix metallopeptidase 11
	MMP12	matrix metallopeptidase 12
	MMP13	matrix metallopeptidase 13
	MMP14	matrix metallopeptidase 14
	MMP15	matrix metallopeptidase 15
	MMP16	matrix metallopeptidase 16
	MMP24	matrix metallopeptidase 24
	MMP26	matrix metallopeptidase 26
TGFβ Signaling	TGFB1	transforming growth factor beta 1
	TGFB2	transforming growth factor beta 2
	TGFB3	transforming growth factor beta 3
Extracellular Matrix Protease Inhibitor	TIMP1	TIMP metallopeptidase inhibitor 1
Housekeeping gene	RPL13	ribosomal protein L13

RESULTS

The proper concentration of hyaluronic acid to be used in treating human fibroblasts cultured in vitro was established by making serial dilutions of the stock solutions and treating the cells for 24 hours. In addition, gene expression of genes belonging to the "Extracellular Matrix and Adhesion Molecules" pathway was investigated in human fibroblasts treated with 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 up-regulated genes belong to "Collagens & Extracellular Matrix Structural constituent" (COL7A1, COL9A1) "Cell Adhesion Molecule" (CTNND2), "Transmembrane Receptor" (HAS1, ILF3, ITGA1, ITGA3, ITGA7, ITGA8, ITGB2, ITGB5), "Basement Membrane Constituent" (LAMA1, LAMB1, LAMB3), "Extracellular matrix protease pathway" (MMP9, MMP11, MMP24), TGFβ Signaling (TGFB3). The down-regulated genes were collagen COL6A1, metalloproteases MMP8, MMP12 and MMP26, and the transmembrane receptor TGFB2. Fig. 1 represents the gene expression profile of treated fibroblasts compared with control (untreated cells).

Table II. *Significant gene expression levels after 24h treatment with high molecular weight hyaluronic acid (HMW-HA)*

Gene	Fold change	SD (+/-)	Gene function
COL6A1	0,45	0,15	Collagens & Extracellular Matrix Structural constituent
COL7A1	2,07	0,15	Collagens & Extracellular Matrix Structural constituent
COL9A1	3,78	1,10	Collagens & Extracellular Matrix Structural constituent
CTNND2	5,43	0,35	Cell Adhesion Molecule
HAS1	3,96	0,54	Transmembrane Receptor
ILF3	3,29	0,22	Transmembrane Receptor
ITGA1	4,98	0,02	Transmembrane Receptor
ITGA3	2,42	0,28	Transmembrane Receptor
ITGA7	2,60	0,38	Transmembrane Receptor
ITGA8	2,44	0,05	Transmembrane Receptor
ITGB2	2,53	0,31	Transmembrane Receptor
ITGB5	3,41	0,25	Transmembrane Receptor
LAMA1	6,17	0,04	Basement Membrane Constituent
LAMB1	7,66	0,34	Basement Membrane Constituent
LAMB3	3,87	0,52	Basement Membrane Constituent
MMP8	0,14	0,01	Extracellular Matrix Protease
MMP9	2,63	0,12	Extracellular Matrix Protease
MMP11	2,28	0,33	Extracellular Matrix Protease

MMP12	0,44	0,04	Extracellular Matrix Protease
MMP24	8,51	1,86	Extracellular Matrix Protease
MMP26	0,35	0,03	Extracellular Matrix Protease
TGFB2	0,38	0,01	TGFβ Signaling
TGFB3	2,42	0,08	TGFβ Signaling

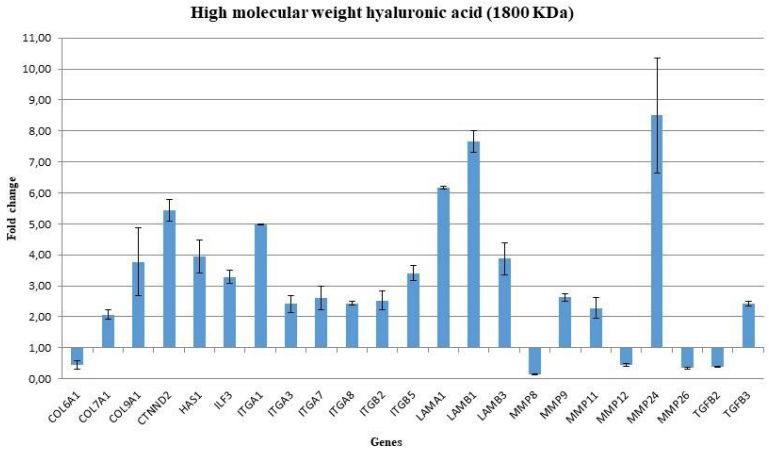

Fig. 1. *Gene expression profile of human fibroblasts treated with HMW-HA 10 mg/ml.*

Table III shows significant gene expression levels after 24h treatment with medium molecular weight hyaluronic acid (MMW-HA) compared with untreated cells. Genes differentially expressed were "Collagens & Extracellular Matrix Structural constituent" (COL4A1, COL9A1), "Cell Adhesion Molecule" (CTNND2), "Transmembrane Receptor" (HAS1), "Basement Membrane Constituent" (LAMA2) and "Extracellular Matrix Protease" (MMP8, MMP10, MMP13). All the genes were up-regulated except MMP2 and MMP15. Fig. 2 shows the expression profile of genes up-and down-regulated in treated fibroblasts with medium molecular weight hyaluronic acid.

Table III. *Significant gene expression levels are reported after 24h treatment with medium molecular weight hyaluronic acid (MMW-HA).*

Gene	Fold change	SD (+/-)	Gene function
COL4A1	2.24	0.19	Collagens & Extracellular Matrix Structural constituents
COL9A1	2.37	0.18	Collagens & Extracellular Matrix Structural constituents
CTNND2	4.91	0.47	Cell Adhesion Molecules
HAS1	4.64	0.03	Transmembrane Receptors
LAMA2	3.14	0.19	Basement Membrane Constituents
MMP2	0.34	0.01	Extracellular Matrix Proteases
MMP8	2.13	0.48	Extracellular Matrix Proteases
MMP10	2.99	0.24	Extracellular Matrix Proteases
MMP13	6.50	1.12	Extracellular Matrix Proteases
MMP15	0,18	0,00	Extracellular Matrix Proteases

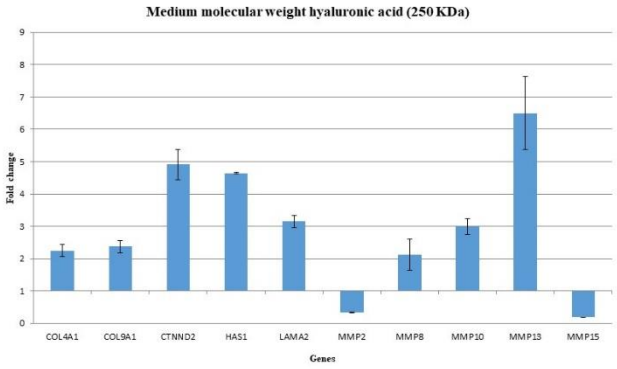

Fig. 2. *Gene expression profile of human fibroblasts treated with MMW-HA 10 mg/ml.*

Table IV reported the significant gene expression levels after 24h treatment with low molecular weight hyaluronic acid (LMW-HA) compared with untreated cells. The treatment induces the up-regulation of catenin delta 2 (CTNND2), laminin subunit beta 1 (LAMB1), and matrix metallopeptidase MMP13 and MMP26. The down-regulated genes were the transmembrane receptors ITGA7 and ITGB4 and the metallopeptidases MMP15 and MMP26. Fig. 3 shows the expression profile of genes up-and down-regulated in treated fibroblasts with low molecular weight hyaluronic acid.

Table IV. *Significant gene expression levels after 24h treatment with low molecular weight hyaluronic acid (LMW-HA).*

Gene	Fold change	SD (+/-)	Gene function
CTNND2	5.82	0.33	Cell Adhesion Molecule
ITGA7	0.44	0.10	Transmembrane Receptor
ITGB4	0.34	0.02	Transmembrane Receptor
LAMB1	7.71	0.09	Basement Membrane Constituent
MMP13	8.04	0.76	Extracellular Matrix Protease
MMP15	0.10	0.03	Extracellular Matrix Protease
MMP24	10.12	1.54	Extracellular Matrix Protease
MMP26	0.39	0.06	Extracellular Matrix Protease

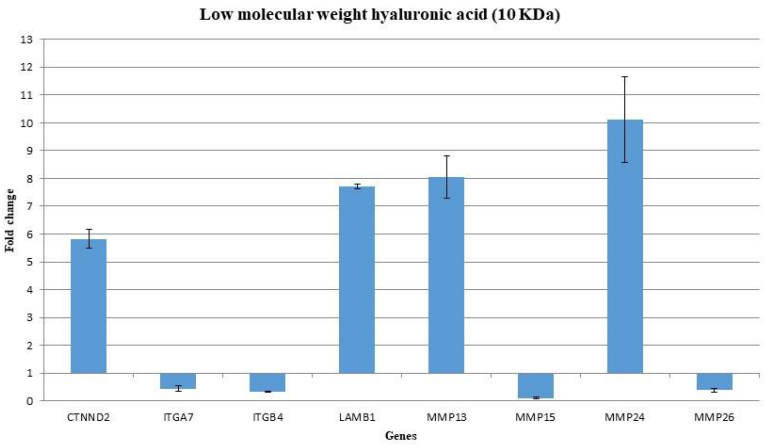

Fig. 3. *Gene expression profile of human fibroblasts treated with LMW-HA 10 mg/ml.*

DISCUSSION

The principal constituent of ECM in the human body is HA, synthesized as HMW-HA, and is degraded very fast by hyaluronidases (8). It plays an important role in supporting cells during wound healing (9, 10), recognizing specific surface receptors during the healing process (11), and favoring collagen deposition and angiogenesis. Here we tested the effect of HA at the different molecular weights (low, medium, and high) on the ECM deposition and remodeling in fibroblasts treated for 24 hours, measuring the gene expression levels of genes belonging to the "Extracellular Matrix and Adhesion Molecules" pathway. High molecular weight hyaluronic acid (HMW-HA) promotes fibrocytes' differentiation, leading to the deposition of extracellular matrix by fibroblasts (14).

In this study, fibroblasts treated with HMW-HA showed a high number of downregulated metallopeptidases, normally involved in extracellular matrix degradation. The administration of high and medium molecular weight hyaluronic acid stimulates the expression in treated fibroblasts of a series of genes involved in synthesizing high molecular weight hyaluronic acid involved in the deposition of extracellular matrix. These genes are the HAS1 enzyme responsible for synthesizing HMW-HA chains, and COL4A and COL9A1 are directly involved in the fibrillar rearrangement component of the extracellular matrix. In the same way, metalloproteinases are also differentially expressed, suggesting a modulation in the remodeling of the matrix.

Low molecular weight hyaluronic acid (LMW-HA) is involved in tissue inflammation mechanisms (15, 16). In fibroblasts treated with this molecule, it would seem that cells respond to the treatment by opposing the inflammatory action of the molecule by down-regulating numerous metalloproteinases, thus trying to stop the degeneration processes of the extracellular matrix. Therefore, the most significant effects in tissue repair and cell proliferation seem to occur with the administration of medium molecular weight hyaluronic acid, which induces the expression of genes such as HAS1, COL4A1, and COL9A1 to synthesise HMW-HA involved in cell proliferation processes. These results demonstrated that HA is involved in the activation of fibroblasts by stimulating the deposition of the extracellular matrix and its remodelling.

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