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Is molecular size a discriminating factor in hyaluronan interaction with human cells?

Antonella D'Agostino, Antonietta Stellavato, Luisana Corsuto, Paola Diana, Rosanna Filosa, Annalisa La Gatta, Mario De Rosa, Chiara Schiraldi *

Second University of Naples, Via L. De Crecchio, 7 80138 Napoli, Italy

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ABSTRACT

Nowadays there is a great interest in investigating the effect of particular hyaluronan fragments in the biomedical field and in cosmeceutical applications. Literature has reported that very low molecular weight HA (Mw < 5 kDa) has an inflammatory effect, whilst HA ranging from 15 to 250 has shown controversial effects. This work aims to give better elucidation on the correlation between the different sized HA fragments and their biological functions. In this respect, a simple and effective degradation strategy is used to obtain several HA fragments. Also, an hydrodynamic and structural characterization was performed in order to obtain samples suitable to evaluate cellular response. In particular an *in vitro* scratch test in time lapse experiments was used to study the effect of HA fragments, ranging from 1800 to 6 kDa on wound dermal reparation based on human keratinocytes. All high and low Mw HA used in this study allowed for faster wound closure compared to the un-treated cells, except for 6 kDa that, on the contrary, prevented repair.

In addition, TGF-β 1, TNFα and IL-6, representative biomarkers of the inflammation phase occurring in wound healing process, were quantified by RT-PCR. A general up-regulation trend of these biomarkers was found with the HA molecular weight reduction. LHA6 kDa was the only treatment that induced a major inflammatory response (over 30 fold increase respect to control) confirming the recent literature outcomes. IL-6 protein level evaluated through ELISA assay corroborated the previous results. Furthermore, activation of key HA receptors, such as CD44, RHAMM, TLR4, with respect to hyaluronan size, was evaluated, at transcriptional level showing selective recognition by HA 1800, 1400, 500 for CD44, whilst the lower Mw fragments activated TLR-4 moderately at 50 and 15 kDa. An increase to "alarm" level was found for 6 kDa fragments. Immunofluorescence staining confirmed this data.

The present research work demonstrated that the diverse pharma grade hyaluronan fragments could modulate cellular processes differently. From 1800 kDa down to 50 kDa, CD44 was the recognized receptor and pro-inflammatory biomarkers were only slightly up-regulated during wound healing in the presence of HA. Finally our outcomes showed that the lower the fragment size the higher the concern for inflammatory cytokines up-regulation; repair process impairment was highlighted only for 6 kDa chains.

Abbreviations: HHA, high molecular weight; LHA, low molecular weight; HaCat, human keratinocytes cell line; NaCl, sodium chloride; TGF-β1, transforming growth factor beta 1; TNFα, tumor necrosis factor α; DMEM, Dulbecco's Modified Eagle Medium; qRT-PCR, quantitative real time PCR; PBS, physiological buffer solution; TLVM, time-lapse video microscopy; RNA, ribonucleic acid; cDNA, complementary DNA; FBS, fetal bovine serum; Mw, molecular weight.

* Corresponding author.
E-mail addresses: antonella.dagostino@unina2.it (A. D'Agostino), antonietta.stellavato@unina2.it (A. Stellavato), luisanacorsuto@hotmail.it (L. Corsuto), paola.diana0@libero.it (P. Diana), rosanna.filosa@unina2.it (R. Filosa), annalisa.lagatta@unina2.it (A. La Gatta), mario.derosa@unina2.it (M. De Rosa), chiara.schiraldi@unina2.it, schiraldi.chiara@gmail.com (C. Schiraldi).

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1. Introduction

In recent years, scientists have been studying the correlation between HA molecular weight and physiological functions (Cyphert, Trempus, & Garantziotis, 2015; Cowman, Lee, Schwertfeger, McCarthy, & Turley, 2015; David-Raoudi et al., 2008; Ferguson, Roberts, Moseley, Griffiths, & Thomas, 2011; Ke, Sun Qiao, Wang, & Zeng, 2011). Generally, native HAs (high molecular weight ranging from 2000 to 1000 kDa) are space-filling molecules with anti-inflammatory and anti-angiogenic effect, while lower Mw HA may be involved in an inflammation process (Frenkel, 2014). In particular, there has been an increasing effort to clarify the role of low molecular weight hyaluronic acid (LHA) in the interaction with both the epidermis/dermis and articular joint functions (Ghosh & Guidolin, 2002). HA has been reported to be a free radical scavenger, presenting antioxidant function. It enhances the wound healing process and presents both angiogenic (Gao et al., 2010) and immunostimulatory activity (Ke et al., 2013). Due to water attracting characteristics in tissue repair, for example, long chain HA has cushioning and visco-elastic properties that create a porous scaffold onto which the cell might migrate; on the contrary, medium-size HA fragments (100–250 kDa) have been found to promote cell migration and contemporarily to stimulate and modulate pro-inflammatory cytokines production; finally very small fragments (4 saccharides) have been found to induce chemotaxis (Frenkel, 2014).

Vigetti et al. have reported that small HA fragments, ranging from 3 to 25 disaccharides (1.2–10 kDa), have inflammatory effects and show pro-angiogenic activity in human cell models (Vigetti et al., 2014). Ranging between 1, 6–4, 8 kDa, Oligo-fragments possess anti-apoptotic activity and induce heat shock proteins. As a matter of fact, they are recognized by different cell surface and receptors both in normal tissue and in cancer cells (Manjunath, Ahmed, Avadni, & Kumar, 2011). Other studies have shown that very low molecular weight HA (10–20 kDa) enhanced cell migration and proliferation and increase the expression of inflammatory mediators acting as an endogenous danger signal (Stern, Kogan, Jedrezejas, & Soltes, 2007).

Especially in wound healing process, the inflammatory response is driven by the primary cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (Campo et al., 2009). Contemporarily in injured tissue, the role of TGF-β1 has been widely investigated for its function in modulating ECM biosynthesis (Eldred, Dawes, & Wormston, 2011).

Another important research study has aimed at investigating HA interaction with membrane receptors, in particular CD44 (the major cell-surface HA binding protein) and TLR-4 the key receptor of the inflammatory cascade induced by dangerous signals (*e.g.* LPS, oligo-HA) (Campo et al., 2010). Besides, it has been proved that the CD44 receptors are over expressed in different tumors: epithelial, colon, leukemia, breast, ovarian and stomach. These cells present increased binding and uptake of HA. Exploiting the activity of HA in relation to cancer cells, hyaluronic acid graft copolymers were characterized for targeted delivery of anticancer drug in human colon cancer cells (HCT-116) that also over-express CD-44 HA receptors (Pitarresi et al. 2010). In addition, scientific community discuss on the importance of the use of polymer constructs based on hyaluronic acid for cartilage repair in alternative to traditional clinical treatments (non steroidal anti-inflammatory drugs). In this respect novel derivatives hydrogels HA based were used potentially to treat articular cartilage damages as recently reported by Fiorica and collaborators (Fiorica et al., 2015).

Relative to this, a recent report has shown that HA fragments stimulate chemokine and cytokine gene expression interacting with TLR-4 (Jiang, Liang, & Noble, 2011). RHAMM receptor was mainly involved in the cell migration. As a matter of fact, RHAMM-HA interaction does play an important function in tissue injury and repair (Viola et al., 2015).

The idea of obtaining a full array of HA fragments has arisen from the consideration that HA residue is sometimes required due to its different chemico-physical characteristics, such as low viscosity and higher solubility in aqueous solutions (Duranti, Salvi, Bovani, Calandra, & Rosati, 1998; Price, Berry, Harshad, & Navsaria, 2007). Slow-releasing gel containing a mixture of HA oligosaccharides (1–4, 8 kDa) was prepared to promote "*in vivo*" repair of tissue injury in an excision dermal wound by increasing neo-blood and lymph vessel formation (Gao et al., 2010). There has been recent interest in obtaining hyaluronan fragments with different chemico-physical characteristics that contribute to increase solubility of HA formulations, contemporarily maintaining an acceptable viscosity similar to those indicated for HA with Mw < 250 kDa(Palumbo et al., 2013). Although many reports have confirmed these differences, there have been no experimental highlights at biological level of these fragments.

Based on this knowledge and shared experience of diffused confusion in assessment and in the relationship between hyaluronan size and function, the goal of this research work, that addressed the question with rigorous methods, has been to identify a final range of HA fragments and their biochemical activities for *in vitro* models. The target of this manuscript is to give better elucidation on the effect of specific HA fragments when they interact with membrane receptors in modulating migration, and cytokines expression. Regarding the latter point, our study used the scratch test, as does the method based on human keratinocytes, to monitor the rate of reparation in time under the effect of different MW HA and contemporarily to observe peculiar inflammation biomarkers of that are widely investigated in injure site models (Joo & Seomun, 2008; Sugawara, Gallucci, Simeonova, & Luster, 2001; Toraldo et al., 2012).

2. Experimental

2.1. Materials

HA fine powder 1800 and 1400 kDa of pharma grade was kindly provided by Altergon s.r.l.

HaCaT cells (Istituto Zooprofilattico, Brescia, Italy), a spontaneously transformed non-tumorigenic human keratinocyte cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/ml streptomycin. All materials were purchased from Flow Laboratories (Milan, Italy). The cells were grown on tissue culture plates (Corning Incorporated, New York, USA), using an incubator with a humidified atmosphere (95% air/5%CO_2 v/v) at 37 °C. Collagen was purchased from Sigma, Aldrich (Milan, Italy).

2.2. Methods

2.2.1. HA fragment preparations: heterogeneous hydrolysis

The strategy used for the heterogeneous acid hydrolysis of hyaluronic acid is described by Melander and Tømmeraas (2010) and has been specifically modified to satisfy our requirements. In particular, HA powder (1400 kDa) hydrolysis in ethanol (EtOH) (93%v/v) was carried out using a HCl-EtOH *vs.* HA ratio 10/1 v/w. The slurry was pre-warmed at 65 °C in a thermostatic bath, then few drops of HCl 37% v/v were added under vigorous stirring, in order to have a final concentration of 0.4 M HCl. The hydrolysis was carried out for 70–120–150–180 min to obtain HA 500, HA 100, HA50 and HA15 kDa respectively. The HA 6 kDa samples derived by further hydrolysis of HA 50 kDa for 24 h using the above mentioned conditions. Each sample was cooled in an ice-bath and

neutralized with an equimolar quantity of NH_3 25%. Samples were washed with ethanolic solution (93%v/v), recovered using a Buchner funnel under vacuum, lyophilized and then stored at −20 °C until characterization was obtained.

2.2.2. Characterization of HA fragments: SEC–TDA analysis

HA fragments were characterized by SEC-TDA (Size Exclusion Chromatography-Triple Detector Array) equipment by Viscotek (Lab Service Analytica, Italy). A detailed description of the system and its analytical conditions are reported elsewhere (La Gatta, Schiraldi, Papa, & De Rosa, 2011; La Gatta et al., 2013). Samples molecular weight (Mw, M_n, Mw/M_n), molecular size (hydrodynamic radius-R_h) and intrinsic viscosity ([η]) distributions were derived for a complete hydrodynamic characterization

2.2.3. Structural characterization using ^1H NMR spectroscopy

HA samples from the acid hydrolysis were evaluated by ^{1}HNMR spectroscopy (conditions 300 MHz, 64 scans, 80 °C). Samples were prepared by dissolving HA samples (10 mg/ml) in D_2O with 5 μl from a 1% stock solution in D_2O. Spectra were acquired on a Brucker AC 300. Chemical shifts are reported in ppm. The abbreviations used are as follows: s, singlet; d, doublet; dd double doublet; bs, broad signal.

2.2.4. Endotoxin content determination

For endotoxin content determination, HA powder, obtained as described in fragment preparation sections, was opportunely dissolved in pyrogen free water. The amount of pyrogens (bacterial endotoxins) in the solution was measured by using Limulus Amebocyte Lysate (LAL) test (chromogenic kinetic method) according to European Pharmacopoeia 01/2005:20614. Specifically, ENDOSAFE®-PTS cartridge US License N.1197 by Charles River Endosafe was used. All operations were performed under conditions avoiding endotoxin contamination. Results were reported as endotoxin units (EU/mg) of HA powder (La Gatta, Papa, Schiraldi & De Rosa, 2016).

2.2.5. In vitro keratinocytes scratch assay using time-lapse videomicroscopy (TLVM)

HaCat cells were used for *in vitro* scratch assay as previously reported in D'Agostino et al. (2015). Briefly 12-wells (pre-coated with collagen) were seeded with HaCat until complete confluence was reached; scratch wounds were created mechanically with a sterile pipette tip (Ø = 0.1 mm). Uniformly sized scratch was carefully obtained approximately 0.7 ± 0.2 mm in width. Detached cells and debris were washed away with PBS solution before placing the multi-well in the stage incubator.

The effect of HA gels on the rate of wound closure was tested by incubating the scratched monolayer with the following solution: HA 1800, 1400, 500, 100, 50, 15, 6 kDa at final concentration of 0.1% w/w. The samples were prepared by dissolving the lyophilized powder directly in the medium. Medium pH and osmolality (7,2–7,4 and 300mosm) containing the treatments were verified to ensure physiological conditions. Protein expressions (Immunofluorescence and enzyme-linked immunosorbent assay, ELISA) were evaluated after 24 h from the scratch and addition of hyaluronan diverse fragments. At least triplicate independent experiments were run.

Briefly the 'wound closure' phenomenon was monitored for 72–96 h using TLVM station, to observe the migration of HaCat cells to repair the wound, in presence of different treatments, allowing us to simultaneously observe the reparation of different wells and successively to perform quail-quantitative analysis of the experiment (D'Agostino et al., 2015).

2.2.6. qRT-PCR analyses of HA receptors and inflammation biomarkers

To reproduce skin inflammation *in vitro*, mechanical injury induced to the cultures was very extensive. With a sterile tip, parallel scratches were inflicted upon the monolayers, estimating damage to at least 40% of the cells. After the wound and the addition of HAs, the cells were directly lysed with TRIzol® (Invitrogen, Milan, Italy). Total RNA was extracted from HA (Mw = 1800,1400,500,100,50,15,6 kDa) treated keratinocytes (HaCat cells) at 3 and 16 h. Following precipitation with isopropyl alcohol and washing with 75% ethanol, the RNA pellets were re-suspended in nuclease-free water. The concentration of the extracted RNA was determined through a Nanodrop spectrophotometer (Celbio, Milan, Italy) and 1 μg of DNase-digested total RNA was retro-transcripted in the cDNA using Reverse Transcription System Kit (Promega, Milan). Quantitative real time PCR was obtained by iQ™ SYBR® Green Supermix (Bio- Rad Laboratories Srl) in order to analyze the gene expression of some hyaluronan key receptors such as CD44 and RHAMM, TLR4 for alert inflammation biomarkers such as TGF-β, TNFα, IL-6. The primer sequences (Table 1) were designed by Beacon Designer™ software. The final melting curve was performed from 55 to 95 °C. Samples were run in triplicate and the expression of specific mRNA relative to the control was determined after normalization with respect to HPRT housekeeping gene (internal control) (Valasek & Repa, 2005). The fold-change of mRNA expression of the genes under evaluation was calculated by using the $2^{-\Delta\Delta Ct}$ comparative threshold method (ΔΔCt = difference of ΔCt between treated cells and non-treated cells used as controls). The results were expressed as normalized fold expression, calculated by the ratio of crossing points of amplification curves of several genes and internal standard, by using the Bio-Rad iQ™5 software (Bio-Rad Laboratories Srl).

2.2.7. Immunofluorescence staining of HA receptor

Immunofluorescence staining was used to investigate the interaction of keratinocytes with CD44 and TLR4 receptors activated on different sized hyaluronan. For immunofluorescence, human keratinocytes were grown on chamber slides, (BD Falcon, Italy) scratched and treated with HA 1800 and 6 kDa respectively. After 24 h, treated cells and the control were washed with PBS, fixed with paraformaldehyde 4% w/v and permeabilized in 0.2% v/v Triton X-100 in PBS. Non-specific sites were blocked by incubation in blocking buffer (PBS containing 10% v/v bovine serum and 1% w/v BSA). Double-labelling studies were performed using a primary rabbit polyclonal Ab against CD44 (1: 400; Abcam) and a mouse monoclonal Ab against TLR4 (1:100; Abcam). For the double-labelling experiments, the cells were incubated with the primary antibodies together overnight. After a 60-min incubation with secondary Abs (FITC-conjugated anti-rabbit 1:2000) and tetramethylrhodamine isomer R [TRITC]-conjugated anti-mouse (1:2000). The cells were then washed three times in PBS and mounted using ProLong Gold Antifade Mountant (Life Technologies, Italy) applied directly to fluorescently labelled cells on microscope slides. Nuclei were stained with 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride hydrate, bisBenzimide (Hoechst 0.5 μg/ml, Sigma-Aldrich). Fluorescence images were captured using by fluorescence microscopy system (Zeiss) and elaborated with AxioVision 4.8.2.

2.2.8. Measurement of IL-6 cytokine concentrations by using ELISA assay

IL-6 levels in cellular supernatants were measured using ELISA assay, in order to investigate whether the different molecular weights of hyaluronan modulated IL-6 different production (amounts). In our study, scratched monolayer of HaCat were treated with HA samples and 24 h later supernatants were collected

Table 1
Oligonucleotide sequences relative to biomarkers used in the study.

Gene	Forward primer	Reverse primer	Primer Cycles
Transforming growth factor, beta 1 (TGFβ-1)	5′TgCggCAgCTgTACATTgA 3′	5′TggTTgTACAgggCCAggA 3′	95 °C 10 s, 55 °C 30 s, 72 °C 3 min, 40 cycles
Tumor necrosis factor alpha (TNFα)	5′CgAgTgACAAgCCTgTAg3′	5′ggTgTgggTgAggAgCACAT3′	94 °C 1 min, 55 °C 2 min, 72 °C 3 min, 40 cycles
Interleukin (IL-6)	5′gCCgCCTTTAACTggAgCAA'3	5′TTCCAggCATCTgCgATgAg 3′	95 °C 10 s, 55 °C 30 s, 72 °C 3 min, 40 cycles
Cluster of differentiation 44 (CD44)	5′gCgCCACCACAgCCAACTATg'3	5′TggATGCCgTCTATgTCgTCTTTA3′	94 °C 1 min, 60 °C for 2 min, 72 °C 3 min, 40 cycles
Toll-like receptors 4 (TLR4)	5′TCCCAggAATTggTgATAAAgTAgA'3	5′CTggCATgACgCgAACAATA'3	95 °C 10 s, 60 °C 30 s, 72 °C 3 min, 40 cycles
Receptor for Hyaluronan Mediated Motility (RHAMM)	5′gATAATCCgCATTCAgTTgTC-3′	5′TAACATCATAAGCACCTGGAC-3′	95 °C 10 s, 60 °C 30 s, 72 °C 3 min, 40 cycles

(Sugawara et al., 2001). The un-treated cells were used as reference (control). ELISA Kit (Enzo Life Sciences, NY USA) was used to quantify IL-6 production in tissue-culture supernatants, following manufacturer's instructions.

3. Results

3.1. HA structural characterization and hydrodynamic characterization

HA samples from the acid hydrolysis were evaluated by ^{1}HNMR spectroscopy.

In order to confirm if HA, degraded by heterogeneous hydrolysis, maintained its structural integrity, the low-molecular weight hyaluronic acid was analyzed by ^{1}H NMR spectroscopy and the spectrum is given in Fig. 1. It can be seen the peaks corresponding to acetamide protons at 1.9 ppm, 2′, 3′, 4′, 5′, and 6′-protons of HA disaccharide unit at 3.2–4.0 ppm, as well as anomeric 1′-protons at 4.4 ppm.

No indication of suspected by-products such as de-*N*-acetylation or ethanolysis at the reducing end were observed. This is in agreement with the previous observation that de-*N*-acetylation of HA does not occur until after extensive degradation (the lowest Mw is 6 kDa) (Tømmeraas & Melander, 2008).

Viscotek data in Table 2 showed that the hydrolysis strategies occurred, thus obtaining a whole array of HA fragments. The poly dispersity index ranged from 1.4 to 1.6 and then was comparable to the one calculated for the HA substrate, confirming an efficient and a simple approach for degrading HA without further complex purification steps. The decrease of intrinsic viscosity and hydrodynamic radius were in accordance with the reduction of chain lengths.

3.2. Endotoxin amount determination

The low endotoxin content crucial in pharma grade requirements is of key importance to better highlight the HA fragment function itself. The endotoxins amount for all hyaluronan powders produced resulted less than 0.05 EU/mg This data proved that LPS and/or endotoxin are below a guard level and therefore cellular phenomenon should be driven by hyaluronan rather than impurities on the tested sample alone because they do not cause inflammation as reported in literature (Ngkelo, Meja, Yeadon, Adcock, & Kirkham, 2012).

3.3. Effect of HA fragments on HaCat wound healing

HHA 1800, 1400, 500, 100, 50, 15, 6 kDa treatments were tested for *in vitro* scratch wound-healing assay and are reported in Fig. 2a and b. HA 1800, 1400 and 500 kDa activities were very similar to one another. The highest Mw HA samples led to complete reparation in shorter time; successively, as the Mw decreased, the completion of reparation was delayed. However, all the HA fragments enhanced the scratch reparation rate compared to the control except for the 6 kDa HA that completely hampered wound healing after 30 h. For this reason, HA 6 kDa was assayed for citotoxicity using MTT test. However, cell viability was >70% compared to the control thus, according to ISO 10993-5, it is not cytoxic at concentrations used.

In the early stage of wound healing (5 h), the curves were similar to each other. 5 h after injuries, the effect of HA fragments became different. In particular, the HA samples with higher molecular weight (1800, 1400, 500) were faster to repair cell monolayers. Similarly, medium size HA samples (100, 50, 15) showed the same trend. At 10 h, both HA 1800 and 50 kDa led to a 50% reparation rate compared to the others that ranged from 30 to 40%. At about

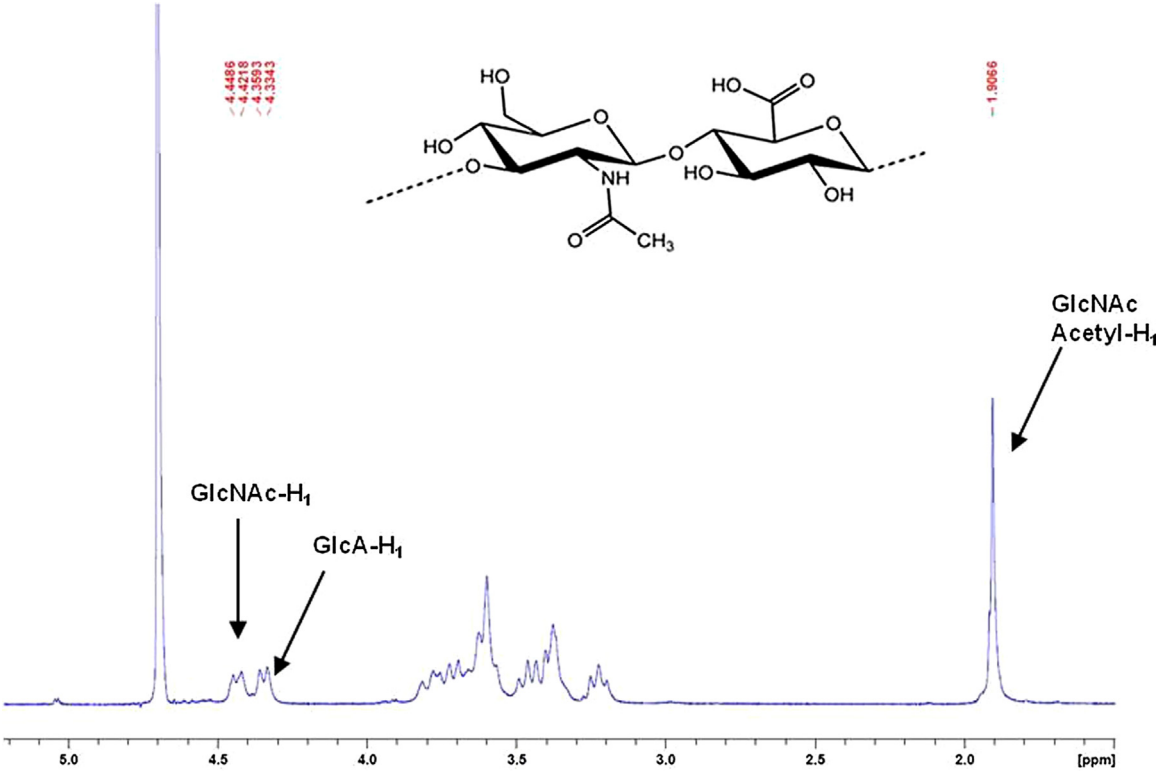

Fig. 1. ^{1}H NMR spectrum of hyaluronic acid sample (6 KDa). Acquisition conditions: 300 MHz, 10 mg/ml in D_2O, 64 scans, 80 °C.

Table 2
The table represented the extrapolated data of Viscotek analysis: molecular weight (Mw), poly dispersion index Mw/Mn and intrinsic viscosity(IV).

Sample	Mw (kDa)	Mw/Mn	IV (dl/g)	Rh (nm)
HHA 1800 kDa	1845 ± 8	1.64 ± 0.15	24.83 ± 0.25	86.17 ± 0.13
HHA 1400 kDa	1388 ± 8	1.460 ± 0.15	23.07 ± 0.87	66.79 ± 0.12
LHA 500 kDa	495 ± 7	1.65 ± 0.13	10.26 ± 0.22	41.08 ± 0.23
LHA 100 kDa	99 ± 1	1.44 ± 0.25	2.90 ± 0.36	14.14 ± 0.41
LHA 50 kDa	54 ± 4	1.56 ± 0.23	1.68 ± 0.22	10.60 ± 0.35
LHA 15 kDa	15 ± 2	1.54 ± 0.11	0.52 ± 0.05	4.72 ± 0.14
LHA 6 kDa	6 ± 2	1.45 ± 0.24	0.31 ± 0.11	2.05 ± 0.32

30 h, reparation occurred for all the samples except for HA 6 kDa that reached only 70% of closure at 40 h.

3.4. Gene expression evaluation of receptor biomarkers

Gene expression data analyses for the main hyaluronan receptors proved that CD44 was to be over expressed for all HA evaluated and only 6 kDa fragment had a lower effect (Fig. 3). All HA samples increased RHAMM expression, however results were significantly higher for 100, 50 and 15 kDa at 16 h (11–14 fold increase compared to control respectively) (Fig. 3). HA 50, 15 and 6 kDa activated significantly ($p < 0.01$) TLR-4 mRNA, interestingly, 100 kDa was not able to over express it as well as the other shorter HA fragments (Fig. 3).

3.5. HA receptors immunofluorescence staining

CD44 and TLR4 protein expression were evaluated by immunofluorescence staining (Fig. 4) in order to estimate the activation degree of the two HA receptors in the presence of 1800 and 6 kDa HA. Staining was performed using specific antibodies against CD44 shown in micrograph Fig. 4 (green) and TLR4 (red). The results showed marked activation of CD44 (Fig. 4a), in the response to HA 1800 kDa treatment (Fig. 4b), compared to the ctr and activation of TLR4 marked only in presence of HA 6 kDa (Fig. 4c). These results demonstrated that high and low HA molecular weight interacted with diverse receptors. The results obtained with other Mw HA are not shown because it was not possible to observe differences between the receptors investigated.

3.6. Gene and protein expression of HA inflammation

Inflammation biomarkers (TGFβ-1, TNF-α and IL-6) involved in epithelial cell migration were evaluated by quantitative RT-PCR. Specifically, TGF-β 1 was up-regulated for all HA fragments investigated (Fig. 5). HA ranging from 500 to 6 kDa, showed a significant increase in TGF-β 1 at 6 h with respect to HA 1800 kDa ($^{*}p < 0.01$). 1800 and 1400 kDa HA chains presented a lower TGF-β 1 expression, of 4 and 2 fold respectively (Fig. 5). In this case the increase prompt a "positive" activation toward the repair. Cell repair activation mechanism, implicate also IL-6 that is regulated by HA/receptor interaction. Our results showed that both TNF-α and IL-6 present similar trend during re-epithelisation process (Figs. 5 and 6a). In particular the expression levels increased with the HA molecular size decreasing. Confirmed by ELISA assay (Fig. 6b), the production of IL-6 protein in cell medium was further induced by small (15 and 6 kDa) HA fragments, in agreement with the gene expression. In fact, protein levels for 15 and 6 kDa were 8 and 9 fold higher respect to HA 1800 kDa, respectively.

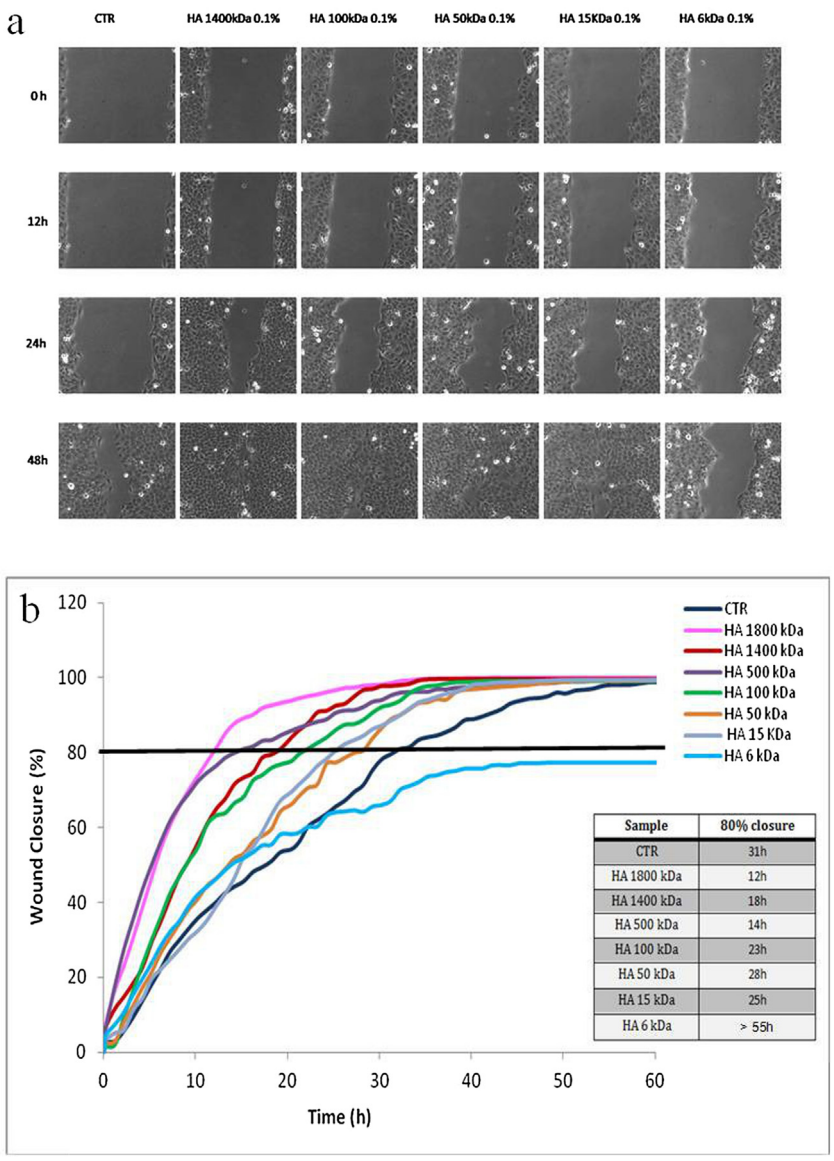

Fig. 2. (a) Representative micrographs pictures of HaCaT scratch assays immediately after the scratches, and in time course of the experiments. Scale bar, 100 μm. (b) Repair area percentage [as] for the control and in presence of following stimuli: H-HA, L-HA100, 15, 6 kDa at 0.1 w/v; the curves are averages of three different experiments with standard deviation within 5% of the value.

4. Discussion

Due to the lack of information, it is very often found that scientists, medical doctors, and even expert product managers confuse the "label" of high and low Mw for hyaluronan fragments. The key issue in this diffused misinterpretation might be related to the demonstrated connection of *in vivo* HA degradation and pathological conditions (*e.g.* ostheochondral diseases, dermal injuries) (Brandt & Cazzaniga, 2008; Fakhari & Berkland, 2013; Goldberg & Buckwalter, 2005). Nevertheless, given H-HA turnover, all HA fragments have a physiological function, as could be expected, that is often very important in the healing processes, biological tissue homeostasis and biosynthesis of ECM. Native HA is reported to be fragmented in smaller molecules during ECM degradation after acute tissue injury in order to activate the host innate immune response by recruiting macrophages and other specific cells to produce chemokines required to begin repair/restoration of tissue integrity (Campo et al., 2010).

In our research, we obtained a full array of HA fragments to analyze their specific functions *in vitro* with respect to their "biorevitalazing" activity. A few models are investigated to evaluate hyaluronan function in the recent and relevant literature (Avantaggiato, Giradi, Palmieri, Pascali, & Carinci, 2015; Farwick et al., 2011; Kage, Tokudama, Matsunaga, Hariya, & Hashimoto, 2014). Successively, interaction between different sized HA and relative specific receptors was investigated. These experiments may improve knowledge of the biochemical basis in the activation/silencing of pathways relative to HA and its degraded products *in vivo*.

Simultaneously we tried to elucidate the biological roles of these fragments by studying specific biomarkers from the preliminary phases of wound healing process.

Improvement and ease of degradation strategies allowed us to obtain fragments of different size with identical structural disaccharides units, thus ensuring that biochemical and biological outcomes has not be ascribed to a modified chemical structure. NMR analysis confirmed these results. The data extrapolated from Viscotek confirmed that products are similar to the ones obtained by hyaluronidase *in vivo* and for this suitable to be tested for *in vitro* biological response.

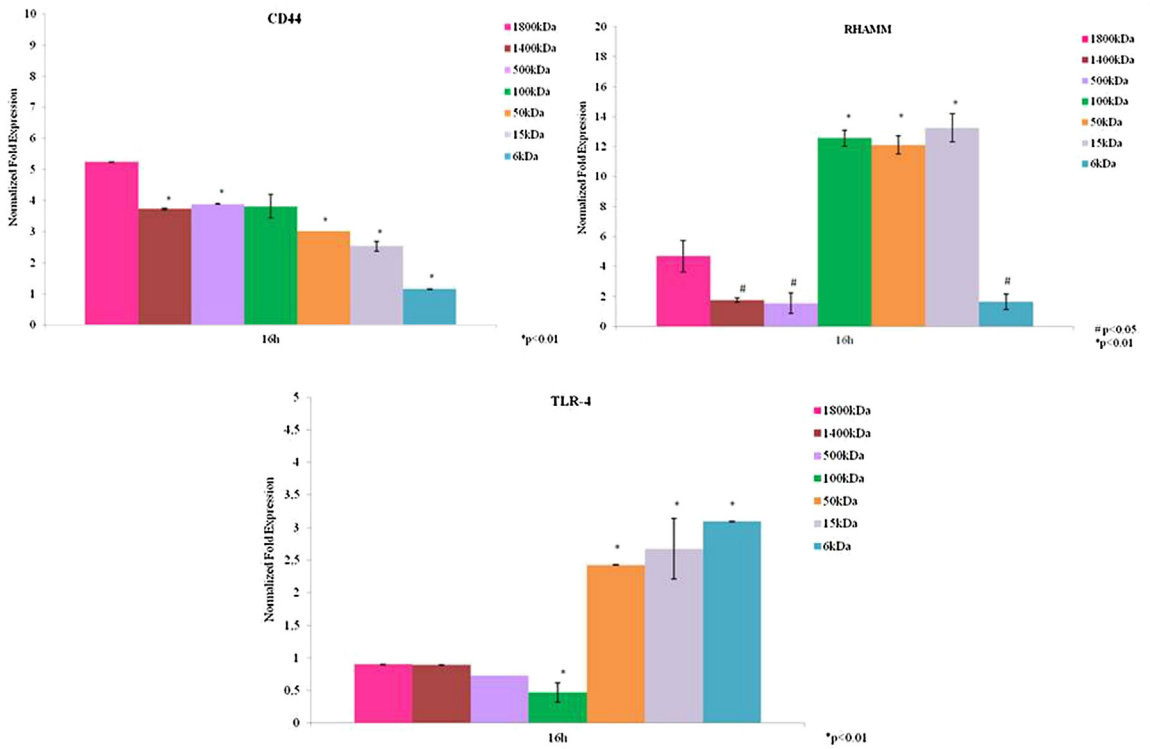

Fig. 3. Cell surface HA receptors: CD44, RHAMM and TLR-4 genes analyzed by quantitative real time PCR (qRT-PCR). Values are the mean ± SD of three different experiments and are expressed as mRNA normalized fold increase respect to CTR. The samples are significantly different (*p < 0.01 and #p < 0.05) respect to HA 1800 kDa, in according to Student's *t*-test.

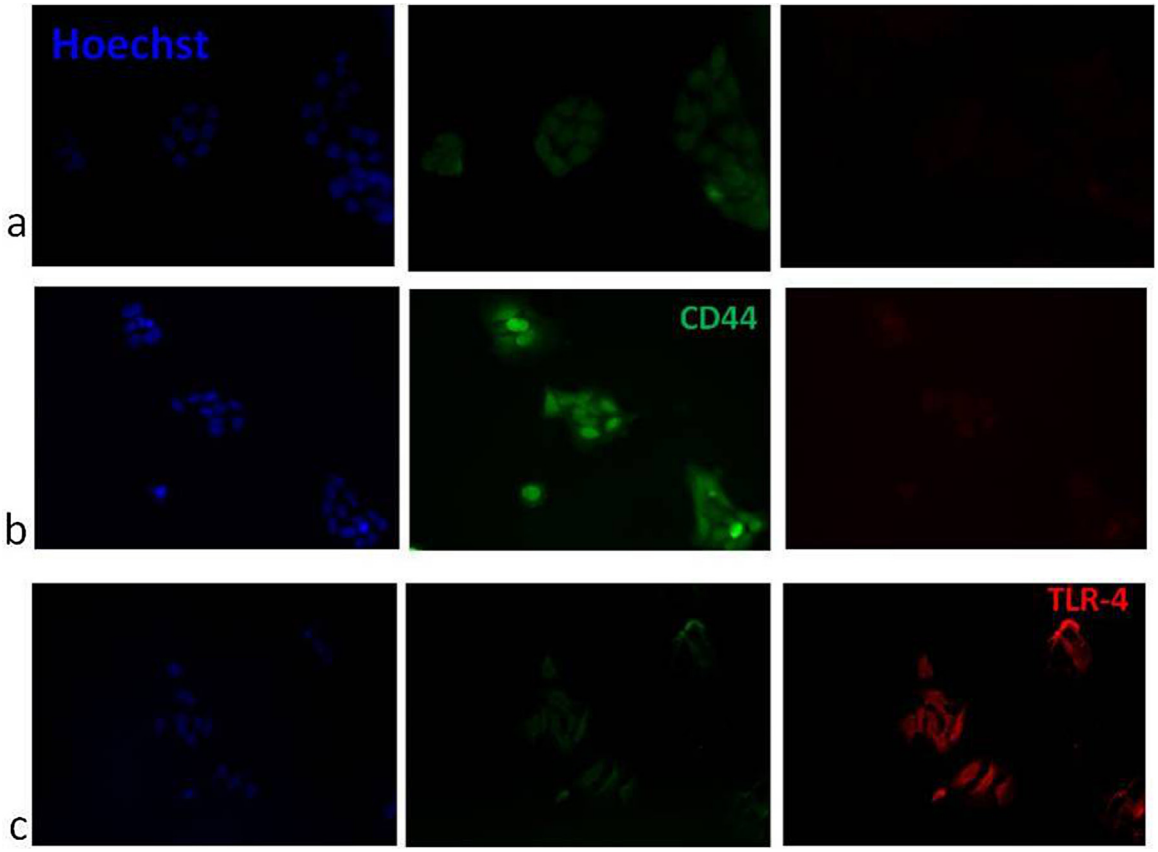

Fig. 4. Immunofluorescence (Hoechts, CD44, TLR-4) panel for receptor activation (a) ctr and treated with(b)1800 kDa (c) 6 kDa. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

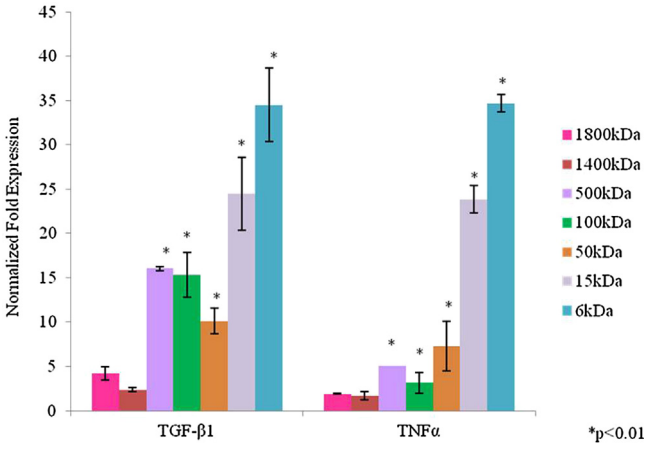

Fig. 5. Biomarkers of inflammation phase: TGF-β1 and TNFα genes analyzed by quantitative real time PCR (qRT-PCR) after 6 h of treatment. Values are the mean ± SD of three different experiments and are expressed as mRNA normalized fold increase respect to CTR. The samples are significantly different (*$p < 0.01$) respect to HA 1800 kDa, in according to Student's *t*-test.

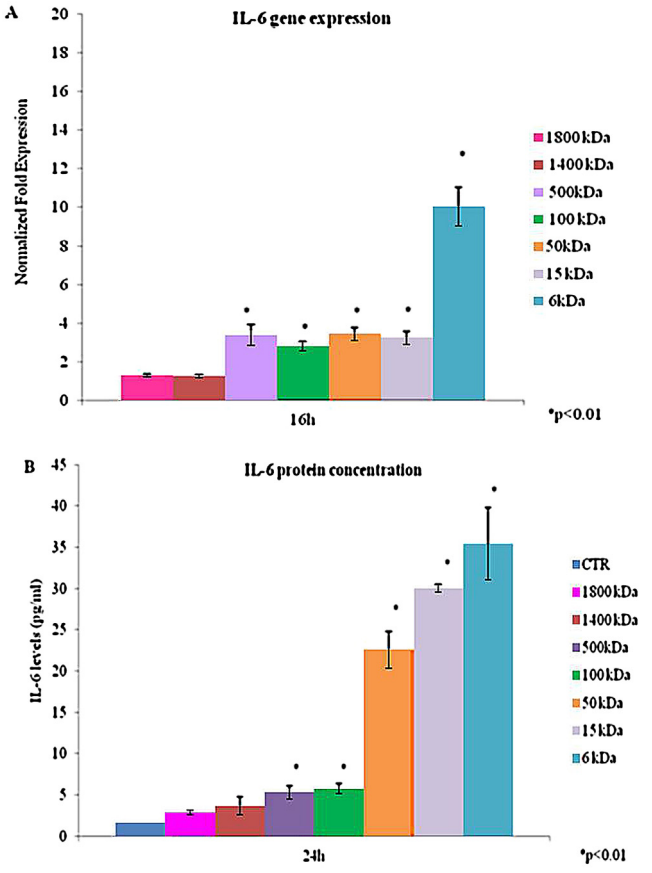

Fig. 6. IL-6 evaluation at gene (A) and protein (B) levels. The IL-6 gene was analyzed by quantitative real time PCR (qRT-PCR) 16 h of treatment, IL-6 levels protein were evaluated by ELISA assay (24 h). Values are the mean ± SD of three different experiments and gene expression results are expressed as mRNA normalized fold increase respect to CTR. For both the assays, the samples are significantly different (*$p < 0.01$) respect to HA 1800 kDa, in according to Student's *t*-test.

In vitro scratch test, widely investigated in a previous work (D'Agostino et al., 2015) is a well-assessed method for studying kinetics of different signaling molecules involved in reparation process. Our results have shown the efficacy of different sized HA to stimulate the migration of keratinocytes.

In particular, the data showed that HA and its intermediate fragments (*e.g.* 500–100–50 kDa) increased the reparation process, thus confirming results reported in literature (Damodarasamy et al., 2014). Similar behavior occurred in presence of HA 1800 and 1400 kDa and represented the treatments that mainly sped up the process. HA 100 and 50 kDa fastened the HaCat migration confirming the biorevitalizing effect of LHA (range 50–250 kDa) in cosmetic, aesthetic, medicinal applications (Avantaggiato et al., 2015). A smaller fragment, (LHA Mw = 15 kDa) was investigated and provided ambiguous results. The only one that hampered reparation was HA 6 kDa. The latter, classified by the scientific community as a very low Mw HA, also proved to cause inflammation in diverse cellular models (Baeva, Lyle, Rios, Langone, & Lightfoote, 2014).

Molecular analyses at gene and protein level corroborated the data derived from time-lapse experiments. To further elucidate our results we tried to depict the biological response of the cells to specific HA sizes in a picture that correlate the binding of HA to receptor and specific signaling (Fig. 7). As a matter of fact, it is known that HA exerts biological activity thought interaction with its receptors on cell surface; in particular CD44, the main HA receptor, interacting with HA, triggers different biological responses ranging from cell proliferation and ECM degradation to angiogenesis and inflammation (Viola et al., 2015). As reported in literature, CD44 is mostly activated by HA at high molecular weight ($Mw \geq 10^7$ Da) (Yang et al., 2012). Our results were also in agreement with Misra and collaborators (Misra, Hascall, Markwald, & Ghatak, 2015) that showed that all HA analyzed, activated CD44, but the major responses were found in presence of HA 1800–500 kDa. Different signaling pathways are triggered by HA-CD44 interaction, activating PKC and ERK1/2 (Viola et al., 2015) as key molecules involved in cellular migration and proliferation process (Fig. 7). In this respect, scratch assay and time lapse video-microscopy were used in our research to correlate HA size and function.

TLRs are receptors of innate immunity and TLR-2 and TLR-4 may bind HA fragments inducing signaling. Differently from CD44 response, TLR-4 activity is correlated to inflammatory process (Vistejnova et al., 2014). In general, it is been suggested that TLR-4 is activated mainly by HA at low molecular weight and its interaction triggers to inflammatory cascade involving a complex signaling that includes myD88, IRAK, TRAF-6 and NF-kB (Fig. 7) (Kenny & O'Neill, 2008). This biochemical cascade result in TGFβ-1, TNF-α and IL-6 expression/production. Expression of TLR-4 showed a trend strongly dependent on HA size. HA at low molecular weight (range 50–6 kDa) significantly increase TLR-4 gene expression respect to control and respect to HA at high and medium molecular weight (range 1800–100 kDa) that on the contrary are significantly down regulated. Gene expression data of HA receptors were confirmed by immunostaining. As reported in materials and methods for immunofluorescence experiments, we considered only HA at the ends of the range (*i.e.* highest Mw1800 kDa and lowest 6 kDa). Pictures showed that both CD44 and TLR-4 activation were dependent on HA polymer size, confirming our mRNA expression data. In addition, RHAMM is one of the cell surface receptors for HA affecting cell migration thus closely correlating to HaCat scratched model (Vistejnova et al., 2014).

In order to follow up the biochemical cascade turned on by HA-receptor interaction, we evaluated the expression profile of key cytokines possibly involved (Fig. 7). Our results showed an up regulation of TGF β-1 and TNF-α in presence of all HA treatments.

In particular the TGFβ-1 was up-regulated also in presence of high molecular weight HA confirming its function in the cell recruitment in migration process. On the contrary, a significant up-regulation of TNF-α (specific inflammation target) was found when the cells were treated only with HA at low molecular weight (MW < 50 kDa). In particular, 6 kDa HA up-regulated both TGFβ-1 and TNF-α 35 fold with respect to the control. Consistent to the

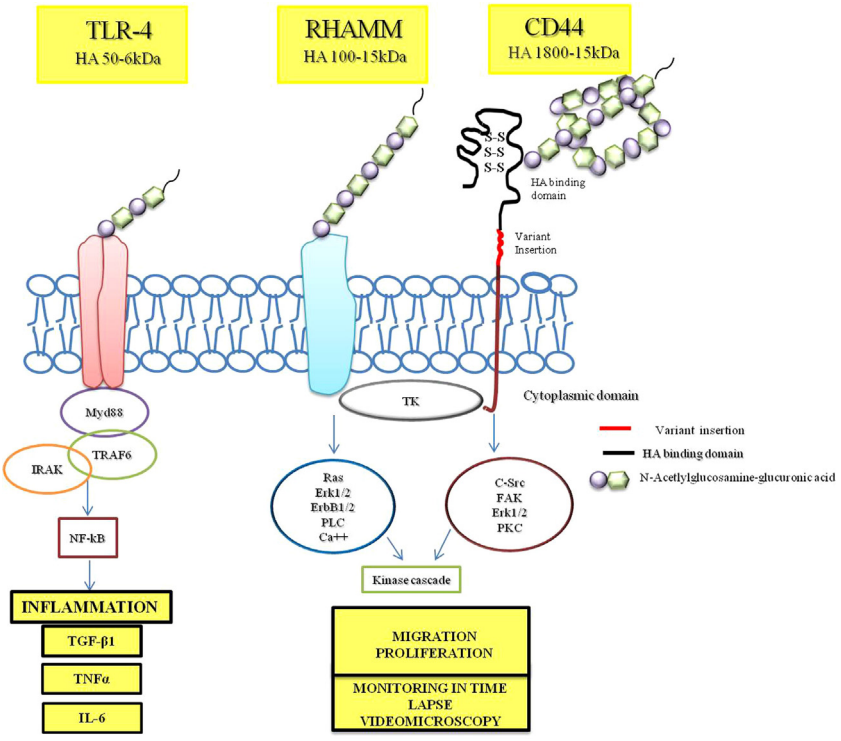

Fig. 7. Schematic diagram of the signaling cascade of HA receptors and involvement in cell functions. In yellow the topic investigated in the framework of this research project. (Misra, Hascall, Markwald, & Ghatak, 2015; Vigetti et al., 2014; Zöller, 2011). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

literature and other cytokines profiles, IL-6 expression both at gene and protein level was up-regulated in presence of HA low molecular weight (50–6 kDa) confirming its pro inflammatory effect (Campo et al., 2012).

5. Conclusions

For the first time, a parallel comparative study has been run on the same model, using specific markers for diverse hyaluronan of pharmaceutical grade ranging from 1800 to 6 kDa in order to eventually assess specific features. All have proved to speed up wound healing except for 6 kDa HA that was also the one to increase outmost inflammatory cytokine levels. Differently from the others, HA, 6 kDa treatments could not prompt CD44 expression but activated the TLR-4 receptor. Overall, down to 100 kDa residues, all hyaluronan behaved similarly, the only differences were highlighted for 15 and 6 kDa HA. Therefore, except for the one below 6 kDa, all hyaluronan fragments studied are safe, biologically active, can support cell activation prompting repair of dermis. On the basis of the experimental results, general concern about low molecular weight hyaluronan is reasonable only when highly concentrated LHA, with average molecular weight lower than 15 kDa, is present. All the other HA chains concur in biochemical activation and therefore may help in skin reparation procedures and biological remodeling.

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