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**Low Molecular Weight Hyaluronic Acid:
Its Effects on Epidermal Gene
Expression and Skin Ageing**



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Introduction

Hyaluronic acid (HA) is a linear polysaccharide with repeating disaccharide units composed of glucuronic acid and *N*-acetyl glucosamine. In contrast to other glucosaminoglycans such as dermatan sulphate or keratin sulphate it does not contain any sulphur. HA is one of the major matrix substances in which cells and fibrous constituents of the matrix such as collagen (1) and elastin (2) are embedded. Another unique characteristic of HA is its enormously high water binding capacity. In solutions HA exists in a flexible, coiled configuration that contains approximately 1000-fold more water than polymer (3). This special feature enables HA to contribute largely to the maintenance of the extracellular space and to control tissue hydration (4). Additionally, HA seems to play a pivotal role in tissue regeneration since recent studies suggest that the integrity and balance of matrix components themselves, which undergo degradation and reconstruction, assure normal tissue function and contribute to the regulation of wound healing (5, 6).

These outstanding properties predispose HA to be a valuable component of cosmetic applications where it could deploy its abilities resulting in anti-ageing and anti-wrinkle effects. However, this plethora of potential beneficial features is limited by the molecular size of HA, which can reach up to 2,000 kDa and thus interferes with efficient skin penetration. This issue could be addressed simply by fragmentation of high molecular weight HA if not recent studies had shown that HA fragments with a molecular weight less than 20 kDa were recognized by so-called Toll-like receptors (TLRs) 2 and 4 resulting in activation of these cells and production of pro-inflammatory mediators (7-10). It was therefore the aim of the present study to identify a low molecular weight (LMW) sized HA molecule that combines strong anti-ageing and moisturizing abilities with efficient skin penetration but is devoid of the negative effects mediated by TLRs.

Materials and Methods

HA Diffusion Assay

Diffusion of tritiated HA with a molecular weight of about 50 kDa, 300 kDa, 800 kDa or 1500 kDa, respectively, through dermatomed porcine ear skin was assessed using Franz diffusion cells as previously described (11). In brief, full thickness skin samples of visually intact skin from the ear of 5-month old female domestic pigs were dermatomed to a thickness of about 750 μm using an Acculan GA 643 (Aesculap, Germany). The samples were mounted in modified Franz static dermal penetration cells with the external surface of the stratum corneum facing the donor chamber. After 5 and 22h of HA application the level of radioactivity in the receptor phase was determined and expressed as $\text{ng}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ which served as a parameter for skin penetration.

Cell culture

Reconstituted human epidermis was purchased from SkinEthic (France) and incubated for 24 h in standard maintenance medium at 37 °C and 5% CO_2 before start of the experiments (12). In order to evaluate the possible pro-inflammatory effects of very low molecular weight (VLMW) HA. Therefore, reconstituted human epidermis was incubated for 48 h with aqueous solutions containing 0.5% 20 kDa, 50 kDa, 130 kDa or 320 kDa HA (Novozymes, Denmark). Another set of experiments was carried out to characterize the effects of HA on reconstituted human epidermis skin models were treated topically with

50 µl aqueous solutions with 0.5% of 50 kDa HA or 800 kDa HA for 48 h.

RNA isolation

Total RNA was extracted from cultured skin models using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's guide. RNA concentration was assessed spectroscopically with the SmartSpec Plus (Biorad, Germany). Purity and integrity of the RNA was determined by Agilent 2100 bioanalyzer with the 6000 Nano LabChip reagent set (Agilent Technologies, USA). RNA samples were stored at -80 °C until analysis.

DNA microarray and data analysis

Gene expression profiles were determined using Affymetrix HGU133 plus 2.0 GeneChips (Affymetrix, USA) using 2 µg of total RNA pooled from three reconstituted human epidermis skin models. Gene chip assays and initial analysis were carried out as described previously (13).

Quantitative RT-PCR

Reverse transcription was performed with the First-Strand-cDNA Synthesis Kit (Super Script III, Invitrogen, USA) according to the guidelines of the manufacturer starting with 100 ng purified RNA from each sample. Quantitative PCR was carried out with a Opticon DNA engine (MJ Research Software, Germany) using the following primers for tumor necrosis factor alpha (TNF- α): forward 5'-CTG TGG CCC AGG CAG TCA GA-3' and reverse 5'-GGC GTT TGG GAA GGT TGG AT-3'. Glyceraldehyde dehydrogenase (GAPDH) served as a house keeping gene with the following primer pair: forward 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse 5'-TCC ACC ACC CTG TTG CTG TA-3'.

In-vivo study

The in-vivo effects of different LMW HA molecules (50 kDa, 130 kDa and 300 kDa) were compared in an eight-week placebo controlled study with an O/W cream containing 0.1% Na-salt of the HA derivatives. 12 female volunteers, aged 30-60 years, applied the cream two times daily for 60 days. After that period several skin roughness parameters were assessed using skin surface replicas (SIL-FLO – Flexico Ltd., UK). In brief, adhesive

discs (3M, USA; 24x40 mm) were applied to the subject's skin in order to delimit the investigated area and to avoid skin stretching during the polymer application. A little amount of polymer was then spread into the internal circular area of the disc and let it *in situ* for 5 minutes until end of polymerisation. The disc was then removed and a duplicate of the skin was faithfully impressed. The skin replicas were then analysed by a designed image processing software (Quantilines, Monaderm, France) which allows a global data analysis of some relief parameters, according to the method described by Corcuff (14).

■ Results and Discussion

At sites of inflammation HA molecules undergo rapid degradation due to massive production of hyaluronidases by infiltrating inflammatory cells and bacterial invaders. These HA fragments have been implicated in the process of injury and repair since they have been shown to activate inflammatory cells such as macrophages and dendritic cells to express pro-inflammatory mediators and enzymes degrading extracellular matrix. Recent studies identified TLR-4 as the HA fragment binding receptor that links HA degradation and activation of the inflammatory cells (a, b). Since keratinocytes also express TLR-4 (c) it could

be possible that downsizing the molecular size of HA could be accompanied by the risk of activating pro-inflammatory responses. In order to determine the threshold that should not be undercut to avoid such unwanted side effects, skin models were incubated with variedly sized LMW HA, more precisely with a molecular weight of 20 kDa, 50 kDa, 130 kDa and 300 kDa.

Expression of TNF- α served as an indicator for the induction of a pro-inflammatory response. As demonstrated in figure X HA molecules with a molecular weight above 50 kDa did not reveal any significant effects. In contrast VLMW HA with a molecular weight of about 20 kDa induced marked up-regulation of TNF- α expression indicating the beginning of an inflammatory response. With this finding we could demonstrate that keratinocytes are not only able to contribute to inflammatory processes by producing pro-inflammatory cytokines such as TNF- α . Furthermore this finding strongly suggests that HA with a molecular weight \leq 20 kDa should not be applied whereas 50 kDa HA is safe (Fig. 1).

After determination of the smallest molecular size of HA that does bare the risk of pro-inflammatory side effects the next set of experiments was aimed to characterize the impact of this active on keratinocytes on a molecular level. For this purpose 50 kDa HA was applied topically to reconstructed human epidermis for

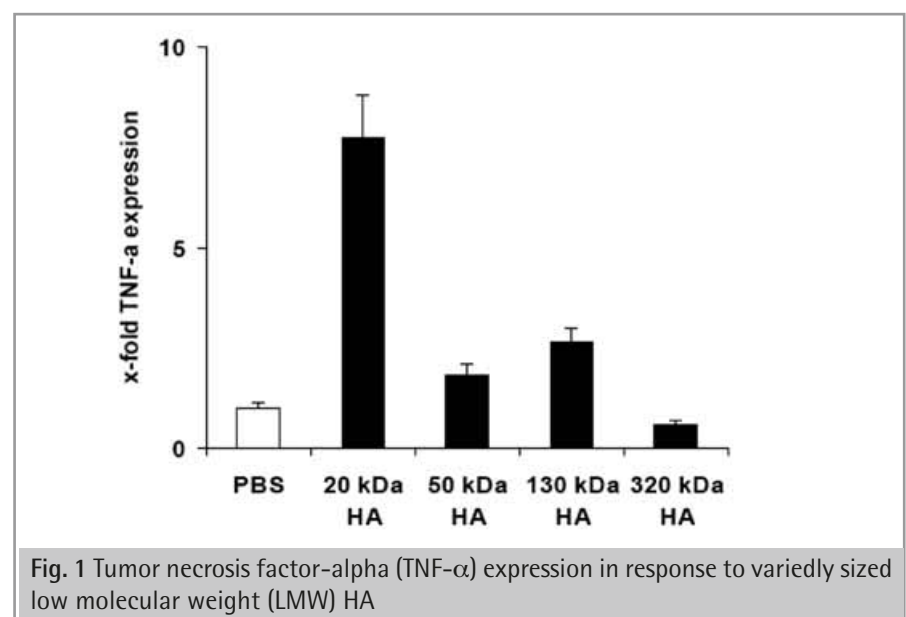


Fig. 1 Tumor necrosis factor-alpha (TNF- α) expression in response to variedly sized low molecular weight (LMW) HA

48 h. Subsequently RNA was extracted for transcriptome analysis using micro array technique. This analysis was performed in comparison to HA with a molecular weight of about 800 kDa, an active ingredient that is used in cosmetic applications for years and that is well known for its strong moisturizing properties. In contrast to 800 kDa HA that induced differential expression of 40 genes, 50 kDa HA lead to significant regulation of about 120 genes including key genes involved in keratinocyte regulation as well as genes that play important roles for the formation of tight junction complexes such as occluding and specific claudins (Fig. 2). No inflammatory response could be observed.

Since 50 kDa HA revealed a much more pronounced gene regulatory activity on keratinocytes than it could be observed for 800 kDa HA we asked the question whether this could be due to better penetration abilities of 50 kDa HA. In order to answer this question HA molecules with different molecular weight, namely 50 kDa, 300 kDa, 800 kDa and 1500 kDa were tested for their ability to penetrate pig ear skin. As shown in Fig. 3 only small amounts of HA with a molecular weight of more than 300 kDa penetrated the skin. In contrast, LMW HA with 50 kDa revealed skin penetration that was even after only 5 h at least three times higher than the penetration of 300 kDa HA. These results indicate that there is an apparent dependence of percutaneous transport on the HA molecular weight, with a better permeation apparent for the lower molecular weight fractions.

The *in-vitro* evaluation revealed that LMW HA with 50 kDa penetrates skin much better than larger sized HA resulting in a gene regulatory activity that is much stronger compared to 800 kDa. In order to investigate whether these *in-vitro* effects are reflected in an *in-vivo* activity an placebo-controlled eight week lasting study with three different LMW HA's was performed. This study demonstrated that 50 kDa is not only able to significantly reduce skin roughness (Fig. 4 a-c) but also provides strong anti-wrinkle properties (Fig. 4 d, e). Furthermore, these seem to be more pronounced for LMW HA with a molecular weight of about 50 kDa since larger sized HA such as 800 kDa does not

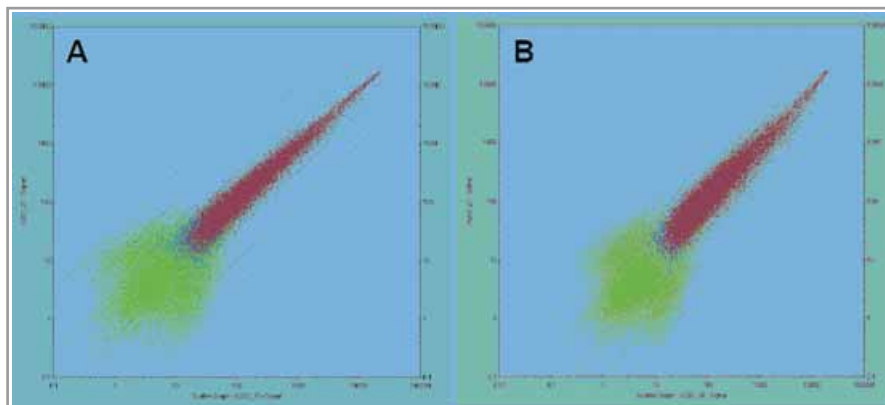


Fig. 2 Differential gene expression of keratinocytes after incubation with 800 kDa HA (A) or 50 kDa HA (B)

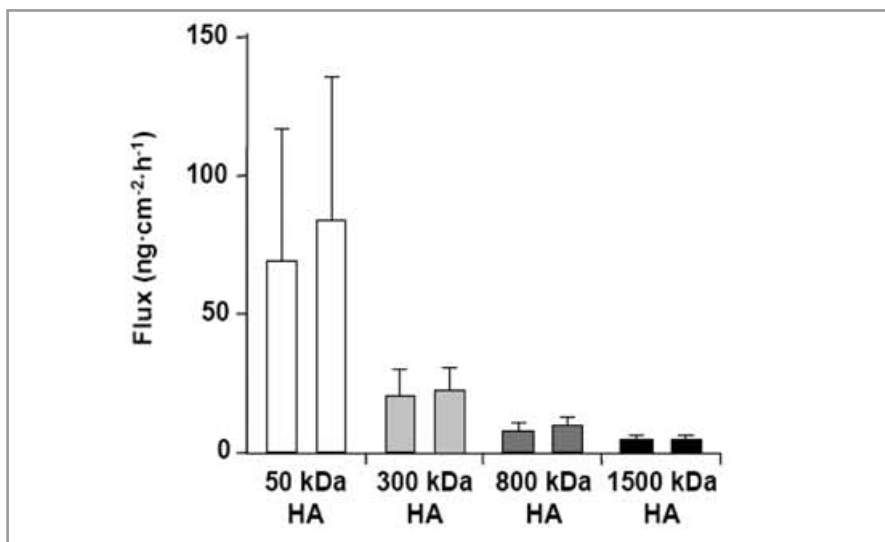


Fig. 3 Penetration of tritiated HA through pig ear skin. The first bar indicates flux after 5 h and the second bar indicates flux after 22 h

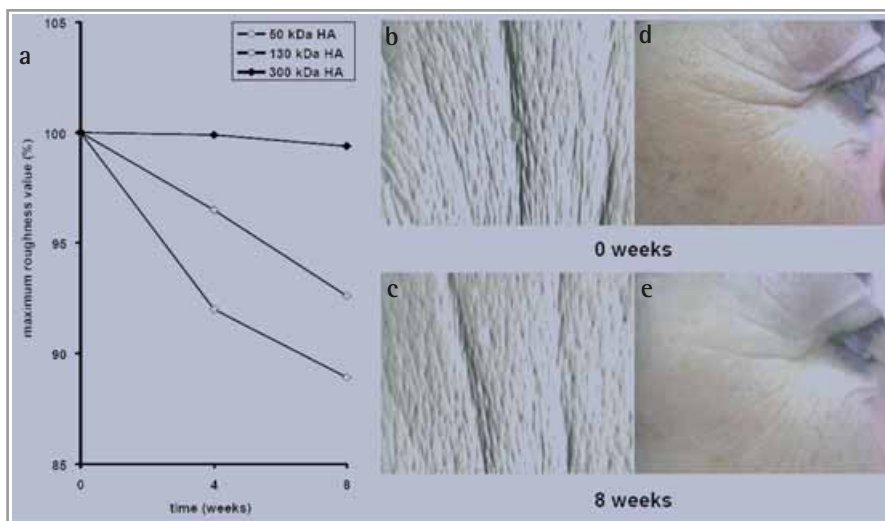


Fig. 4 *In-vivo* effects of LMW HA with a) demonstrating the effects 3 different LMW HA's on skin roughness after 4 and 8 weeks of topical application and with b-e) showing the strong skin roughness and wrinkle reducing properties of 50 kDa HA

display such properties, but is commonly used due to its strong moisturizing effects.

■ Conclusions

With the results of the present study it was clearly demonstrated that HA offers not only beneficial effects to the skin but also that these effects can be controlled by varying the molecular size. It was found that LMW HA provides better penetration abilities than larger sized HA and, accordingly, influenced the expression of many genes including those contributing to keratinocyte differentiation and formation of intercellular tight junction complexes which are reported to be reduced in aged and photodamaged skin. These different molecular properties of high and low MW HA generated different *in-vivo* effects with pronounced moisturization and elasticity properties shown for high MW HA and marked reduction of wrinkles demonstrated for LMW HA's. The increased activity at decreasing molecular weight can be explained by the more efficient skin penetration of the smaller HA molecules. These data show for the first time that topical application of LMW HA improves skin functioning and provides anti-aging effects which could be achieved by strengthening its penetration abilities based on decreasing the molecular size.

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