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The effect of N-acetyl-glucosamine on stratum corneum desquamation and water content in human skin

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Synopsis

Alpha-hydroxy acids have been used topically to treat skin for both dermatological and cosmetic problems for many years. Though there are many known benefits of the use of alpha-hydroxy acids on skin, there have been recent reports that topical treatments with alpha-hydroxy acids increase skin damage resulting from UVB. Additionally, high concentrations of alpha-hydroxy acids by themselves have also been found to cause skin irritation.

In order to find alternatives to alpha-hydroxy acids, we investigated a variety of amino sugar compounds that were previously reported to inhibit the reaggregation of dissociated corneocytes by modulating cellular adhesion. *In vivo*, we observed that topical treatments with a formulation containing N-acetyl-glucosamine (NAG) led to an increase in skin moisturization, a decrease in skin flakiness, and the normalization of stratum corneum exfoliation. *In vitro*, we observed an upregulation of differentiation markers, keratin 10 and involucrin, in keratinocytes treated with NAG. CD44 is a lectin cell adhesion molecule that is also expressed in keratinocytes. Amino sugars such as NAG may competitively bind to CD44, modulating keratinocyte cellular adhesion. We hypothesize that these amino sugars modulate keratinocyte cellular adhesion and differentiation, leading to the normalization of stratum corneum exfoliation. We propose the use of amino sugars such as NAG as alternative compounds to replace the use of alpha-hydroxy acids in skin care.

INTRODUCTION

Alpha-hydroxy acids have been used for a number of years to treat skin for both dermatological and cosmetic problems (1). Topical treatments with alpha-hydroxy acids have clearly demonstrated an anti-aging effect (2), photoprotection (3), and anti-inflammatory activity (3).

However, the mechanism by which alpha-hydroxy acids operate is largely unknown. One hypothesis for their mechanism of action is that they lower corneocyte cohesion (4) and thereby enhance the stratum corneum exfoliation process. This mechanism of action suggests, however, a purely denaturing effect on the proteins of the epidermis and represents a low specific functionality. This is demonstrated by the fact that high concentrations, 5–10%, of alpha hydroxy acids have a direct irritating effect.

In order to develop alternatives to the use of alpha-hydroxy acids with higher specific activity and fewer side effects, we have been investigating a variety of materials. One such class of compounds is amino sugars. These have been reported by Brysk *et al.* (5) to inhibit

the reaggregation of dissociated corneocytes. These same authors reported on the ability of n-acetylglucosamine (NAG), n-acetylneuraminic acid, and n-acetylgalactosamine to affect the dissociation aggregate of extracted skin corneocytes. These amino sugars bind to the lectin-like (sugar-binding) glycoproteins that bind corneocytes together. This lectin-binding protein has more recently been shown to be CD44, the receptor for hyaluronic acid (6). Hyaluronic acid has been shown to be present in the epidermis (7) and even in the stratum corneum (8).

We hypothesized that amino sugars, by disrupting corneocyte bonds, like alpha-hydroxy acids may work topically to promote desquamation. This desquamation will be similar to that caused by alpha-hydroxy acids, with the benefits associated with them but more gentle to the skin.

METHODS

CELL CULTURE AND VIABILITY

HaCaT cells were grown to confluence in six-well plates (Costar, Corning Corp., Corning, NY). These cells are a spontaneously immortalized human keratinocyte line (9), kindly supplied to us by Dr. Norbert E. Fusenig of the German Cancer Research Center, Heidelberg. Cells were grown in Dulbecco's Modified Eagle's Medium, DMEM, (Gibco BRL, Grand Island, NY). Cells were treated with 50 mM, 125 mM, and 250 mM NAG (Sigma) for 24 hours in whole media.

MICROSCOPIC VISUALIZATION

Cell cultures were viewed at ×100 magnification by phase contrast and reflectance microscopy with an Olympus BX60 microscope (Olympus, Melville, New York).

GEL ELECTROPHORESIS AND WESTERN BLOT

Tissue culture plates were scraped 24 hours after treatment with NAG into media and centrifuged at 3,000 rpm for ten minutes. Harvested cells were then resuspended in lysing buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP40) and sonicated for three one-minute intervals with a cone attachment on a model W-225 sonicator (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) set at 100 watts/minute. Samples were then directly applied to sodium lauryl sulfate polyacrylamide gels (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and electrophoresed in a Phastsystem gel electrophoresis unit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Immediately after electrophoresis, the gels were overlaid with Immobilon-P transfer membranes (Millipore Corp., MA), wet with distilled water and two layers of wet Whatman filter paper. This sandwich was then heated to 50 degrees for 30 minutes to allow transfer to take place. The membranes were then immediately blocked by placing them in 3% milk protein (BioRad Laboratories, CA) in 50 mM Tris HCl pH 8.0, 0.138 M NaCl, 2.7 mM KCl (TBS) for 18 hours. Following this blocking step, the membranes were washed three times with TTBS (Triton-X100

containing TBS). These membranes were then incubated for 120 minutes with an antibody for involucrin (Biomedical Techniques Inc., MA) and keratin K1 and K10 (Chemicon, Temecula, CA). Following this incubation the membranes were again washed three times with TTBS. The membranes were then incubated with a secondary antibody (either goat or rabbit anti-mouse antibody) conjugated to alkaline phosphatase. The enzyme activity was then visualized by reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium tablets (Sigma, St. Louis, MO). The blots were scanned with a Hewlett-Packard ScanJet IIc and analyzed with the densitometric software package SigmaGel 1.0.

CLINICAL STUDY DESIGN

The subjects included in this study were 45 females between the ages of 21 and 65 years, all meeting the screening criteria of good health and not pregnant or lactating. The subjects reported for testing without moisturizers or any other products on their faces and hands, and their baseline measurements were taken. They were given the product to take home and self-administer for four weeks to their right hand only, twice a day, in the morning after washing and in the evening at least 15 minutes before bedtime. The left hand served as the untreated control site. The subjects were only allowed to use the test product and specifically log its use in a daily diary we provided. At the end of two and four weeks the subjects returned for testing without applying the product for at least 12 hours and they were re-evaluated under the same conditions.

SKIN EXFOLIATION VIA THE D-SQUAME DISCS METHOD AND IMAGE ANALYSIS

Skin exfoliation was evaluated by measuring the amount of flakes removed from the skin surface using D-Squame discs and analyzing them by image analysis. Four D-Squame discs were firmly and evenly pressed on the face and the back of each hand with a hand-held uniform pressure device and removed by gently pulling them away from the skin. The D-Squame discs were mounted on clear microscope slides and labeled according to the panelist's name and date of visit. Desquamation was evaluated from the D-Squame discs using the image analyzer. Skin evaluation was carried out before treatment and after two and four weeks of treatment. The OPTIMA image analyzer (Optimas 6.5 from Media Cybernetics, Bethesda, MD) was used to evaluate skin flakiness. The D-Squame samples containing the corneocytes were placed under a camera on top of a light table and each image was imported into the image analyzer. The average gray value corresponding to the sample density was measured: the denser the sample, the higher the gray value difference.

ASSESSMENT OF SKIN MOISTURIZATION WITH A DERMAL PHASE METER

Skin surface capacitance measurements were made with a NOVA dermal phase meter (DPM 9003; Nova Technology, Portsmouth, NH). The DPM is an electronic instrument that non-invasively measures skin capacitance *in vivo*. The capacitance readings are directly related to picoFarads of capacitance in the volume of skin that is effectively measured,

and are correlated to skin water content. A uniform-pressure sensor probe was placed on the surface of the hand skin for approximately five seconds and a reading was taken. Four measurements were taken at each site at every time point. Measurements were automatically acquired via a computer software package, ensuring standardization of the measurements.

STATISTICAL ANALYSIS

Data were given as means ± SEM. Excel (Microsoft) software was used to evaluate statistical significance by using the Student's two-tailed *t*-test function in the software package. A p value of < 0.05, at least, was considered significant.

RESULTS

IN VITRO EFFECTS OF N-ACETYL-GLUCOSAMINE ON KERATINOCYTES IN CULTURE

Human keratinocyte (HaCaT) monolayers were treated with increasing amounts of amino sugars to assess their effect on cellular morphology. HaCaT keratinocytes treated with 50 mM, 125 mM, and 250 mM of NAG appeared to be released from culture dishes and dissociated (Figure 1). Similar effects were observed with other amino sugars such as N-acetylneuraminic acid and N-acetyl-galactosamine (data not shown). Suspension or release of keratinocytes from the substrate has been reported to promote keratinocyte differentiation (10). We therefore investigated the levels of two different protein markers of keratinocyte differentiation in these N-acetyl-glucosamine treatments. Increasing concentrations of N-acetyl-glucosamine resulted in increased accumulation of involucrin, keratin K1, and keratin K10 in cultured keratinocytes (Figure 2). These data supported the observation that the keratinocytes had reduced adhesion to the substratum.

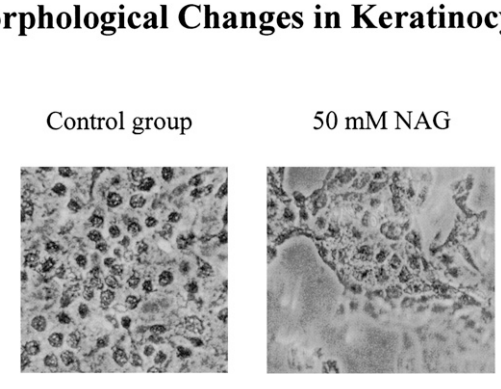

Figure 1. Effect of NAG on human keratinocyte morphology. HaCaT cells were treated for 24 hours with 50 mM, 125 mM, and 250 mM of NAG in whole media. Cell cultures were then viewed at ×100 magnificaion by phase contrast and reflectance microscopy with an Olympus BX60 microscope (Olympus, Melville, NY).

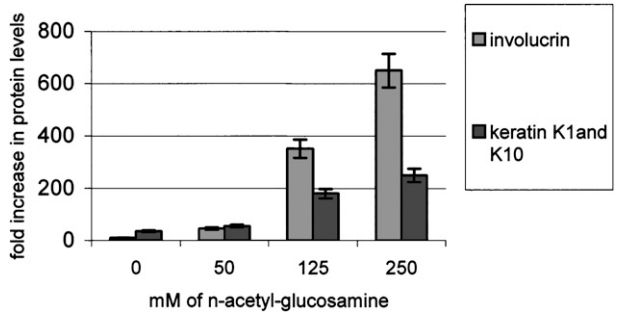

Figure 2. Protein levels of involucrin and keratin K1 and K10 in human keratinocytes treated with NAG. HaCaT cells were grown in six-well culture dishes to confluence and treated with 50 mM, 125 mM, and 250 mM NAG for 24 hours. Each dose was run in triplicate. Cells were then scraped and processed as detailed in Methods. Experiments were repeated three times. Data is representative of one experiment.

CLINICAL EFFECTS OF N-ACETYL-GLUCOSAMINE ON SKIN MOISTURATION AND DESQUAMATION

Skin moisturization was evaluated using a Nova dermal phase meter (Table I). Moisturization was observed to increase by 12% and 18% over controls, at two and four weeks, respectively, in human skin. The two-week change was not statistically significant, but this became significant after four weeks.

NAG was tested at 1% vs the placebo, on the back of the hands for their effect on skin desquamation and reduction of skin flakiness via the D-squame disc method. After two and four weeks of treatment, the group using 1% NAG showed 39% and 38% decreases in flakiness, whereas the placebo use resulted in a 30% increase (Table II).

Table I
Effect of 1% NAG on Skin Moisturization via the Dermal Phase Meter

	DPM values at			*p* values at baseline vs	
Product	Baseline	2 Weeks	4 Weeks	2 Weeks	4 Weeks
1% Glucosamine	145	131	131	0.16	0.11
Untreated control	159	125	116	0.06	0.01
p value (treated vs control)	0.11	0.16	0.009		
% Increase (treated–control)		12	18		

Four measurements were taken at each site at every time point. Measurements were performed on 45 panelists.

Table II
Desquamation Efficacy of NAG in Vehicle via the D-Squame Disk Method

	% Decrease in flakiness	
Product treatment group	2 Weeks	4 Weeks
Placebo	6.0	–29.9
1% N-acetyl glucosamine	38.8	38.0

Four D-Squame discs were used on the face and hands of each of 45 panelists. Desquamation was evaluated from the D-Squame discs via image analysis as described in Methods. The average gray value corresponding to the sample density was measured.

CONCLUSION

There are many known benefits for the use of alpha-hydroxy acids on skin. There have also been recent reports that topical treatments with alpha-hydroxy acids increase skin damage resulting from UVB. Additionally, high concentrations of alpha-hydroxy acids have also been found to promote irritation. In order to find alternatives to alpha-hydroxy acids, we investigated a variety of amino sugar compounds that were previously reported to inhibit the reaggregation of dissociated corneocytes by modulating cellular adhesion. Human keratinocytes in culture treated with 50 mM to 250 mM of NAG were released from the culture dish. This treatment appears to dissociate the keratinocytes from each other and from substrates. In addition, we observed an upregulation of differentiation markers, keratin 10 and involucrin, in keratinocytes treated with NAG. *In vivo*, we observed that topical treatments with a formulation containing NAG led to an increase in skin moisture levels and the normalization of stratum corneum exfoliation.

In addition, high concentrations of NAG do not cause any noticeable irritation (data not shown). Topical doses as high as 5% and 10% did not produce any skin irritation. The benefits of topical N-acetyl-glucosamine are numerous and have been well documented by Bissett (11). These include accelerated wound healing, reduction of hyperpigmentation, and improved skin hydration. We have added to these benefits by showing an exfoliation enhancement. We propose the use of amino sugars, such as N-acetyl-glucosamine, as alternative compounds to replace the use of alpha-hydroxy acids in skin care.

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