"Skin-Omics": Use of Genomics, Proteomics and Lipidomics to Assess Effects of Low Molecular Weight Scleroglucan

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Introduction

The stratum corneum, representing the outmost layer of the skin, is a vitally important barrier of the body and protects it from various environmental stress factors. In order to maintain its elasticity, suppleness and barrier function, the skin requires optimal water content. In order to guarantee these functions skin moisture is tightly regulated by two factors, namely by the integrity of the water impermeable barrier of the skin itself and by the content of water-binding substances in the stratum corneum.

In order to compensate epidermal water loss, topical application of substances with large water binding capacity such as polyvalent alcohols and polysaccharides can be a solution. Scleroglucan is a glucose polymer that forms a triple helical structure and represents a major constituent of the cell wall of fungi. These polymer chains essentially consist of (1→3)-β-linked glucose units wherein each 3rd unit is additionally (1→6)-β-linked to another glucose unit to form side branches [1]. Glucans in general are described to stimulate cells of innate immunity, namely monocytes and macrophages, to provide anti-infective potential, and to promote anti-tumour responses as well as wound-repair [2-5]. Recent studies identified several receptors including Dectin-1, Type 3 complement receptor, class A scavenger receptor and TLR-2 [6,7] that recognize glucans and play a pivotal role in the mediation of its biological effects. Since expression of these receptors is not only limited to cells of the innate and adaptive immunity but could also be demonstrated for epithelial cells, fibroblasts and vascular endothelial cells, it seems that glucan receptors are widely distributed throughout the human body [8-10].

Scleroglucan is a water-soluble beta-glucan secreted by *Sclerotium rolfsii* that protects the cells from dehydration by creating an extra-cellular matrix capsule. The large water binding capacity and its numerous biological activities predispose Scleroglucan as an active ingredient for dermatological and cosmeceutical applications. However, due to its high molecular weight, up to 1.200 kDa and following from this it's marked thickening abilities, the usage concentration of scleroglucan is limited. Depolimerisation of scleroglucan polymers is accompanied by decreased viscosity and allows usage at higher concentrations up to 1%. The present study was aimed at characterising the effects of low molecular weight Scleroglucan (LSG) on keratinocytes and included *in-vitro* analysis of gene expression, protein and lipid formation from reconstructed human epidermis as well as *in-vivo* determination of its effects on skin moisturisation.

Materials and Methods

Cell culture

Reconstituted human epidermis was incubated for 24h in standard maintenance medium at 37°C and 5% CO_2 before the start of the experiments. [11]. In order to characterise the effects of HA on reconstituted human epidermis, skin models were treated topically with 50 µl aqueous solutions with 0.5% of LMW Scleroglucan for 48h. As a positive control retinol was utilised.

RNA isolation

Total RNA was extracted from cultured skin models using RNeasy Mini following the manufacturer's guide. RNA concentration was assessed spectroscopically with the SmartSpec Plus. Purity and integrity of the RNA was

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determined by a 2100 bioanalyser with the reagent set. RNA samples were stored at -80°C until analysis.

DNA microarray and data analysis

Gene expression profiles were determined using HGU133 plus 2.0 GeneChips 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 [12]. In the first step the number of approx. 50,000 probe-sets from the raw data set was reduced to approx. 7,500 probe-sets using the "Best Probe-Sets Filter" and a "2-Fold-Filter". In a second step for the most active compounds the data from the hierarchical clustering were further analysed bioinformatically using Bibliosphere software. This software looks specifically at the genes associated with the probe-sets on the microarray.

Protein sample preparation

The cellular protein extracts of human skin models were obtained by sonication of the sample in lysis buffer containing 4% CHAPS and protease inhibitor. Subsequently, urea and thiourea were added to the cellular lysate until a final concentration of 7 M and 2 M, respectively. After dissolving these additives by vortexing, 65mM DTT was added. Prior to 2D gel electrophoresis the protein concentration of each sample was determined according to the method of Popov [13].

2D PAGE

The 2D PAGE was performed in a modified form of the method of Klose [14]. For isoelectric focusing carrier ampholytes pH 2-11 were applied. The proteins were focused under non-equilibrium pH gradient electrophoresis (NEPHGE) conditions in 20cm gel rods. The IEF gels were applied onto 15% SDS gels of 25x30 cm^2 size. Subsequently, the proteins were separated according to their apparent molecular weight in a continuous buffer system. The separated proteins were stained with silver to achieve highest sensitivity according to the method of Heukeshoven [15].

Protein identification

The selected protein spots were cut out of the gels. The gel plugs were washed and dried. Trypsin solution was added to digest the protein for several hours at 37°C. The protein identification was performed using a TOF/TOF Mass Spectrometer. Peptide mass fingerprint spectra (PMF, MS) were acquired from all samples. The resulting mass lists were sent to the Proteinscape™ database for protein identification. Peptide fragmentation spectra (PFF, MS/MS) were acquired where possible. The peaks for fragmentation were selected by the Proteinscape database based upon the results of the protein identification by PMF. Protein identification was achieved by searching the mass spectra against the NCBI protein database using several external search algorithms (ProFound™, Mascot™, Sequest™). PFF spectra were either used to confirm the protein already identified by PMF or for identification of proteins that eluded the PMF identification.

Lipid analysis

Extracted lipids were analysed using electrospray ionisation mass spectrometry (ESI-MS) as described previously [16].

In-vivo study

The *in-vivo* effects of LMW Scleroglucan were analysed in two placebo controlled studies (n=10-12) with an O/W cream containing 0.5% or 1% LMW Scleroglucan. In the first study skin moisturisation was analysed before and 2 hours after topical application of the placebo or the verum formulation. Corneometer measurement was carried out with a Corneometer CM 825 in a climatic room with 50% air humidity at 24°C. In the second study the volunteers applied the cream twice daily for 4 weeks. After that period skin roughness was analysed using D-Squame tapes according to the manufacturer's instructions. Additionally the sensory properties were evaluated.

Results and Discussion

Gene array analysis of scleroglucan treated reconstituted human epidermis revealed a significant gene regulatory activity of this compound, since 39 genes appeared to be at least 2-fold up- or down-regulated. Figure. 1, see next page, shows the

Bibliosphere-network of up- (orange) and down-regulated (blue) genes which were selected form the hierarchical clustering tree. The results of the Bibliosphere-analysis were compared with the Affymetrix validation values. From this data it can be interpreted that scleroglucan seems to activate detoxification as indicated by regulation of several peptide genes from the cytochrome 450 family. Other functional pathways include junctional control

Promotion of keratinocyte differentiation could also be confirmed by protein analysis, since genes such as caspase-14, filaggrin, keratin-1 and -10 were detectable in significantly increased amounts in protein extracts from skin models. Furthermore, 15 other proteins appeared to be markedly up-regulated whereas twelve proteins were downregulated, among them several actins.

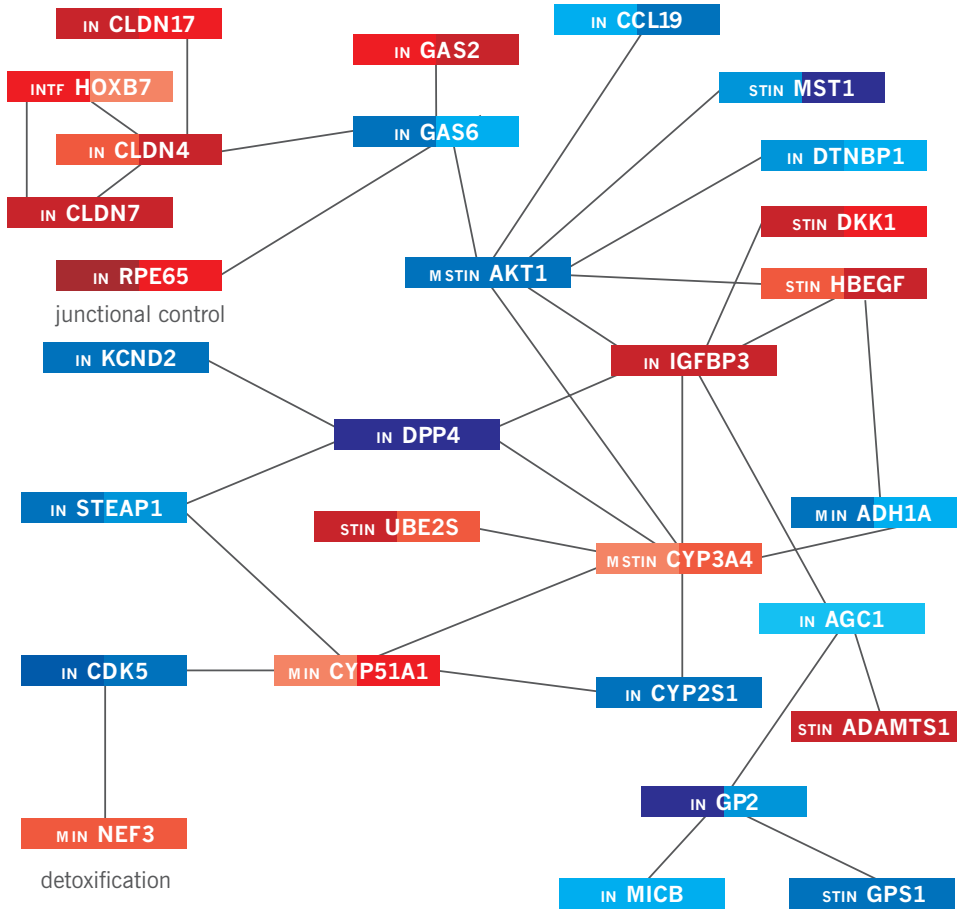

Figure 1 Network view of genes up and down regulated in keratinocytes by scleroglucan.
A line between two genes means at least one co-citation in the same abstract

including regulation of claudin 4 and 7 as well as keratinocyte differentiation mediated by induction of the phosphoinositide 3-kinase (PI3K)/Akt pathway, one of the main promoters of keratinocyte differentiation. This receives further support by markedly enhanced calbindin expression, since calcium represents a strong signal for keratinocyte differentiation as well.

Figure 2, see next page, shows representative photomicrographs of 2D PAGEs of protein extracts from scleroglucan treated and untreated reconstituted human epidermis. Interestingly, a novel extra-cellular nuclease was also identified, that could give a hint towards induction of an anti-microbial response in the epidermis.

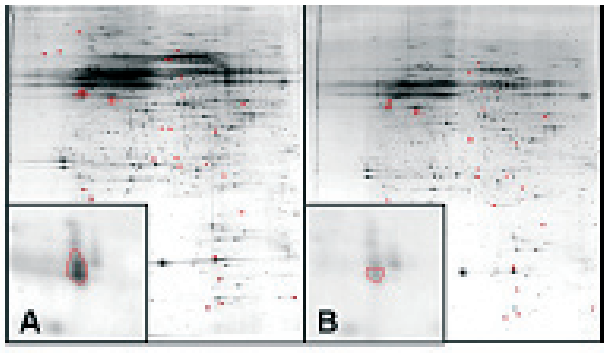

Figure 2 2D PAGE of protein extracs from keratinocytes after treatment with Scleroglucan (A) or untreated cells (B) with special focus on caspase-14 production (magnified)

ESI-MS analysis of the total lipid composition of reconstituted human epidermis identified that in untreated cells cholesterol and phosphatidylcholine represent the main constituents, whereas ceramides, glucosylceramides and cholesterylesters are only found in minor fractions. In contrast, after treatment with Scleroglucan the fraction of phosphatidylcholine is significantly decreased, whereas fractions of cholesterol and ceramides, representing the main parts of the epidermal barrier lipids, appeared to be markedly increased.

The *in-vivo* studies revealed that a treatment with a cosmetic formulation containing 1% LMW Scleroglucan has significant moisturising properties compared to placebo 2 hours after application. Additionally the silky, supple skin feel provided by the LMW Scleroglucan led to a high preference of the volunteers towards the LMW Scleroglucan containing the formulation. After 4 weeks of application of either a placebo or a cream containing 0.5% LMW Scleroglucan, D-Squame tape analysis showed an 8% overall improvement of skin roughness whereas the vehicle itself had no effect.

Conclusion

Here we present data derived from a comprehensive *in vitro* study in which effects of LMW Scleroglucan were assessed using state of the art analysis of gene expression, protein production and lipid formation. Using the genomics approach LMW Scleroglucan was proven to trigger keratinocyte differentiation as indicated by expression of one of its most potent promotors, PI3K/Akt, that further acts as a protector from premature cell death. These findings about the induction of differentiation gained further support by the identification of a huge variety of structural proteins such as several keratins and filaggrin. Additionally increased caspase 14 production, which is exclusively expressed in late differentiated keratinocytes, was monitored by the proteomics approach. Interestingly, also a novel extra-cellular nuclease was identified, which suggests stimulation of epidermal defence mechanisms since it is able to counteract bacterial attachment to cellular surfaces and to target viral nucleic acids. LMW Scleroglucan was further shown to stimulate barrier lipid formation by increasing cholesterol and ceramide levels in living skin equivalents as clearly figured out by the lipidomics approach. Within this study, no linear changes in the same genes/proteins but similar expression patterns and affected pathways were observed. Clearly Scleroglucan has many different effects on the epidermis and this report highlights the value of using a 'skinomics' study in understanding the overall biological effects of an active ingredients on skin by a system biology approach. Further *in-vivo* analysis revealed that depolymerised Scleroglucan leads to improved moisturising properties and improved skin feeling.

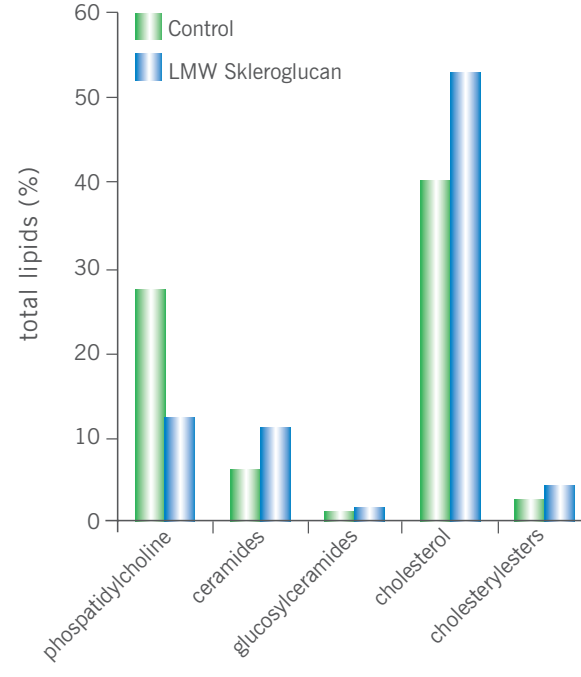

Figure 3 Total lipid composition from reconstituted human epidermis as analysed by electrospray ionisation mass spectrometry (ESI-MS)

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Authors' Biographies

Mike Farwick graduated in Biology from University of Düsseldorf, Germany. He earned his Ph.D. degree in Molecular Biology. After this, Dr Farwick joined Evonik Industries, formerly known as Degussa. He started in the field of fermentative amino acid production for the Feed Additives Business Unit, where he was responsible for functional genome analysis including DNA-Chips, proteomics and bioinformatics. From there he moved to a position as R&D Manager for Active Ingredients in Goldschmidt Personal Care. Since 2006 Mike has been the Head of the Active Ingredients R&D department. Fields of activity are: *in vitro* claim support with a focus on molecular processes using DNA-Chip technology and other "omics"; penetration and stability analysis of Actives; encapsulation technologies; and *in vivo* studies for the demonstration of cosmetic efficacy.

Peter Lersch. Following completion of his studies at the University of Essen, Germany, he earned his doctorate in 1989 at the Institute for Technical Chemistry. In the same year he started his professional career as a laboratory manager at the then Th. Goldschmidt AG in Essen. After holding various R&D positions in the silicone chemistry unit, Peter moved to the United States in 1996, where he worked as Technology Transfer Manager at the Hopewell, VA site. After his return to Germany in 1999, Peter set up a new department, focussing on active ingredients for cosmetics, wherein he then was responsible for R&D before accepting his current position in early 2006. He currently heads the department R&D Care Ingredients/Biotechnology in the R&D organization of Evonik's Consumer Specialties Business Unit.