Proteomic analysis of the psoriatic skin, and the effect of hyperosmotic stress on keratinocytes

Summary of the PhD Thesis

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1. INTRODUCTION

1.1 Psoriasis

Psoriasis is a chronic inflammatory skin disease, mainly characterised by abnormalities in the immunological response of the skin to various internal and external stimuli. It affects approximately 2% of the human population world-wide with no gender differences. The most common form (90%) is plaque-type psoriasis. The exact cause of psoriasis is unknown, however, genome-wide association studies (GWAS) have identified over 50 regions associated with the risk of the disease. The coding genes of these regions are connected to antigen presentation, the interleukin-23 (IL-23) axis, T-cell development and polarisation, innate immunity and negative regulators of immune responses. Psoriasis is not only a skin disorder; it is associated with systemic inflammation. Comorbid conditions are psoriatic arthritis, Crohn's disease, cancer, depression, non-alcoholic fatty liver disease, metabolic syndrome and cardiovascular disorders. Understanding alterations behind the disease is crucially important for developing new therapies for better disease management.

1.1.1 Proteomic studies of the psoriatic skin

One of the most effective ways to study different diseases with such enormous complexity and to elucidate related mechanisms is to perform a comparative proteomic analysis of protein extracts derived from affected tissues. Until now, none of the full-scale psoriatic proteomic studies, to the best of our knowledge, have compared lesional and non-lesional psoriatic skin regions (both epidermis and dermis) with the inclusion of biopsies from healthy individuals as a reference in the comparison.

1.1.2 The psoriatic non-lesional skin

The psoriatic non-lesional skin is characterised by increased stress response to several external and internal factors, such as mechanical stress and alcohol, which results in plaque formation. The central features of psoriatic plaque formation include abnormal keratinocyte proliferation and differentiation leading to the disturbance of the barrier function of the skin, among others.

1.2 Impaired barrier function of the skin and related osmotic challenge

Impaired barrier function is the main pathologic finding in irritant contact dermatitis (ICD), resulting in increased skin permeability and transepidermal water loss (TEWL). Water

evaporation can lead to a higher osmotic pressure in the superficial layers of the skin, therefore local hyperosmotic challenge may contribute to the development of the disease.

Hyperosmolarity is known to reduce cellular viability and elevates intracellular calcium (Ca^{2+}) concentration in HaCaT keratinocytes. Osmotic stress induces further intracellular responses. At mRNA level, the expression of tumour necrosis factor-alpha (TNF-α), IL-1β, IL-8, IL-6 and the nuclear factor of activated T cells 5 (NFAT5) was found to be higher in epithelial cells under hyperosmotic condition. Elevated mRNA expression of TNF-α, IL-1β, IL-6 and IL-8 was observed in normal human epidermal keratinocytes.

1.3 Beneficial effects of polyols on the skin

Glycerol and xylitol have well-known beneficial effects on the skin. Previously, we have shown their anti-inflammatory and anti-irritant effects. Other *in vitro* experiments have revealed that glycerol suppresses human leukocyte antigen-DR (HLA-DR) mRNA level, while xylitol inhibits inflammatory cytokine expression and upregulates filaggrin mRNA expression. In addition, glycerol is known to compose the principal osmolyte system of several bacterial species. However, the role of glycerol and xylitol as osmolytes in the skin has not yet been fully clarified.

2. AIMS

We aimed to extend previous proteomic studies, in order to get more information regarding the putative alterations in psoriasis. Therefore, a complex comparison was performed, where in addition to non-lesional and lesional skin, samples from healthy skin were also included, in a label-free, semi-quantitative proteomic analysis.

Furthermore, we aimed to investigate whether glycerol and xylitol, as known effective anti-irritant agents, provide protection against hyperosmotic stress *in vitro*. Their effects on cellular viability, cytotoxicity, intracellular Ca^{2+} concentration and expression of pro-inflammatory cytokines were studied in hyperosmotic condition.

3. MATERIALS AND METHODS

- **Patients and skin samples:** A total of 9 (3x3) patients suffering from chronic plaque psoriasis and the same number of healthy donors were involved in our study. Healthy, non-lesional and lesional 6 mm skin punch biopsies containing the epidermis and the dermis were collected from an area of the upper-middle gluteal region.
- **Comparative proteomic workflow of healthy, non-lesional and lesional skin:** Samples were cut with a razor blade. Skin proteins were extracted sequentially in four consecutive, solubility-based extraction steps. The same protein extracts of three donors were pooled in each investigated group (healthy, non-lesional, lesional). The extraction procedure was carried out three times and each contained extracted proteins of three donors. Samples were then subjected to two-dimensional liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis. To compare protein abundance from healthy, lesional and non-lesional skin extracts, significant differences were determined based on relative peptide ion chromatograms and spectrum counting, and evaluated using two different approaches: 1) modified t-test (limma) and 2) rank product test following a t-test. We considered a protein amount to be different between two samples if at least one test was significant ($p<0.05$) and the absolute fold change was at least two or higher.
- **Immunofluorescent staining for UDP-glucose 6-dehydrogenase (UGDH):** 5 μm sections of frozen embedded skin biopsies from psoriatic patients (non-lesional and lesional skin) and healthy individuals were used. After fixation, permeabilisation and blocking, the sections were incubated with rabbit polyclonal primary antibody against UGDH. Following washing, AF546 (goat anti-rabbit IgG) secondary antibody was applied.
- **Bioinformatic analysis:** To identify proteins not yet linked with the pathomechanism of psoriasis, literature mining was carried out using protein names or the encoding gene's symbol(s). Each protein or gene name was searched together with "psoriasis" as a keyword using the RISmed R package. For the identification of cellular mechanisms that may be associated with the proteins that were detected in altered amounts in the proteomic approach, we used the Ingenuity Pathway Analysis (IPA) software.
- **Cell culture:** HaCaT cells were cultured in Dulbecco's modified Eagle's medium supplemented with the appropriate agents (DMEM-HG).

- **Measurement of cellular viability and cytotoxicity:** For 60 min., cells were incubated with or without 0.27% glycerol or 0.45% xylitol in serum-free DMEM-HG, followed by incubation with 450, 500 or 600 mOsm, sorbitol-containing culture medium with or without 0.27% glycerol or 0.45% xylitol for 24 h. For the cell viability assay, 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to the cells, then the produced formazan crystals were solubilised and the optical density (OD) was measured. Cytotoxicity was assessed by using the Cytotoxicity Detection Kit PLUS (Roche Diagnostics, Risch, Switzerland) according to the manufacturer's instructions.
- **Determination of changes in intracellular Ca^{2+} concentration:** HaCaT keratinocytes were plated onto 13 mm diameter uncoated sterile coverslips. Coverslips with the attached cells were transferred and incubated in Tyrode's solution, followed by loading with Ca^{2+}-sensitive fluorescent dye (Fluo-4). Subsequently, cells were incubated with 0.027 or 0.27% glycerol and 0.045 or 0.45% xylitol in Tyrode's solution, respectively. The coverslips were placed into a low-volume imaging chamber at the Zeiss Axiovert 100 microscope stage. Hyperosmotic stimulus was added to the cells in rapid perfusion of 450 mOsm, sorbitol-containing solution, followed by the addition of A23187 ionophor.
- **RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (RT qRT-PCR):** Cells were pre-incubated for 60 min. with 0.27% glycerol or 0.45% xylitol, followed by incubation with 450 mOsm, sorbitol-containing medium for 2 h. Untreated or only sorbitol-treated cells served as negative or positive controls, respectively. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions, and DNase treatment was performed. cDNA was synthesised from 1 μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California, USA). RT qRT-PCR experiments were carried out with the Universal Probe Library system. Relative mRNA levels were calculated
by the ΔΔCt method.

4. RESULTS

4.1 Proteomic results

4.1.1 Comparison of healthy and lesional skin, and the associated biological processes

The relative abundance of 249 proteins was found to be different comparing lesional and healthy skin samples. A protein–protein interaction-based enrichment analysis performed with these proteins led to the identification of biological processes in the following categories: development, proliferation, regulation of expression and response to stimulus.

Since the major characteristics of psoriatic alterations include altered stress and immune responses, as well as dysregulation of proliferation and differentiation, we screened among proteins expressed differentially in lesion compared to healthy skin for central regulators participating in all four of these mechanisms. As a result, four central proteins — MYBBP1A, PML, PRKDC and STAT1 — were identified, however, only STAT1 had previously been associated with psoriasis.

To determine which lesional alterations and to what extent are manifest in non-lesional skin, we selected the 249 proteins that exhibited differential expression in healthy and lesional skin, and their expression level was compared to those in non-lesional skin. In non-lesional skin, the expression of 79.9% (199) of the 249 proteins differed from the expression in healthy and lesional skin by less than two-fold. Therefore, this category was termed as intermediate, as they may represent a discrete step in the healthy-to-lesional transition.

4.1.2 Differential protein expression in non-lesional and lesional skin, and the biological processes associated with these proteins

Comparison of non-lesional and lesional skin proteomes led to the identification of 60 proteins exhibiting at least two-fold differences in relative abundances. Of these proteins, 34 exhibited lower protein abundance in non-lesional skin compared to lesions, whereas 26 exhibited higher abundance. Functional enrichment analysis of these 60 proteins revealed several biological processes identified in psoriasis pathomechanism, including development and response to stimulus. We also found a subset of proteins to be differentially expressed in non-lesional and lesional skin, that were not differentially expressed in healthy skin and lesions. The levels of 8 proteins were greater in non-lesional skin and lower in lesional skin compared to the levels in healthy skin (non-lesional>healthy>lesional), and 1 protein exhibited the opposite trend (non-lesional<healthy<lesional).

4.1.3 Comparison of protein expression in non-lesional and healthy skin

7 proteins exhibited higher expression levels in non-lesional skin compared to healthy skin and 1 showed lower expression. Out of the 8 proteins GBP1, KLK10 and S100A7 have already been associated with psoriasis pathogenesis, while the other 5 are potential novel, early markers of the disease. The relative amount of 4 proteins (GART, CSE1L, GBP1 and UGDH) was similar in the non-lesional and lesional skin samples.

4.1.3.1 Verification of the proteomic results: UGDH expression in healthy, non-lesional and lesional skin

UGDH had the largest expression differences in non-lesional and healthy skin. As UGDH had not been linked to psoriasis previously, this protein was chosen for further analysis. Immunofluorescent staining of UGDH showed similar epidermal distribution in all three sample types, but clear differences in staining intensities were observed: the non-lesional and lesional psoriatic samples displayed more robust intensities compared to that of healthy samples, confirming our proteomic results.

4.1.4 Known and novel psoriasis-associated trigger proteins

We also identified 44 proteins that had altered expression only in the comparison of lesional skin to either non-lesional or healthy skin. It is anticipated that these proteins play a role in the manifestation and/or maintenance of lesions. The results of a computer-aided, keyword-based literature search suggests that, of these 44 proteins, 23 are already associated with the disease, whereas 21 have not yet been associated with psoriasis pathogenesis.

4.1.5 Psoriatic biomarkers, biological functions, canonical pathways and annotation of diseases associated with the detected alterations in protein amounts

Out of biomarkers identified previously in large-scale genomic, transcriptomic and/or proteomic studies, AKR1B10, CSTA, FABP5, PI3, SCCA2, STAT1, STAT3 and members of the S100 family, including S100A2 and S100A7-9 were also found in our study. These molecules exhibited elevated expression levels in psoriatic lesions, compared to healthy control skin.

Further analysis was performed to identify the cellular mechanisms that may be associated with the proteins that were detected in altered amounts in the proteomic approach, using the IPA software. Diseases annotation revealed “psoriasis” as the first hit when lesional and healthy, or lesional and non-lesional differences were compared. Annotation of biological

functions by IPA highlighted “initiation of protein translation” and “killing of *Staphylococcus aureus*” as the main functions likely to be affected, respectively. Ingenuity canonical pathway screening identified the “role of IL-17A in psoriasis” among the top ten most significant canonical pathways, when either lesional or non-lesional protein expression was compared to healthy samples. In addition, several cancer, neurological and neuromuscular canonical pathways were also highlighted.

4.2 *In vitro* results

4.2.1 Effects of glycerol and xylitol on the viability of HaCaT cells exposed to hyperosmotic stress

During a period of 24 h, 600 mOsm, sorbitol-containing medium significantly reduced viability. The reduction was also considerable in the additional polyol-treated groups, but glycerol treatment resulted in significantly higher viability as compared to the positive control (DMEM-HG + sorbitol (600 mOsm)) group. The average cytotoxicity value of the 0.27% glycerol + sorbitol (600 mOsm)-treated group was somewhat lower than that of its control group (DMEM-HG + sorbitol (600 mOsm)), but the difference was not significant.

4.2.2 0.45% xylitol protected against the hyperosmotic stimulus-induced increase in intracellular Ca^{2+} concentration

Hyperosmotic stress induced by 450 mOsm, sorbitol-containing solution was accompanied by a short elevation of intracellular Ca^{2+} concentration. This elevation was suppressed by the higher concentration (0.45%) of xylitol.

4.2.3 Preventive effects of polyols on the elevation in the mRNA expression of inflammatory cytokines and NFAT5 induced by osmotic stress

450 mOsm hyperosmotic stress induced considerable increase in IL-1α, IL-1β, IL-8 and NFAT5 mRNA expression. Both 0.27% glycerol and 0.45% xylitol prevented the elevation in the expression of IL-1α. Furthermore, both 0.27% glycerol and 0.45% xylitol led to considerably lower expression of IL-8; the expression levels did not differ significantly from those of the untreated control group. As concerns IL-1β and NFAT5, only 0.27% glycerol diminished considerably their expression.

5. DISCUSSION

The comparison of healthy, non-lesional and lesional skin at the proteomic level has been missing from the large-scale comparative proteomic studies of psoriasis. To fill this gap, our proteomic analysis included healthy skin as well as non-lesional and lesional psoriatic samples.

Comparing non-lesional and lesional skin, we identified 60 proteins with differential expression, which represent only 24.1% of the number of proteins, which showed altered expression in the comparison of healthy and lesional skin (60 vs. 249). This highlights the importance of studying healthy skin in comparisons using patient samples for pinpointing disease-associated alterations.

Based on differentially expressed proteins in either lesional vs. healthy or in lesional vs. non-lesional comparison, annotation of diseases resulted in the identification of psoriasis with the strongest correlation. Canonical pathway analysis of either lesional or non-lesional differences compared to healthy skin resulted in the identification of the "role of IL-17A in psoriasis". However, these annotations also highlighted cancer, neurological, neuromuscular or muscular disease-related mechanisms, suggesting their potential involvement in disease pathomechanism, or some similarities between these diseases. Since our proteomic and *in silico* analysis cannot distinguish between cell types and provide information whether mechanistically linked alterations take place within the same or different cell types, further experiments are required to clarify the exact relevance of these predicted connections to psoriasis pathomechanism.

We searched for potential central proteins in disease pathogenesis participating in key mechanisms of psoriasis including regulation of stress and immune response, proliferation and differentiation. Out of the central proteins, STAT1 had already been linked to psoriasis, however, we also identified 3 proteins, MYBBP1A, PML and PRKDC, which had not previously been highlighted in context with the disease. The suggested altered expression by our results of the transcription factor MYBBP1A may also be among the potentially important proteins implicated in the pathogenesis of psoriasis since it functions as a co-repressor of NF-κB that may regulate responses to stress and cytokines. An isoform of PML (PML-4) is known to regulate apoptosis and growth suppression, while the nuclear isoforms are involved in gene expression regulation at the MHC-I locus. The PRKDC may play a role in the

detection and repair of breaks in double-stranded DNA and mediates the phosphorylation of c-Myc and p53 suggesting a potentially important role in psoriasis.

Proteins whose expression was affected only in lesions are often considered “trigger” proteins, as changes in the expression of these proteins are linked to the shift of the disease state. The proteins that had not previously been associated with psoriasis were categorised into two groups. The first group of proteins might contribute to the mechanosensitivity of the tissue (SGCD, SYNM, MYH11 and ATP1B1). The second group functions within the nervous system (MPZ, PRX, CSPG4, CNTN1 ITGA8 and ATP1B1), which could suggest the involvement of the peripheral nervous system in psoriasis.

By comparing non-lesional and healthy skin, differential expression was observed for 8 proteins (CSE1L, GART, GBP1, KLK10, MYO18A, S1007A, SMARCA5 and UGDH). 4 of these proteins (CSE1L, GART, GBP1 and UGDH) might be predisposing factors, as their expression was similar in non-lesional and lesional skin, and their significance would have been missed in comparisons in which healthy samples were not included. Of these 4 proteins, UGDH was detected with the highest relative difference. UGDH had not been highlighted previously in association with psoriasis. Elevated UGDH levels may increase chondrocyte proliferation indirectly, probably through increased hyaluronan production that binds different cytokines. However, *in vitro* downregulation of UGDH and consequently decreased hyaluronan amounts did not influence keratinocyte proliferation. These results are in line with our observation, suggesting that elevated UGDH levels observed in non-lesional keratinocytes are not sufficient to modify their proliferation.

A further analysis focused on gaining insight about the extent to which alterations are manifest in lesions and in non-lesional skin. Strikingly, nearly 80% of the 249 proteins exhibiting differential expression in lesional and healthy skin exhibited an intermediate expression level in the non-lesional skin, suggesting the possible presence of early, lesional-like alterations in the non-lesional skin. Divergence from this trend was only observed in two small protein groups. 10 proteins — CHCHD6, CHMP5, COLEC12, FLOT2, ITGA7, LEMD2, NOP56, PLVAP, RRAS and SMARCA5 — differed in relative protein amounts in non-lesional and lesional skin, but the amounts of these proteins were similar in healthy and lesional samples. These 10 proteins are likely to represent a group of non-lesional characteristic alterations. For 9 proteins — CD207, COLEC12, CTSV, ITGA7, ITGA8,

PLVAP, PSAPL1, SMARCA5 and XP32 — the direction of the expressional changes was different in non-lesional and lesional samples compared to healthy skin. It might represent proteins that contribute to maintaining the non-lesional state. Next, with the proteins in these two groups, we performed an extensive literature search to suggest potential mechanisms by which they may influence disease pathogenesis. Interestingly, all the identified proteins may play a role in signaling at different levels starting from the cell surface all the way to the nucleus or mitochondria. The alteration of these systems is likely to lead to increased reaction to external signals that could contribute to the maintenance of psoriatic plaques.

During psoriatic plaque formation, abnormal proliferation, differentiation and thereby, skin barrier function are key processes. Among proteins identified with altered amount in the proteomic analysis, SMARCA5 is a component of the nucleosome remodelling factor complex. Decreasing SMARCA5 levels are required for basal keratinocytes to shift from proliferation toward differentiation. XP32 is also a component of the epidermal differentiation complex and it is associated with skin barrier function. Further proteins related to mechanical stress (for example SYNM) and disturbances of osmoregulation (such as ATP1B1) were also identified. These alterations with the abnormal barrier function are characteristic not only of psoriasis, but also of irritant contact dermatitis (ICD).

ICD is a frequent occupational disorder, which is characterised by impaired barrier function leading to increased TEWL. Water evaporation exposes keratinocytes to a condition of high osmotic pressure. The present study was aimed at the investigation of osmotic challenge potentially accompanying ICD.

Osmotic challenge influences cellular viability in an osmolarity- and time-dependent manner. In our experiments, 24 h exposure to 450 and 500 mOsm, sorbitol-containing medium did not influence cellular viability and cytotoxicity, but 600 mOsm resulted in a significant decrease in viability as compared to untreated control cells.

However, instead of 600 mOsm, 450 mOsm osmotic stress was applied to measure intracellular Ca^{2+}concentration, in order to examine the protective effects of polyols, with the elimination of cell death. Our results show that xylitol prevented the elevation of intracellular Ca^{2+} concentration induced by the hyperosmotic sorbitol solution, while glycerol did not influence this parameter. Further investigations are necessary to reveal the reason for this difference and the exact mechanism via which xylitol inhibits Ca^{2+} signal.

In addition to the rapid Ca^{2+} response, the applied osmotic challenge has longer effects, as well. 2 h of exposure to 450 mOsm, sorbitol-containing medium increased the expression of IL-1α, IL-1β and IL-8 in HaCaT cells. Hyperosmotic stress also induces the expression of the transcription factor NFAT5, which regulates TNF-α and can bind the promoter of IL-1 and IL-6. Thus, elevated expression of NFAT5 might have contributed to the increased cytokine response in the present study. Several factors may explain the beneficial effects of polyols on cytokine expression. In addition to the prevention of Ca^{2+} signal and the inhibition of NFAT5 expression, glycerol and xylitol as chaperon osmolytes may affect the inflammatory process via stabilising protein structure, as well.

SUMMARY

In this study, a complex comparison of the psoriatic lesional, non-lesional and healthy skin was performed by semi-quantitative proteomic analysis. In addition, the effects of glycerol and xylitol were tested under hyperosmotic conditions as an *in vitro* model of osmotic stress accompanying psoriasis, ICD and other xerotic skin diseases.

- Our comparative proteomic approach of healthy, non-lesional and lesional skin led to the identification of various proteins, which may function in psoriasis pathogenesis, providing a strong base for future studies.
- Proteins exhibiting opposite expression changes in lesional and non-lesional samples compared to healthy skin may function in the maintenance of the non-lesional stage and may represent future targets for therapeutic purposes.
- Glycerol supported cell viability, while xylitol prevented the hyperosmosis-induced Ca^{2+} signal, and both polyol protected against the increased expression of some inflammatory cytokines in hyperosmotic stress.
- Despite the similar chemical structure of glycerol and xylitol, their effects displayed some differences. Hence, joint application of glycerol and xylitol may be a useful therapeutic approach for different skin disorders.

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