SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the immune and permeability barrier in hidradenitis suppurativa

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INTRODUCTION

Hidradenitis suppurativa (HS) is an immune-mediated inflammatory skin disease localized to the apocrine gland-rich (AGR) skin region. In this disease the Th1/17 cytokine milieu has been well characterized; however, the stages and time course of the development of the immune milieu characteristic of the disease are not yet known. In a previous study, we have investigated the immunological characteristics of healthy AGR skin and HS lesional skin at the protein and gene levels. According to these results, the number of CD4+ T cells, CD11c+ myeloid dendritic cells (DC) and CD163+ macrophages, as well as the expression of molecules related to the dermal Th1/Th17 milieu, keratinocyte (KC)-derived proinflammatory cytokines and chemokines, were significantly different in HS lesional skin compared to AGR skin. However, little is known about the initial causes of inflammation and the first steps of disease development. Increasing evidence suggests that the apparently healthy non-lesional skin next to the lesion represents an intermediate state between the lesional skin of patients with immune-mediated skin diseases (e.g. psoriasis) and healthy skin, a subclinical state that can reveal the initial steps of disease development. In the first part of my thesis, I describe in detail the results of our research aimed at revealing the immunological characteristics of non-lesional HS skin.

In several chronic inflammatory skin diseases, such as atopic dermatitis (AD) or papulopustular rosacea (PPR), damage to the permeability barrier is likely to trigger the initial stages of the disease through activation of KCs. However, the possible role of permeability barrier alteration as a KC-activating event in HS has not yet been investigated. Our research group was the first to investigate permeability barrier abnormalities at the molecular level in HS, and I will report on this in the second half of my thesis.

OBJECTIVES

Investigation of the immune barrier in HS

Hidradenitis suppurativa is an immune-mediated inflammatory skin disease located on the apocrine gland-rich skin region, in which the Th1/17 cytokine milieu is well characterized; however, the stages of this immune milieu development and the time course of the disease are not known.

In our study, we aimed to perform the following investigations in non-lesional HS skin without clinical symptoms, lesional HS and healthy apocrine gland-rich moist skin:

1. investigation of immune cells, Th1/Th17 cell-associated molecules and keratinocyte-associated sensors and mediators by RNAseq

2. analysis of immune cells at gene (RT-qPCR) and protein (immunohistochemistry-IHC) level

3. analysis of Th1/Th17 cell-associated molecules at gene (RT-qPCR) and protein (IHC) level

4. analysis of keratinocyte-associated sensors and mediators at gene (RT-qPCR) and protein (IHC) level

5. immunofluorescence (IF) staining of TNFα and IL23 in HS skin

Investigation of the permeability barrier in HS

The results of our previous study suggest that KCs play an important role in the early stages of HS development. However, it is not yet known which factors activate KCs. One of the main exogenous factors in the activation of KCs may be changes in the permeability barrier. However, the possible role of permeability barrier alteration as a KC-activating event in HS has not yet been investigated in detail. We aimed to study the permeability barrier in HS at the molecular level compared to the AGR region.

1. Investigation of transepidermal water loss to study permeability barrier function (functional studies)

2. Investigation of SC molecules at the gene and protein levels

a) CE intracellular structural molecules

b) Components involved in corneocyte desquamation

c) Components involved in corneodesmosome formation

3. Analysis of TJ molecules at the gene and protein level

4. Visualization of the distribution of the coupling structures using confocal microscopy

MATERIALS AND METHODS

Skin samples

For our studies, punch biopsies were collected from the lesional and non-lesional (perilesional area ≥3 cm from the lesion) skin of patients with HS and from the apocrine gland-rich (AGR) skin region of healthy individuals who had plastic surgery as controls. The research was approved by the Scientific and Research Ethics Committee of the Health Scientific Council (ETT-TUKEB) and the Regional Ethics Committee of the University of Debrecen. Before sample collection, all patients gave written informed consent for the use of their skin samples for research purposes in accordance with the principles of the Declaration of Helsinki. The inclusion criteria for HS patients were as follows: individuals aged 18 years or older, diagnosed with moderate to severe HS and with a history of at least 6 months of HS. Clinical severity was defined by the Sartorius score and the dermatological quality of life index (DLQI). None of the patients had received biological therapy prior to skin biopsy sampling and all previous conventional systemic treatments were discontinued 4 weeks prior to sampling. Topical treatments were discontinued 4 days before sampling. One half of each biopsy was stored in an RNAlater (Qiagen, Hilden, Germany) at -70°C until RNA isolation for RT-PCR studies, the other half of the biopsy was fixed in formalin and embedded in paraffin for IHC and IF studies. Following hematoxylin-eosin (H&E) staining, samples were classified according to the number of apocrine glands and defined as AGR skin if n≥2 apocrine glands were identified in the field of view at 100x magnification.

RNAseq

The complementary DNA (cDNA) library for RNASeq analysis was generated from 1 µg of total RNA using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's protocol. A 50 base-pair sequencing was performed on the Illumina HiScan SQ instrument (Illumina) and 16-18 million reads per sample were obtained. The sequenced reads were aligned to the Human Genome v19 using the TopHat and Cufflinks algorithms and bam files were generated. StrandNGS software was used for further statistical analysis. A non-parametric Mann-Whitney test was used to identify statistically significant gene expression patterns. Library preparation, sequencing and data analysis were performed by the Genomic Medicine and Bioinformatics Services Laboratory, University of Debrecen.

Matrix data containing expression levels of target molecules were collected manually. The heatmap was generated using the Morpheus program.

RNA isolation, Reverse Transcription

All samples were homogenized in TriReagent solution (Sigma-Aldrich, Dorset, UK) using Tissue Lyser (QIAGEN, Hilden, Germany), prefilled with metal beads (Analytik Jena, 845-CS-1020050) using innuSPEED lysis tubes and total RNA was isolated. The concentration and purity of RNA samples were determined using a NanoDrop spectrophotometer (Thermo Scientific, Bioscience, Budapest, Hungary). RNA quality was checked using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA, USA). Samples containing RNA were transcribed to cDNA according to the manufacturer's instructions and the specified heat treatment protocol using High Capacity cDNA Archive Kit (Invitrogen, Life Technologies, San Francisco, CA, USA). Prior to this, samples were treated with DNase I enzyme (Applied Biosystems, Foster City, CA, USA).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

RT-qPCR measurements were performed in triplicates using the manufacturer's designed FAM-MGB assay and the TaqMan® Gene Expression Master Mix (Applied Biosystem, Life Technologies, San Francisco, CA). Reactions were performed on a LightCycler® 480 System (Roche, Grenzach-Wyhlen, Germany). Relative mRNA levels of target genes were determined by the 2-ΔΔCT method, in all cases normalized to PPIA mRNA expression.

Immunohistochemistry (IHC), immunofluorescence (IF) and routine staining

For IHC studies, paraffin-embedded sections of skin samples from HS patients and healthy controls were deparaffinized and rehydrated. A 15 min treatment with 3% H_2O_2 was used to inhibit endogenous peroxidases, followed by heat-induced antigen digestion. Then, after blocking with 5% bovine serum albumin (BSA) solution, sections were incubated overnight with primary antibodies at 4°C. The next day, sections were incubated with anti-mouse/rabbit (Dako) HRP-conjugated secondary antibody. Before and after incubation with antibodies, samples were washed for 3x5 min and ImmPACT™ NovaRED™ Kit (VECTOR Laboratories, Burlingame, CA, USA) was used to detect staining. Background staining was performed with methyl green. Each protein was extracted in parallel and simultaneously in all sections, in order to be able to compare protein levels when evaluating each other. Positive, IgG

and isotype controls were also used to normalize staining against all proteins [mouse and rabbit IgG (Covalab), rabbit immunoglobulin fraction (Dako)]. AGR skin samples were also stained with H&E. Visual scoring of apocrine glands was performed by a pathologist. IHC sections were digitized using a Zeiss Mirax Midi scanner (Zeiss, Oberkochen, Germany). Sections were evaluated using the Pannoramic Viewer software HistoQuant (3DHistech, Budapest, Hungary). We trained the application to separate the positive area (which pixel is positive) and background staining for each molecule. In each section, we assigned three unit lengths within the epidermis (valuable area=ROI) that represented the staining and evaluated the amount of protein within these units. The pre-trained algorithm evaluated the ROIs at each stage. Finally, the total staining intensity was determined and compared between sample groups.

Trans-epidermal waterloss measurement

Measurements were performed on HS patients (n=16) and healthy subjects (n=20) under standardised laboratory conditions at 22-25°C and 40-60% humidity. Before measurements, individuals were acclimatized for 15 min in the above room conditions. A Dermalab Combo (Cortex Technology, Hadsund, Denmark) was used to determine TEWL (g/hm2) in the axillae (representing the AGR area) of healthy individuals and in the lesional and non-lesional areas of HS patients. Three parallel measurements were performed, each lasting 30 seconds.

Confocal microscopy

The confocal microscopic analysis was using 3 to 3 skin samples embedded in paraffin-embedded paraffin at 30 μm thickness from HS-NL and healthy AGR sample groups. As a first step, the floating sections were deparaffinized, followed by heat-induced antigen digestion. The floating samples were incubated with primary antibodies (anti-human CLDN1: rabbit polyclonal IgG, ab15098, Abcam; anti-human DSG1: mouse monoclonal IgG, 129204, Novus) for 48 h at 4°C and with secondary antibodies (Alexa FluorTM 488 and 555 goat anti-mouse IgG (H+L), Thermo Fisher Scientific) for 2 h at room temperature. Finally, the samples were placed on slides and covered with VECTASHIELD® HardSet™ Antifade Mounting Medium (Vector Laboratories) containing DAPI. The immunostained samples were examined under an Olympus FV3000 confocal microscope with a 40x or 60x oil-immersion lens (NA: 1.40-1.42). The settings (laser power, confocal aperture and gain, detector parameters) were identical for all samples. A series of optical sections with a thickness of 1 um were taken at 0.5 μm in the Z axis. The number of optical sections/photographs was 6 for each sample. The pixels corresponding to the immunostained points were not saturated. Images of the figures were

created using Adobe Photoshop CS5 software. The regularity and pattern of the distribution of CLDN and DSG1 were analyzed by measuring the distance between two adjacent immunostained spots along the cross-section of the cell membrane.

Statistical analysis

Data normality was tested using the Shapiro-Wilk test. To determine statistical differences between groups, one-way ANOVA and Tukey post hoc test (normal distribution) or Kruskal-Wallis test with Dunn post hoc test (non-normal distribution) were used (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Figures show median and corresponding 95% upper and lower confidence intervals, as well as maximum and minimum values. Statistical analyses were performed using GraphPad Prism software version 7 (GraphPad Soft-ware Inc. San Diego, CA, USA).

RESULTS

Immune barrier investigation in HS

In a previous study, our research group studied how the immune milieu characteristic of healthy AGR skin is altered in HS lesional skin. In our current study, we aim to investigate in detail the immunological characteristics of non-lesional HS skin without clinical symptoms, in order to understand the first steps in the development of HS. We investigated the number of CD4+ T cells, CD11c+ myeloid DCs and CD163+ macrophages, as well as the expression of Th1/Th17 immune-associated molecules, KC-derived factors, proinflammatory molecules and chemokines at gene and/or protein level.

Immunological characterization of healthy AGR, non-lesional and lesional HS skin samples by RNASeq

We collected normalized gene expression levels of the previously mentioned target molecules from the RNASeq dataset from the 3 sample groups. Based on the heatmap, mRNA expression of most target molecules was similar at first glance between non-lesional HS and healthy AGR skin. Among the KC-derived mediators and sensors, S100A7, S100A8 and S100A9 AMPs showed a significant and significant increase in mRNA levels, while TLR4 expression was significantly lower in non-lesional HS skin compared to healthy AGR skin. No significant differences were found between the two groups with respect to immune cell surface markers (CD4, CD11c, CD83 and CD163), Th1/Th17-related mediators and transcription factors, except for TGFB1, whose expression was slightly but statistically significantly decreased in non-lesional HS skin compared to healthy AGR skin.

When comparing the lesion HS and non-lesion HS samples, the differential expression of target molecules was clearly detected. The levels of KC-derived mediators and sensors were highly and significantly increased in lesion HS, except for LCN2 and cathelicidin (CAMP), where the difference was not significant. Similarly, mRNA levels of cell surface markers and Th1/Th17-related mediators and transcription factors were significantly higher in lesion HS compared to non-lesion HS samples, including TBX21, a major transcription factor for inflammatory Th17 and Th1 cells. Notably, the expression of RORC, the major transcription factor in non-inflammatory Th17 cells, was in the opposite direction.

Analysis of immune cells in HS and healthy AGR skin by RT-qPCR and IHC

To further investigate the initial factors for the development of HS inflammation, healthy AGR and non-lesional and lesional HS skin samples were compared by RT-qPCR and IHC. We first performed gene and protein analysis of T cells, DCs and macrophages. Based on these results, mRNA expression of the CD83 DC activation marker CD83 was significantly higher in lesional HS skin compared to non-lesional HS, but similar levels were observed in AGR and HSNL skin. Based on our IHC assays, the numbers of CD4+ T cells, CD11c+ DC and CD163 macrophages were similar in healthy AGR and non-lesional HS skin, while their abundance was significantly increased in lesional HS skin samples.

Analysis of Th1/Th17-related molecules in HS and healthy AGR skin by RT-qPCR and IHC

Expression of Th1/Th17 signaling-related mediators at both mRNA and protein levels was similar in AGR and non-lesional HS skin. The mRNA expression of IFNG, IL17A, IL10, IL6, IL23A, TGFB and IL1B was significantly higher in lesional HS skin samples than in non-lesional HS. However, no significant difference was detected for IL12B, TBX21, TNFA, CCL20 molecules when comparing HS samples, but a trend increase was observed in all cases. An inverse trend is seen for the RORC gene, which is a transcription factor for the non-pathogenic IL17. At the protein level, all dermal Th1/Th17 mediators tested showed significantly elevated expression in lesion HS samples.

Analysis of KC-related sensors and mediators in HS and healthy AGR skin by RT-qPCR and IHC

The mRNA-based analysis of TLR2 and TLR4 sensors revealed that they were similarly expressed in healthy and non-lesional HS skin, whereas they were significantly higher expressed in lesional HS skin. In contrast, the mRNA levels of all KC-derived AMP mRNAs tested were already significantly elevated in non-lesional HS skin compared to AGR, with S100A7, S100A8, S100A9 and CAMP reaching significant levels, and their levels further increased in lesional HS skin. We then examined these molecules at the protein level. The presence of S100A8, LCN2 and hBD-2 was striking in the interfollicular epidermis of non-lesional HS skin; however, the differences were not statistically significant. Among the proinflammatory cytokines, in addition to significantly increased epidermal expression of IL-1β, TNFα and IL-23 levels were also significantly elevated in the nonlesional HS interfollicular epidermis but not in the dermis. In the lesional HS interfollicular epidermis, IL-23 levels tended to increase, while TNFα and IL-1β expression remained at high levels without further increase

compared to non-lesional skin, probably indicating a decreasing rate of their expression. However, dermal immunostaining for IL-23 and TNFα showed significantly higher levels in lesional HS skin.

Regarding the staining pattern of follicular epidermis, the above molecules showed strong positivity in non-lesional HS skin. Although it was not possible to quantify the protein levels of follicular epidermis in skin samples due to the different distribution of hair follicles, their levels showed an upward trend compared to interfollicular epidermis. Since hair follicles in lesional HS skin are destroyed by strong immune activation, we could not evaluate the staining pattern of follicles.

Immunofluorescent staining of IL-23 and TNFα in HS skin epidermis

The surprising early epidermal presence of IL-23 and TNFα in non-lesional HS samples motivated us to confirm these results using IF to demonstrate the staining pattern of these proteins in a horseradish peroxidase-independent system. IF assays further confirmed the increased epidermal expression of IL-23 and TNFα in non-lesional HS samples, whereas their dermal occurrence was less striking. In terms of staining pattern, TNFα was mainly localized in the upper apical part of the epidermis, with increased levels decreasing towards the basal epidermal layer. In contrast, proliferating KC basal layers showed strong cytoplasmic staining for IL-23, while slight positivity was detected throughout the epidermis. In lesional HS, strikingly high levels of IL-23+ and TNFα+ dermal infiltrates were present, with similar epidermal staining.

Investigation of the permeability barrier in hidradenitis suppurativa

In our study, we investigated the main permeability barrier elements, namely stratum corneum and tight junction at the mRNA level by RT-qPCR. Among the SC components, we studied molecules related to CE formation (FLG, LOR, KRT1, KRT10, TGM5), desquamation (KLK5, KLK7, KLK14) and corneodesmosome organization (DSG1, DSC1, PKP1, CDSN), and TJ molecules (CDH1, CLDN1, OCLN) and barrier sub-armins (KRT6A, KRT16) were analysed in lesional and non-lesional HS skin and in healthy skin samples as controls (AGR). As gene expression levels do not always reflect protein levels, representative representatives involved in SC and TJ formation were also analysed at protein level by IHC. It is important to note that HS is a follicular disease, therefore in non-lesional HS samples, barrier molecules were analysed separately in the interfollicular and follicular epidermis. In lesional HS samples, the follicular epidermis could not be quantified due to the uneven distribution of hair follicles.

Functional analysis of the permeability barrier

To investigate the permeability barrier function, TEWL was measured in lesional and non-lesional regions of HS patients (n = 16) and compared to AGR (axillary) regions of healthy volunteers (n = 20). In each case, three parallel measurements were performed. TEWL values in lesional HS (58.48 ± 20.26 g/m2h) and non-lesional HS (41.5 ± 20.15 g/m2h) skin were not significantly different from those in AGR skin (49.61 ± 20.81 g/m2h), suggesting no detectable barrier dysfunction in HS.

Analysis of the expression of molecules related to CE formation in HS and AGR skin

First, we investigated the intracellular structural molecules of CE from SC components at the mRNA and then protein level. No significant difference was found between non-lesional HS and AGR samples at either mRNA or protein level for molecules involved in CE formation (FLG, KRT1, KRT10, LOR, TGM5). Although most CE molecules showed significantly lower gene expression levels in lesional HS samples compared to non-lesional HS and AGR samples; at the protein level, we could only confirm a significant decrease in KRT1 in lesional HS skin compared to healthy skin regions.

Analysis of KLK proteases in HS and healthy skin regions

No significant differences were detected between non-lesional HS and AGR samples when KLK enzymes, which play an important role in skin desquamation, were examined at the

mRNA and protein levels. However, the expression of KLK5 and KLK7 at mRNA level was significantly decreased in lesional HS skin compared to non-lesional HS skin. In contrast, at protein level, KLK7 enzyme was significantly increased in lesion HS samples compared to non-lesion HS and AGR skin samples. However, KLK5 levels were significantly decreased in lesion HS skin samples compared to AGR skin samples based on immunostaining.

Analysis of corneodesmosome and TJ components in HS and healthy skin regions

Analysis of corneodesmosome molecule mRNA revealed similar expression levels in non-lesional HS and AGR skin samples. When comparing non-lesion and lesion HS skin samples, we found that gene expression levels of CDSN, DSC1 and DSG1 desmosome components were significantly decreased in lesion HS samples compared to non-lesion HS samples. And compared to healthy skin, all desmosome molecules showed significantly lower expression of mRNA levels in lesion HS skin. At the protein level, DSG1 was significantly more abundant in lesional HS skin compared to AGR, while CDSN protein expression did not differ between the three skin regions examined.

When CDH1, CLDN1 and OCLN TJ molecules were analyzed at the gene level, no major differences were found between non-lesional HS and AGR skin samples, but CDH1 and CLDN1 mRNA expression was significantly decreased in lesional HS skin compared to healthy and non-lesional HS skin. However, the protein expression of TJ molecules was similar when comparing the interfollicular and follicular epidermis of the three groups of samples tested.

Analysis of barrier alarmins in HS and AGR skin

The mRNA expression of KRT6 and KRT16 barrier alarmins were significantly increased in lesion HS samples compared to AGR samples. The amount of KRT6 protein was prominently but not significantly increased in non-lesion HS samples compared to healthy samples, but its expression in lesion HS skin was remarkably high compared to non-lesion HS and AGR skin, which was reflected in the staining pattern.

Analysis of the organization of cell-junctional structures in non-lesional HS and AGR skin

Due to the fact that our analyses at the mRNA and protein levels did not always show the same results (several molecules showed a decrease at the mRNA level but no change at the protein level), we investigated the organisation of the cell-junction structures by confocal microscopy. Immunofluorescence staining of DSG1 and CLDN1 was used to visualize (corneo)desmosome and TJ structures in the epidermis of healthy and non-lesioned HS

specimens. Lesional HS samples were not examined here because of their severely damaged hair follicles.

A similar and well-organized expression pattern of CLDN1 was detected in the follicular and interfollicular epidermis of both non-lesional HS and AGR samples. In the interfollicular area, the mean of the distances between puncta was significantly smaller in the non-lesional HS than in the AGR samples, whereas the distances measured in the follicular epidermis were similar in the two groups of samples, but the distribution of distances mostly overlapped.

DSG1 expression along the cell membrane showed a less organized distribution than CLDN in all four samples, and its staining pattern appeared similar in all conditions. The mean of the interpunctate distances was significantly greater in both the follicular and interfollicular regions of non-lesional HS skin, and the variance of the data points (i.e., the variability of interpunctate distances) was also greater in the follicular and interfollicular regions of non-lesional HS skin. In conclusion, the staining pattern of TJs and corneodesmosomes was similar in non-lesional HS and AGR samples, and the differences in the distance of cell-connected structures were very small and in opposite directions (FC HS-NLvs AGR=-1.08 and 1.1, respectively), confirming our previous results.

DISCUSSION

Our current results on the first focus of my dissertation confirm, in agreement with our previous study, that the activation of epidermal KCs triggers immunological events in the development of HS, as all AMPs tested, as well as IL-1β, IL-23 and TNF-α, are already significantly expressed in the epidermis of non-lesional HS skin. On the other hand, the expression of dermal IL-23 and TNF-α showed a significant increase only in lesional HS skin, together with an influx of T cells, dendritic cells and macrophages, and an increase in the expression levels of IL-12, IFNγ, IL 17A, IL-10, TGFβ and CCL20. In lesional HS skin, epidermal IL-1β, IL-23, TNFα and CCL2 protein levels also remained elevated (without further significant increase), while S100A8 and LCN2 levels increased even further compared to non-lesional HS. Together, these results confirm that KCs play a key role in the pathomechanism of HS already at the disease early stage.

The prominent role of KCs in the early stages of HS lesion formation has been suggested by other research groups. Hotz and colleagues have also demonstrated increased gene expression of certain inflammatory cytokines, chemokines and AMPs using RT-qPCR and Luminex assay, although their results were based primarily on KCs isolated from hair follicles of HS patients. Similarly, Coates et al. found elevated AMP levels in HSL skin and hypothesized that the development of HS may be caused by altered AMP expression. Other research groups have also studied changes in AMPs at the mRNA or protein level in non-lesional and lesional HS skin, but these studies did not use healthy skin samples as controls. Regarding the presence of IL-1β, TNFα and IL-23, several studies focused only on their dermal presence without highlighting their epidermal expression, while other studies used methods that were not suitable for the *in-situ* detection of these molecules.

In our present study, the activity of KCs in non-lesional HS skin was found to be strong not only in the interfollicular epidermis but also in the follicular epidermis. The prominent role of follicular KCs in the pathogenesis of HS has been suggested in a previous study. These previous results provided the first evidence that dysregulation of the immune response of follicular KCs may be a major cause of HS. This study focused on follicular AMP production in lesional and non-lesional HS.

Our present results are consistent with the above-mentioned studies and, in fact, our results suggest that molecular changes in KCs occur before dermal activation. By comparing healthy non-lesional HS and lesional HS skin samples, using RNASeq and RT-qPCR methods and parallel morphological staining methods, we observed the localization of immunological

changes that characterize the three stages of disease pathogenesis. In psoriasis, it is already accepted that healthy-appearing non-lesional skin represents an intermediate stage between healthy and lesional skin and shows a predictive phenotype. Applying the same concept, in the present study we were able not only to isolate the epidermal and dermal localization of mediators within the dermal tissue, but also to highlight a possible sequence of immunological changes during the development of HS. Our results also demonstrate the importance of using RNA and in situ protein-based methods in combination, rather than separately, as they complement each other and provide valuable, comprehensive data.

In our previous study, we detected a high number of non-inflammatory Th17 cells in healthy AGR skin, whereas in lesional HS skin we found a predominant presence of inflammatory Th17 cells. Consistent with previous studies, our present results suggest that increased epidermal production of IL-1β and IL-23 may trigger a phenotype switch of resident T cells from IL-17/IL-10-producing non-inflammatory Th17 [Th17(β)] cells to IL-17/IFNγ-producing inflammatory Th17 [Th17(23)] cells. This shift was confirmed by RNASeq data (heatmap), which showed highly downregulated mRNA levels of the major transcription factor RORC in Th17(β) cells and significantly elevated gene expression levels of TBX21, the major transcription factor in Th17(23) cells, in HS. Several studies have described an increase in the number of inflammatory Th17 cells in HS, with a concomitant increase in the effector Th17/Treg ratio, which is considered an important step in the pathogenesis of the disease. On the other hand, the epidermal production of TNF-α may increase the inflammatory potential of IL-17, as recent immunological data suggest that the combined presence of TNFα and IL-17 induces inflammatory mediators in a synergistic manner and more potently, whereas IL-17 alone is not able to induce inflammation efficiently.

The important role of the TNFα and IL-23 cytokines is demonstrated by the fact that biological agents against TNFα have already been approved for the treatment of HS and clinical trials of monoclonal antibodies against IL-23 are ongoing. Although these therapeutic agents have significant efficacy in HS patients, they are not as potent as those seen in psoriasis patients. The reason for this phenomenon is as yet unknown. The extremely high dermal TNFα and IL-23 levels detected in HS may explain this, while another explanation may be their high epidermal production. It would be useful to study in detail the retrograde penetration of these high molecular weight (anti-TNF: 148 kDa; anti-IL-23: 143 kDa) monoclonal antibodies in the HS epidermis, which may be very low and thus lead to therapeutic failure.

In summary, to better understand the pathogenesis of the disease, we performed a comprehensive analysis of healthy AGR, non-lesional HS and lesional HS in skin using

multiple mRNA and protein-based methods. Our results highlighted the importance of KCs in the pathology of HS, as their prominent mediator production plays a key role in the transformation of non-inflammatory Th17 cells into inflammatory Th17 cells and enhances inflammation.

In the second study, which became the base of my dissertation, we investigated the permeability barrier characteristics in HS. One of the many functions of the skin is the establishment and maintenance of the permeability barrier, which provides the primary line of defence against various exogenous physical, biological and chemical stressors. At the same time, the permeability barrier prevents endogenous water loss. The SC layer and the TJ network in the stratum granulosum are primarily responsible for the permeability barrier function of the skin.

In several chronic inflammatory skin diseases, such as atopic dermatitis (AD) and possibly also papulopustular rosacea (PPR), damage to the permeability barrier is thought to trigger the primary steps of the disease through activation of KCs. In AD, both genetic and acquired barrier damage have been shown to activate KCs, and cytokine production (TSLP, IL-33, IL-25) by KCs triggers activation of DCs, thereby triggering type 2 adaptive immune responses and inflammation. Recently, a very similar permeability barrier impairment has been described in PPR, and the role of this barrier alteration in disease development has been pointed out. In HS, the role of KCs as a primary immune activator was demonstrated in the study that formed the first part of my dissertation, but the role of barrier damage as a KC activating factor has not been thoroughly investigated by us or other groups to date. Therefore, we examined the permeability barrier characteristics in lesional and non-lesional HS skin samples and compared them with healthy AGR skin. Our aim was to investigate SC and TJ as the two main barrier elements of the permeability barrier. We analyzed molecules related to CE formation, SC desquamation, intercellular lipid lamellae formation and corneodesmosome organization, as well as TJ molecules and barrier alarmins at the mRNA and protein levels.

According to our results, in non-lesional HS skin, neither mRNA nor protein expression of the molecules tested differed significantly from normal skin. In lesional HS skin, the mRNA levels of several molecules were significantly different compared to AGR skin; however, more relevant protein-level studies could not confirm these differences. Lesion HS showed only slight and opposite changes at the protein level (KRT1 and KLK5 decreased, KLK7, KRT6 and DSG1 increased), of which only KRT6 showed a large change in the same direction in lesion HS skin at both mRNA and protein levels. KRT6 expression in HS has been reported by other

research groups. Since KRT6 is a marker of highly activated and proliferative KCs under pathological conditions rather than a barrier subarmin molecule, our results suggest abnormal KC proliferation/inflammation rather than barrier failure. Our functional and confocal microscopy studies confirmed our immunohistochemical results, as both lesional HS and non-lesional HS samples lacked severe barrier abnormalities; neither TEWL nor confocal microscopy results indicated significant impairment of permeability barrier function or expression of junctional structures.

Only a few papers have so far investigated the permeability barrier in HS. However, they have not used simultaneous RNA- and protein-based assays or confocal methods, and have not investigated all major barrier elements. Furthermore, previous publications have only studied lesional skin, not non-lesional skin. In HS, healthy-appearing non-lesional skin, which represents an intermediate state between healthy and lesional skin of patients, is often used to detect early events in disease pathogenesis (early mediators and cellular components). We therefore included non-lesional HS skin samples in our study.

Among the other available studies investigating barrier molecules, Navrazhina et al. investigated the structural components of CE, FLG and LOR, and obtained similar results, with no significant differences between the lesion HS and control groups. Note that protein levels were not quantified in this case. The results of two publications investigating the expression levels of desmosome-forming molecules in HS were also consistent with our study, as increased levels of DSG1 were found in inflamed HS skin. However, they did not investigate the mRNA level of DSG1 and did not examine non-lesional HS samples. To the best of our knowledge, changes in TJ molecules in HS have not been previously investigated.

In conclusion, our results suggest that barrier damage does not play a prominent role in the development of the disease. Since HS is a follicular disease, we performed our protein level studies (using IHC and confocal microscopy) in both interfollicular and follicular epidermis and did not find severe barrier damage in either case. Based on these results, we conclude that other triggers, such as mutations, mechanical stress, hormonal changes, altered sweat production or pH, and dysregulated microbiota, rather than barrier damage, may activate genetically sensitive KCs and trigger inflammation through the innate and then adaptive immune system, leading to clinical manifestation of HS.

SUMMARY

Hidradenitis suppurativa is an inflammatory skin disease of the apocrine gland-rich skin region, whereas the pathomechanism of the Th1/17 cytokine environment is well understood; however, the stages of immune milieu development and the time course of the disease are not yet known. To understand the initial steps of HS development, we investigated the number of CD4+ T cells, CD11c+ myeloid DCs and CD163+ macrophages, as well as the molecules related to the Th1/Th17 immune milieu, KC-derived factors, pro-inflammatory molecules and chemokines were first investigated at the mRNA level by RNASeq, quantitative real-time PCR (RT-qPCR) and at the protein level by IHC and IF. According to our results, all the investigated AMPs as well as IL-1β, IL-23 and TNF-α were already significantly expressed in the epidermis of non-lesional HS skin. On the other hand, dermal IL-23 and TNF-α showed a significant increase only in lesional HS skin, together with an infiltration of T cells, DCs and macrophages, and an increase in the expression levels of IL-12, IFNγ, IL 17A, IL-10, TGFβ and CCL20. In conclusion, these results confirmed that KCs play a key role in the pathomechanism of HS.

Our second study investigated the potential role of the permeability barrier of HS as a factor in KC activation, because damage to the permeability barrier in many chronic inflammatory skin diseases is thought to trigger the primary steps of disease through activation of KCs. First, we measured TEWL in lesional and non-lesional regions of HS patients and compared it with AGR regions of healthy volunteers. TEWL measurements in lesional HS and non-lesional HS skin showed no significantly different levels from those in AGR skin, suggesting that there is no detectable barrier dysfunction in HS. We investigated the two major barrier elements of the permeability barrier, SC and TJ, at the mRNA and protein levels. Molecules involved in cornified envelope formation, stratum corneum desquamation, intercellular lipid lamellae formation, corneodesmosome organization, and barrier alarmins were also investigated. Our results showed that in non-lesional HS skin, neither mRNA nor protein expression of the studied molecules differed significantly from normal skin. In HS lesional skin, although the mRNA levels of some molecules showed significant differences compared to AGR skin, only slight changes in the opposite direction were detected at the protein level (KRT1 and KLK5 decreased, KLK7, KRT6 and DSG1 increased). Among them, only KRT6 showed a large and consistent change in lesional HS skin at both mRNA and protein level. The confocal microscopy analysis confirmed our immunohistochemical results, as it did not indicate any significant impairment of permeability barrier function or junctional structure

expression. Our results suggest that it is likely that it is not barrier damage that activates genetically sensitive KCs, but other triggers, such as mutations, mechanical stress, hormonal changes, altered sweat production or pH, and dysregulated microbiota, which may combine to trigger inflammation leading to the clinical presentation of HS.

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Registry number: DEENK/120/2024.PL
Subject: PhD Publication List

Candidate: Orsolya Somogyi
Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology

List of publications related to the dissertation

1. **Somogyi, O.**, Dajnoki, Z., Szabó, L., Gáspár, K., Hendrik, Z., Zouboulis, C. C., Dócs, K., Szűcs, P., Dull, K., Töröcsik, D., Kapitány, A., Szegedi, A.: New Data on the Features of Skin Barrier in Hidradenitis Suppurativa.
   Biomedicines. 11 (1), 1-12, 2023.
   DOI: http://dx.doi.org/10.3390/biomedicines11010127
   IF: 4.7 (2022)
2. Dajnoki, Z., **Somogyi, O.**, Retzlerné Medgyesi, B., Jenei, A., Szabó, L., Gáspár, K., Hendrik, Z., Gergely, P., Imre, D., Póliska, S., Töröcsik, D., Zouboulis, C. C., Prens, E. P., Kapitány, A., Szegedi, A.: Primary alterations during the development of hidradenitis suppurativa.
   J. Eur. Acad. Dermatol. Venereol. 36 (3), 462-471, 2022.
   DOI: http://dx.doi.org/10.1111/jdv.17779
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List of other publications

3. Szabó, L., Kapitány, A., **Somogyi, O.**, Alhafez, I., Gáspár, K., Palatka, R., Soltész, L., Töröcsik, D., Hendrik, Z., Dajnoki, Z., Szegedi, A.: Antimicrobial Peptide Loss, Except for LL-37, is not Characteristic of Atopic Dermatitis.
   Acta Derm.-Venereol. 103, adv9413, 2023.
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4. Szabó, L., Dajnoki, Z., **Somogyi, O.**, Gáspár, K., Hendrik, Z., Szabó, I. L., Szöllősi, A. G., Dinya, T., Töröcsik, D., Kapitány, A., Szegedi, A.: Cytokine profile of the epidermis is region specific and may determine the characteristics of inflammation.
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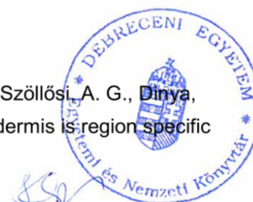
   IF: 3.6 (2022)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5. Kapitány, A., Retzlerné Medgyesi, B., Jenei, A., **Somogyi, O.**, Szabó, L., Gáspár, K., Méhes, G., Hendrik, Z., Dócs, K., Szűcs, P., Dajnoki, Z., Szegedi, A.: Regional Differences in the Permeability Barrier of the Skin: implications in Acantholytic Skin Diseases.
Int. J. Mol. Sci. 22 (19), 1-15, 2021.
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