Title: Increased expression of the interleukin-36 cytokines in lesions of hidradenitis suppurativa

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SUMMARY

Background

Hidradenitis suppurativa (HS) is a recalcitrant chronic skin disease with poorly understood immunopathogenic mechanisms. Previous studies reported that the interleukin-36 (IL-36) cytokines (IL-36α, IL-36β, IL-36γ, and IL-36 receptor antagonist [IL-36RA]) are important players in the pathogenesis of psoriasis (PS).

Objective

We aim to determine whether the IL-36 cytokines are upregulated in HS patients. For this purpose, we analysed local expression and systemic levels of the IL-36 cytokines in HS patients and compared the results to healthy donors and PS patients.

Methods

Skin biopsies from healthy donors and HS and PS patients were analysed for expression of the IL-36 cytokines by immunohistochemistry and semiquantitative real-time PCR. The enzyme-linked immunosorbent assay (ELISA) was used to measure systemic levels of the IL-36 cytokines in the serum of the three donor groups.

Results

The agonists IL-36α, IL-36β, and IL-36γ were found to be upregulated in HS both systemically and lesionally, while the IL-36RA was not differently regulated in comparison to healthy donors.

Conclusion

Our findings suggest that the agonistic IL-36 isoforms are upregulated in HS. The relevance of the enhanced production of IL-36 cytokines in HS pathogenesis remains to be determined.

INTRODUCTION

Treatment of hidradenitis suppurativa (HS), also called acne inversa, a chronic inflammatory skin disease, is challenging and tedious because of the poorly understood pathogenesis. HS is painful and lowers the patient's quality of life considerably.[1] It manifests through subcutaneous nodules and deep dermal abscesses in axillary, inguinal, and anogenital areas of the body and is accompanied by malodorous suppuration.[2] The initial starting point of HS is thought to be follicular keratinisation leading to the occlusion, dilation, and rupture of the pilosebaceous unit. This results in an infiltration of inflammatory cells into the dermis with a subsequent dysregulation of the immune system.[3,4]

The IL-36 cytokines (IL-36α, IL-36β, IL-36γ, and IL-36RA) are interleukins of the IL-1 cytokine family and are mainly produced by keratinocytes.[5] The three different agonistic isoforms of IL-36 (IL-36α, IL-36β, IL-36γ) are proinflammatory and are induced by themselves[6,7] as well as by other cytokines, such as IFN-γ, IL-1α, IL-1β, IL-17, IL-22, IL-23, and TNFα.[5,8-10] IL-36α, IL-36β, and IL-36γ have similar functions. In more detail, IL-36α stimulates the production of IL-17 and IL-23.[8] The isoform IL-36β induces antimicrobial peptides, matrix metalloproteinases, and IL-8 expression in keratinocytes.[9] IL-36γ is induced in macrophages by *mycobacterium tuberculosis*,[11] and it upregulates not only chemokines (CXCL8, CCL2, CCL5, and CCL20) but also the adhesion molecules VCAM-1 and ICAM-1.[7,12] Furthermore, IL-36γ is inducible by cathelicidin in human keratinocytes and supports its alarmin function.[13] The IL-36RA blocks the agonistic IL-36 isoforms from binding onto the IL-36 receptor, which is expressed on keratinocytes, other endothelial cells, dendritic cells, monocytes, and dermal M2 macrophages.[7,12,14] IL-36 signals through MAPK, JNK, ERK1/2, and NFκB.[15]

IL-36 cytokines play an important role in the pathogenesis of PS,[5,9,10,16] which is why an anti-IL-36-receptor antibody (WO2013074569) has been developed recently.[17] The function of IL-36 in this inflammatory skin disease is the regulation of the IL-23/IL-17 axis. This was shown in the imiquimod mouse model, where the absence of IL-36R made it impossible to induce psoriasiform dermatitis.[6] We sought to investigate whether the IL-36 cytokines are also implicated in HS pathogenesis, because HS shares a lot of proinflammatory pathways with PS,[18] such as the upregulation of the proinflammatory cytokines TNFα, IL-1β, IL-17, IL-12, and IL-23[19-25] and the antimicrobial peptides cathelicidin, psoriasin, and β-defensins 2 and 3.[26-32] Therefore, we wanted to determine whether similar pathogenetic mechanisms are present in HS. To this aim, we not only compared levels of the IL-36 cytokines to healthy donors but also to PS patients. We stained and quantified the protein levels of the three agonistic IL-36 isoforms and their antagonist by immunohistochemistry (IHC). Additionally, we compared mRNA levels of the IL-36 cytokines between the three donor groups and analysed systemic levels of the IL-36 cytokines by sandwich ELISA.

MATERIAL AND METHODS

Samples from patients

This study was approved by the Medical Ethics Committee of the Canton of Berne, Switzerland, and followed the Declaration of Helsinki protocols. All patients signed a written informed consent before enrolment. Lesional skin biopsies were obtained from 25 HS patients (16 women, 9 men, median age 36 years, range 18–51 years) with a mean Hurley stage of 2.16 ± 0.55. Furthermore, biopsies from 6 psoriasis patients (5 men, 1 woman, median age 48 years, range 30–61 years) and 7 healthy donors (3 women, 4 men, median age 66 years, range 24–94 years) were taken. Biopsy specimens were either snap frozen and stored at -70°C or formalin fixed and embedded in paraffin (FFPE). Additionally, serum was taken from 5 HS patients (2 women, 3 men, median age 40 years, range 30–55 years), 5 PS patients (1 woman, 4 men, median age 43 years, range 20–56 years) and 4 healthy donors (1 woman, 3 men, median age 33 years, range 24–51 years). Serum was stored at -20°C. Study subjects were never treated with biologics or immunomodulatory drugs, and systemic antibiotics were stopped at least 3 weeks before skin biopsy or blood donation.

Immunohistochemical staining

Immunohistochemistry for IL-36α, IL-36β, IL-36γ, and IL-36RA (see list of primary antibodies in Table 1) was performed using the streptavidin-biotin complex/alkaline phosphatase method.[19] Briefly, FFPE tissue sections were deparaffinised, rehydrated, and pretreated in citrate puffer (pH6) for either 10 minutes in the pressure cooker (IL-36β and IL-36γ) or 5 minutes in the microwave (400 Watt) (IL-36RA). Sections for IL-36α were pretreated with TE-buffer (pH 9) for 10 minutes in the pressure cooker, then all sections were rehydrated in 0.1% saponin containing

Tris-buffered saline. After blocking (Roti-ImmunoBlock, Roth GmbH, Karlsruhe, Germany) for 15 minutes, sections were incubated with the primary antibody for 1 hour at room temperature, followed for 1 hour by a biotinylated rabbit anti-goat secondary antibody (Dako, E0466, Santa Clara, CA, USA) and thereafter sections were treated with the streptavidin-biotin complex/alkaline phosphatase method (551008; BD Biosciences, San Diego, CA, USA). Finally, the sections were developed with the Fuchsin Substrate-Chromogen System (KO625, Dako) and counterstained with hematoxylin for 3 minutes. To ensure specific detection, the same tissue was stained with the secondary antibody only. To quantify the expression of the IL-36 cytokines in the epidermis, a semiquantitative score was used.[33] In short, density and intensity of the staining as well as spread over the epidermis was assessed and included in the allocation of the score, which ranged from 0 (no expression) to 3 (very high expression).

RNA isolation and complementary DNA synthesis

As previously described,[19] around 1 mm of snap frozen tissue was cut into 10 µm sections and collected in a frozen tube. RNA was extracted by means of the RNeasy lipid tissue mini kit (Qiagen, Valencia, CA, USA). Possible contaminating DNA was removed with an RNase-free DNase step (Qiagen) following the manufacturer's protocol. A total of 500 ng of RNA was used to synthesise complementary DNA using the BD Reverse Transcriptase Kit (BD Bioscience) according to the manufacturer's indications.

Semiquantitative real-time PCR analysis

PCR primers and probes were purchased (Applied Biosystems, Foster City, CA) as premade Taqman© gene expression assays. The following primer pairs, spanning exon-exon borders, were used: IL-36α (Hs00205367_m1), IL-36β (Hs00205359_m1), IL-36γ (Hs00219742_m1), and IL-36RA (Hs01104220_g1); and the following two reference genes: β_2-microglobulin (B2M) (Hs99999907_m1) and hypoxanthine phosphoribosyltransferase-1 (HPRT-1) (Hs99999909_m1). The semiquantitative real-time PCR was performed with ABI-Prism 7300 Sequence Detector System (Applied Biosystems) as previously described.[19] In short, for complementary DNA amplification, a 10-minute incubation at 95°C was performed to activate AmpliTaqGold (Applied Biosystems), followed by 45 cycles with 15 seconds at 95°C and 1 minute at 60°C. The results are expressed as fold difference. To determine these differences, relative units were calculated by the $2^{-\Delta\Delta CT}$ method: The threshold cycle (CT) for the target amplicon and the CT for the reference genes were determined for each sample. Differences in the CT of the target and the geometric mean of the CT of the two reference genes (see Ref.[34]), called ΔCT, were calculated to normalise for the difference in the amount of total nucleic acid added to each reaction. The ΔCT of control skin (calibrator) was subtracted from the ΔCT of each sample and termed as ΔΔCT. The amount of target normalised to the endogenous control and relative to the calibrator was then calculated by the equation $2^{-\Delta\Delta CT}$. In samples, where no CT value could be determined, the CT value was set as 42, as described in Ref.[35]

Enzyme-linked immunosorbent assay (ELISA)

To detect IL-36α, IL-36β, and IL-36γ in the serum, the antibodies and standards in Table 1 were used. IL-36RA was detected by the IL-36RA DuoSet ELISA kit (RnD Systems, Minneapolis, MN, USA, DY1275-05). All ELISAs were performed according to the manufacturer's instructions. The results are expressed either in ng/mL or in pg/mL.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software, version 5.01 for Windows, San Diego, CA, USA. Quantification of IHC staining, semiquantitative real-time PCR, and ELISA was analysed by means of the non-parametric Mann-Whitney test. A *p*-value lower than 0.05 was considered significant, with $p < 0.05$ = *, $p < 0.01$ = **, and $p < 0.001$ = ***.

RESULTS

IL-36 cytokines are expressed in lesional HS skin

Analysis of protein expression of the IL-36 cytokines was performed by immunohistochemistry on FFPE tissue sections of lesional HS and PS skin as well as on healthy skin. Representative examples of all four stainings are shown in Figure 1a–d, and analysis with a semiquantitative score of six sections per donor group is shown in Figure 1e–h. As expected, lesional PS skin showed high epidermal protein expression of all IL-36 cytokines, while healthy skin expresses the IL-36 cytokines only minimally. Lesional HS skin shows a trend towards elevated protein expression of IL-36α (p-value = 0.26) and IL-36RA (p-value = 0.06) and a significant upregulation of IL-36β (p-value = 0.02) and IL-36γ (p-value = 0.02), even though levels are not as high as in lesional PS skin.

IL-36α mRNA is significantly upregulated in lesional HS skin

After investigating IL-36 expression on the protein level, we analysed the mRNA levels of IL-36α, IL-36β, IL-36γ, and IL-36RA by semiquantitative RT-PCR. Relative changes in gene expression between lesional patient skin (HS and PS) and healthy skin are shown in Figure 2a–d. In accordance with our IHC results and with the literature, all IL-36 cytokines are significantly elevated in lesional PS skin compared to healthy skin. In HS, only the agonistic isoform IL-36α shows a statistically significant increase (mean increase = 45.07, p-value = 0.01) in comparison to healthy skin, with both IL-36β (mean increase = 1.45, p-value = 0.25) and IL-36γ (mean increase = 1.96, p-value=0.07) showing a trend to elevation, while IL-36RA levels are comparable to healthy skin.

Systemic levels of agonistic IL-36 cytokines trend to be elevated in HS patients

To investigate whether systemic levels of the IL-36 cytokines correlate with lesional expression in HS patients, sandwich ELISA was performed with serum from the three donor groups (healthy donors and HS and PS patients) (see Fig. 3a–d). In healthy donors, no or only low concentrations of the IL-36 cytokines could be detected, while HS patients show a trend towards elevated levels of isoforms IL-36α (p-value = 0.16), IL-36β (p-value = 0.31), and IL-36γ (p-value = 0.12) with no detection of the IL-36RA. PS patients have high IL-36 cytokine levels in their serum, even though no statistical significant differences could be detected.

Discussion

We show that IL-36α, IL-36β, and IL-36γ are significantly upregulated locally (on the mRNA or the protein level) and also show a tendency towards a systemic upregulation in patients suffering from the recalcitrant skin disease HS, while the IL-36RA is neither significantly elevated on the protein and mRNA level nor on the systemic level. Furthermore, we found high local and systemic levels of all IL-36 cytokines in psoriatic skin.

IL-36 cytokine expression in lesional HS skin was found to be elevated compared to healthy skin but lower than in lesional PS skin, which suggests different pathogenetic events in those two chronic inflammatory skin diseases. This is especially remarkable as previous studies could not find any striking differences between these two conditions when analysing the expression of various cytokines and antimicrobial peptides.[19-32] One of the reasons for the lower IL-36 levels in HS compared to PS could be that HS is not a disease of the epidermis but rather of the hair follicle, and inflammation is mostly present in the dermis. Even though hyperkeratinisation can be detected in HS,[36] this effect is much more pronounced in PS, and as keratinocytes are the major producers of IL-36, this could explain why we see lower levels in HS than in PS.

Additionally, the importance of other members of the IL-1 cytokine family in HS pathogenesis is controversial. In vivo evidence with blockade of IL-1β in HS patients yielded conflicting results.[37-39] Interestingly, when Boutet et al. compared mouse models of psoriasis, rheumatoid arthritis, and Crohn's disease, IL-36 levels were elevated in all the models, but in the psoriasis model they were the highest.[40] Still, it is difficult to speculate about the relevance of IL-36 in HS pathogenesis and how HS immunopathogenesis differs from that of PS. What we can show with our data is that

in HS the IL-36 cytokines are upregulated, which fits well with the reported Th1/Th17 phenotype present in lesional HS skin.[19,41]

Dysregulated IL-36 levels have also been implicated in other inflammatory diseases. Levels of the IL-36 cytokines have been reported to be comparable to healthy skin in atopic dermatitis, lichen planus, subacute cutaneous lupus erythematosus and mycosis fungoides [42] and high in allergic contact dermatitis.[43]

Moreover, IL-36 was shown to be an important cytokine for mucosal immunity.[44] In a mouse model for Crohn's disease, IL-36 was upregulated in the intestinal inflammation, and IL36R-/- mice show reduced mucosal inflammation.[45] In humans, two recent studies analysing IL-36 in patients with inflammatory bowels disease (IBD), and in particular ulcerative colitis, showed increased local expression of IL-36.[45,46] This shared upregulation of the IL-36 cytokines would suggest that common pathomechanisms exist in the development of HS and IBD, which may at least in part explain the reported association between the two inflammatory conditions.[47]

Taken together, the agonistic isoforms IL-36α, IL-36β, and IL-36γ are upregulated in lesional HS skin, while IL-36RA does not seem to be upregulated. Future studies are required to determine the cellular and cytokinic background of HS pathogenesis and to clarify the exact role of IL-36 in this process.

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FIGURE LEGENDS

Figure 1

IL-36 cytokines are expressed in lesional HS skin

a–d) These panels show representative examples of FFPE skin sections immunohistochemically stained for IL-36α, IL-36β, IL-36γ, and IL-36RA in lesional HS and PS skin and healthy skin. Sections were counterstained with haematoxylin; 200x magnification, scale bars indicate 50µm.

e–h) Quantification of the intensity and density of IHC stainings for the IL-36 cytokines. Score ranges from 0–3 in the three donor groups; n(healthy skin) = 6, n(HS) = 6, n(PS) = 6. Data are presented as mean with SEM and were statistically analysed with the Mann-Whitney test, with * indicating a *p*-value <0.05, ** indicating a *p*-value <0.01, *** indicating a *p*-value <0.001.

Figure 2

IL-36α messenger RNA levels are elevated in lesional HS skin

a–d) Results of analyses of IL-36 cytokines mRNA expression in HS and PS compared to healthy skin. Ct-values were normalised with housekeeping genes HPRT-1 and B2M to healthy skin (= 1); n(healthy skin) = 7, n(HS) = 25, and n(PS) = 6. Data are presented as mean with SEM and were statistically analysed with the Mann-Whitney test, with * indicating a *p*-value <0.05, ** indicating a *p*-value <0.01, and *** indicating a *p*-value <0.001.

Figure 3

Systemic expression of the IL-36 cytokines in HS patients

a–d) Sandwich ELISA was performed to detect systemic IL-36α, IL-36β, IL-36γ, and IL-36RA levels in the serum of healthy donors and HS and PS patients. Data are indicated with the absolute concentration either in pg/ml (IL-36α, IL-36β, and IL-36RA) or in ng/mL (IL-36γ). Results are presented with mean and SEM; n(healthy skin) = 4, n(HS) = 5, n(PS) = 5.

TABLES

Table 1

Antibodies and standards used in the study

Primary antibodies (IHC and ELISA)	
IL-36α	AF1078, RnD
IL-36β	AF1099, RnD
IL-36γ	AF2320, RnD
IL-36Ra	AF1275, RnD
Biotinylated antibodies (ELISA)	
IL-36α	BAF1078, RnD
IL-36β	BAF1099, RnD
IL-36γ	BAF2320, RnD
ELISA standards	
IL-36α	1078-IL, RnD
IL-36β	1099-IL, RnD
IL-36γ	2320-IL, RnD