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Mast cell interleukin-1 beta, neutrophil interleukin-17 and epidermal antimicrobial proteins in the neutrophilic urticarial dermatosis in Schnitzler's syndrome

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Running head: Dermal mast cells produce IL-1 β in Schnitzler's syndrome

Key words: Schnitzler's syndrome, interleukin-1 beta, chronic urticarial rash, monoclonal component, neutrophilic urticarial dermatosis

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Bulleted statements

What's already known about this topic?

- Interleukin-1 beta (IL-1 β) is pivotal in the pathophysiology of Schnitzler's syndrome
- Dermal mast cells produce IL-1 β in the cryopyrin-associated periodic syndrome, which has clinical similarities to Schnitzler's syndrome

What does this study add?

- Dermal mast cells produce IL-1 β in Schnitzler's syndrome
- Dermal neutrophils produce IL-17 in Schnitzler's syndrome
- Antimicrobial protein expression is increased in lesional epidermis in Schnitzler's syndrome

Abstract

Background

Schnitzler's syndrome (SchS) is an autoinflammatory disease characterized by a chronic urticarial rash, a monoclonal component, and signs of systemic inflammation. Interleukin-1 beta (IL-1 β) is pivotal in the pathophysiology.

Objectives

Here we investigated the cellular source of pro-inflammatory mediators in skin of SchS patients.

Methods

Skin biopsies of lesional and non-lesional skin from eight SchS patients and healthy controls, cryopyrin-associated periodic syndrome (CAPS), delayed-pressure urticaria (DPU) and cold contact urticaria (CCU) patients were used. We studied *in vivo* IL-1 β , IL-17 and antimicrobial protein (AMP) expression in resident skin cells and infiltrating cells. In addition we investigated the *in vitro* effect of IL-1 β , IL-17 and poly:IC stimulation on cultured epidermal keratinocytes.

Results

Remarkably, we found IL-1 β -positive dermal mast cells both in lesional and non-lesional skin of SchS patients, but not in normal control skin, CCU, and fewer in CAPS. IL-17-positive neutrophils were only observed in lesional SchS and DPU skin. In lesional SchS epidermis, mRNA and protein expression levels of AMPs were strongly increased compared to non-lesional skin and that of healthy controls. When exposed to IL-1 β , poly:IC, or IL-17, patient and control primary human keratinocytes produced AMPs in similar amounts.

Conclusions

To conclude, dermal mast cells of SchS patients produce IL-1 β . This presumably leads to activation of keratinocytes and neutrophil influx, and further amplification of inflammation by IL-17 (from neutrophils and mast cells) and epidermal AMP production leading to chronic histamine-independent neutrophilic urticarial dermatosis.

Introduction

Schnitzler's syndrome (SchS) is a chronic disabling autoinflammatory disorder, characterized by chronic urticaria, a monoclonal component, fever and other signs of systemic inflammation.¹⁻³ Recent advances in our understanding of the pathophysiology of SchS have focused on systemic IL-1 β production⁴⁻⁶, based on the successful suppression of inflammation by inhibition of interleukin-1 (IL-1)^{4,5,7-14}, IL-1 β in particular.^{5,13,14} The cardinal skin features of SchS are erythematous macules or maculopapules that are often non-pruritic. Histopathologically the disease can be classified as neutrophilic urticarial dermatosis.¹⁵ SchS has clinical similarities to the cryopyrin-associated periodic syndrome (CAPS) which is caused by activating *nucleotide-binding oligomerization domain–leucine-rich repeats containing pyrin domain 3 (NLRP3)* mutations. In CAPS, dermal mast cells were identified as IL-1 β producers and inducers of neutrophil recruitment.¹⁶ We recently described myeloid-lineage-restricted *NLRP3* mutations in two patients with variant-type SchS.¹⁷

Studies of SchS skin lesions have so far mainly been confined to histopathological description. The presence of IL-1 β -positive cells in the dermis of one SchS patient was reported, without specification of the cell type.¹⁸ The epidermal compartment and the influence of IL-1 β in SchS skin lesions have not been studied to date. In several skin diseases, both infiltrating inflammatory cells and resident skin cells like keratinocytes are an important source of proinflammatory mediators. Keratinocytes secrete large amounts of cytokines and antimicrobial peptides (AMPs), many of which have immunomodulatory properties. AMPs are increased in lesional epidermis in other inflammatory skin diseases, such as psoriasis, and can be induced by several cytokines. We hypothesized that AMPs were involved in the perpetuation of inflammation in SchS skin lesions,

also because of the abundance of neutrophils, which are rich in AMPs. Taken together, this provided the rationale of the current study, which aims to analyze the expression of cytokines and AMPs in SchS skin.

Materials and methods

Patients and patient samples

Eight patients with SchS, 4 with IgM monoclonal gammopathy (classical type), and 4 with IgG (variant type), 4 patients with cold contact urticaria (CCU), 2 CAPS patients (1 *NLRP3* mutation-negative with chronic infantile neurological, cutaneous and articular (CINCA) syndrome-like phenotype including skin involvement, and 1 with an I313V *NLRP3* mutation and lack of urticaria), 1 patient with delayed pressure urticaria (DPU) and 17 healthy controls provided written informed consent after medical ethical approval was obtained. All SchS patients had been on treatment with anakinra. This treatment was stopped in the run-in phase to a clinical trial with canakinumab, as described elsewhere ⁵, and they became symptomatic within days. Skin biopsies were taken when they were symptomatic: lesional skin for mRNA and histology, and clinically uninvolved skin for mRNA, histology and keratinocyte cultures. The extent of infiltrating immune cells was rated mild (<10 cells in field of view (200x magnification)), moderate (10-50) or extensive (>50). Serum samples were collected from symptomatic SchS patients and healthy volunteers. In the CCU patients, lesional and non-lesional skin biopsies were taken 15 minutes after the application of an ice cube to the skin. DPU was inflicted by 15 minutes application of a 250-gram weight on the skin, which was biopsied after 6 hours. Non-lesional skin biopsies were taken from the two CAPS patients when they were asymptomatic during anakinra treatment.

Isolation epidermal sheets, RNA isolation and real-time quantitative PCR

Isolation of epidermal sheets for mRNA isolation, mRNA extraction, cDNA synthesis, qPCR, and normalization to the reference gene *ribosomal phosphoprotein P0* were performed as previously described.^{19,20} Primers for qPCR (Biolegio, Nijmegen, the Netherlands) had an efficiency of 100 ± 10%, and corrections were made for primer efficiency. The delta-delta Ct method was used for the calculation of the relative mRNA expression levels.²¹

Immunohistochemistry and immunofluorescence

For immunofluorescence (IF), paraffin-embedded formalin-fixed skin sections were blocked for 15 minutes with 20% normal goat serum (for IL-1 β), normal donkey serum (for IL-17, MPO, tryptase) in PBS and subsequently incubated with the antibodies rabbit anti-IL-1 β 1:50 (Abcam), goat anti-IL-17 1:500 (R&D Systems, Minneapolis, MN), mouse anti-myeloperoxidase (MPO) 1:400 (R&D), and mouse anti-tryptase 1:2000 (DAKO, Heverlee, Belgium). Skin sections for IL-1 β staining were pretreated with a citrate pH 6.0 buffer, and for IL-17 staining with a Tris/EDTA pH 9.0 buffer. The following fluorochromes were used: Alexa fluor donkey anti-mouse Fab 488 1:200 for MPO and tryptase, Alexa fluor donkey anti-goat Fab 594 1:200 for IL-17, and Alexa fluor goat anti-rabbit Fab 594 1:200 for IL-1 β . For immunohistochemistry, paraffin-embedded formalin-fixed skin sections were blocked for 15 minutes with 20% normal rabbit serum (for hBD-2 and IL-1 β) or normal goat serum (elafin, S100A7/psoriasin and S100A8/MRP8) in PBS and subsequently incubated with anti-IL-1 β 1:50 (Abcam, Cambridge, UK; stains both cleaved and uncleaved IL-1 β), anti-hBD-2 1:100 (Abcam), anti-elafin 1:500²², anti-S100A7/psoriasin 1:00000 (kind gift of Dr P. Madsen, University of Aarhus, Denmark), and anti-S100A8/MRP8 1:10000 (kind gift of Dr G. Siegenthaler, University Hospital, Geneva, Switzerland) antibodies for 1 hour at room temperature. Next, sections were incubated for 30 minutes with a secondary antibody (biotinylated rabbit anti-goat or biotinylated goat anti-rabbit in PBS containing 1% BSA, Vector laboratories, Burlingame, CA), and for 30 minutes with Avidin-Biotin complex (Vector Laboratories).

Cell culture

Primary human epidermal keratinocytes from skin biopsies of 7 SchS patients, and 6 age- and gender-matched healthy volunteers were cultured and induced to differentiate by growth factor depletion as described before.²³ At 100% confluence, differentiated cell cultures were exposed to either of the following stimuli: poly:IC 5ng/ml, IL-1 β 50ng/ml, IL-17 30ng/ml or interferon gamma (IFN- γ) 500 U/ml (Peprotech). We also performed a cross-over experiment using the stimuli 10% control serum or 10% serum from the symptomatic patient. After 24 hours the cells were harvested for mRNA isolation.

Statistical analysis

The number of IL-1 β -positive cells was counted by one blinded and one non-blinded observer, and it was analyzed by means of a Kruskal-Wallis test. A repeated-measures analysis of variance using SPSS v20.0 with LSD post-hoc testing was performed on the delta-Ct (dCt) values of the qPCR data corrected for primer efficiency.

Results

Dermal mast cells express IL-1 β in Schnitzler's syndrome

Numerous IL-1 β -positive (mature and uncleaved IL-1 β) cells were found throughout the dermis of lesional and non-lesional SchS skin, whereas fewer IL-1 β -positive cells were found in non-lesional skin of CAPS patients, and hardly any in skin of healthy controls and lesional or non-lesional skin of CCU patients (Figure 1). Overall, the presence of IL-1 β was significantly higher in all pooled SchS skin samples compared to healthy skin (p 0.001), as did the subgroups mild and moderate infiltrate (Figure 1B, p 0.008, and p 0.014, respectively). The extent of the cellular infiltrate in lesional SchS skin did not correlate with the number of IL-1 β -positive cells (statistics not shown). In SchS skin, the IL-1 β -positive cells contained granules and IF double staining showed that most of them were mast cells, and that almost all mast cells contained IL-1 β . However, in healthy skin or CCU, hardly any mast cells were IL-1 β positive, whereas in non-lesional CAPS skin, part of them were (Figure 1). Neutrophils were negative for IL-1 β (data not shown).

Dermal neutrophils and mast cells express IL-17

IL-1 β induces IL-17. Neutrophils are the dominant cells in the cellular infiltrate in SchS. Recently, it was reported that neutrophils express IL-17 in psoriatic lesional skin.²⁴ Here we found strong IL-17 expression in neutrophils in SchS urticaria as well as in DPU lesions (Figure 2). Neutrophils were absent in CCU. The remaining IL-17 positive cells in SchS and DPU skin samples were mast cells, which also expressed IL-17 in non-lesional skin and in CCU lesional and non-lesional skin (Figure 3).

Epidermal antimicrobial protein expression in SchS lesions

mRNA expression levels of *IL1B*, *IL6*, *IL8*, *IL23* and *IL33* did not differ in lesional and non-lesional SchS epidermis (N=8). However, mRNA expression levels of several AMPs were elevated in

lesional epidermis (Figure 4A), especially *DEFB4*, *PI3*, *S100A7*, *S100A8* and *S100A9*. Three symptomatic patients whose clinically seemingly uninvolved skin showed microscopic neutrophilic infiltrates, indicated that urticaria had been present on this part of their skin on the previous day; the other patients did not recall if the sampled skin site had been symptomatic. Depending on the extent of the infiltrate, these samples had similar changes in epidermal gene and protein expression levels as the overtly involved skin samples. Hence, for statistical analyses, we grouped the lesions according to the size of the infiltrate rather than the clinical appearance at the time of biopsy-taking. Validation at the protein level showed that psoriasin (*S100A7*), elafin (*PI3*) and human beta-defensin 2 (*DEFB4*) protein expression was increased in the keratinocytes, whereas MRP8 (*S100A8*) and MRP14 (*S100A9*) were only detected in neutrophils (Figure 4B; MRP14 not shown). Truly uninvolved skin (both clinically and histopathologically) resembled normal skin of healthy volunteers (Figure 4B). Such a correlation of AMP mRNA expression and neutrophil infiltrate was not seen in the DPU skin sample (data not shown). We also isolated mRNA from the dermal compartment from lesional and non-lesional skin of four patients, in which we did not detect *IL1B*, *IL-6*, and *IL-17*, but did detect *S100A8* and *S100A9* mRNA in lesional dermis.

IL-1 β , IL-17 and poly:IC induce antimicrobial proteins similarly in patient and control primary keratinocytes

Next, we studied the influence of IL-1 β and IL-17 on epidermal inflammation, and especially epidermal AMP expression, as these are implicated in the pathophysiology of several other inflammatory skin diseases with epidermal involvement, such as psoriasis.²⁴ We also used the TLR3 agonist poly:IC, which induces potent responses in keratinocytes. Moreover, to find out whether keratinocytes from SchS patients respond differently to inflammatory stimuli *in vitro*, we isolated primary human keratinocytes from 7 SchS patients and 6 age- and gender-matched healthy controls. IL-1 β , IL-17 and poly:IC induced mRNA expression levels of *DEFB4* and *S100A7*, and poly:IC induced *IL1B* mRNA in keratinocytes. There was no difference between patient and control cells. A cross-over experiment with 10% serum from either a healthy control or serum from the symptomatic patient from whom the keratinocytes were isolated did not show any differences in response between patient and control cells (Figure 5). The most important *in-vitro* finding,

however, was that AMP expression can be induced by IL-1 β and IL-17 in keratinocytes, thereby offering an explanation for the *in-vivo* findings.

Discussion

In this paper, we report the presence of IL-1 β -positive mast cells in lesional and non-lesional skin of SchS patients. In addition, we detected IL-17-positive neutrophils in the dermis and enhanced AMP expression in the epidermis of SchS skin lesions. SchS has clinical similarities to CAPS which is caused by activating *NLRP3* mutations, and in which mast cells were identified as the main source of IL-1 β in the skin.¹⁶ This is in line with the constitutive IL-1 β production found in peripheral blood mononuclear cells (PBMCs) of CAPS patients²⁵ and of our two SchS patients with myeloid-restricted *NLRP3* mosaicism.¹⁷ Mast cell degranulation is not triggered by inflammasome activation, underlining the histamine-independent nature of the CAPS (and SchS) skin lesions.¹⁶ Further, upon transplantation into mouse skin, *NLRP3*-mutant mast cells produced IL-1 β and induced neutrophil migration and vascular leakage.¹⁶ Given our *NLRP3* findings in SchS, we speculate that a low threshold for NLRP3 activation leads to an exaggerated IL-1 β response. Possibly, the lower number of IL-1 β -positive mast cells in our two CAPS patients can be accounted for by the effect of their IL-1Ra treatment when biopsies were taken, whereas the SchS patients were symptomatic.

But what drives the NLRP3 activation, as we found IL-1 β -positive mast cells in both lesional and non-lesional SchS skin? An explanation might lie in the balance between triggering factors and negative feedback mechanisms, such as microbial products and IL-1ra, respectively. Indeed, primary mast cells secrete IL-1 β in an NLRP3-dependent fashion upon stimulation with microbial constituents.¹⁶ Intriguingly, depletion of the maternal microbiota prevented skin disease in neonates of mice carrying *Nlrp3* mutations, and antibiotic treatment of these neonatal mice reduced disease severity.²⁶ Hence, microbiome constituents may inflict skin inflammation in SchS too, and might be responsible for the variation in (skin) disease severity as the skin microbiome varies greatly among healthy individuals as well as skin locations.²⁷ Moreover, PBMCs from

symptomatic SchS patients were hyperresponsive to lipopolysaccharide (LPS), resulting in enhanced IL-1 β secretion.^{6,28,29}

In a previous study, elevated IL6 and IL1B mRNA expression levels were found in full-thickness skin biopsies of CAPS patients. Protein expression of IL-1 β and IL-6 was found in dermal cells in involved skin by means of immunohistochemistry.³⁰ Here, we analyzed epidermal and dermal mRNA expression levels separately. In purified epidermal sheets from lesional and non-lesional skin of SchS patients we observed no difference in mRNA cytokine expression levels of *IL1B*, *IL6*, *IL8*, *IL23* and *IL33*. In the dermal compartment of lesional and non-lesional skin these patients we did not find *IL1B*, *IL-6*, and *IL-17*, but we detected *S100A8* and *S100A9* mRNA in lesional dermis. In view of the considerable difference in abundance of IL-1 β - and MRP8-positive cells in the dermis in lesional skin, and the relatively low mRNA yields from the dermis, we think the mRNA levels of the cytokines were below the level of detection. Another explanation could be the timing of measurement: two hours after cold exposure in the aforementioned CAPS study and at several days after disease onset in our SchS study. Interestingly, IL-1 β -positive cells (with dendritic shapes like that of mast cells) were only found in lesional skin in the CAPS patients in the study of Hofmann *et al.*³⁰, whereas we found IL-1 β -positive mast cells in both lesional and non-lesional skin of SchS patients and a few IL-1 β -positive mast cells in uninvolved skin of two CAPS patients. Possibly, the presence of (higher number of) IL-1 β -positive mast cells in non-lesional skin of SchS is another factor that distinguishes it from CAPS, but the timing of sampling during systemic disease activity in SchS might also explain this difference.

In lesional skin of SchS patients, the DPU patient and the reported psoriasis patients neutrophils were IL-17-positive, which may be true for other neutrophilic dermatoses as well. Indeed, IL-17 positive neutrophils were reported in one FCAS patient so far.³¹ IL-1 β , IL-17 and poly:IC strongly induced AMPs in SchS and controls keratinocytes. *In vivo*, only lesional SchS skin displayed strong AMP expression, which correlated with the extent of the cellular infiltrate. This suggests that neutrophil-derived IL-17 may cause epidermal activation in SchS skin lesions. AMPs have several functions, including leukocyte chemotaxis. Hence, AMP induction in SchS lesional

epidermis could lead to a vicious circle of inflammation in both the epidermal and dermal compartments.

Limitations of the study are that we used relatively small sample numbers, and for a few analyses semi-quantitative immunohistology. However, a sample size of eight patients with SchS is quite large for such a rare disease.

To conclude, dermal mast cells of SchS patients produce IL-1 β in lesional and non-lesional skin. We propose the following course of events causing cutaneous inflammation in SchS: mast cells that are hypersensitive to pathogen- or microbiome-associated molecular patterns secrete IL-1 β , which leads to recruitment of neutrophils, that in turn secrete IL-17 which induces epidermal AMP production, whereupon AMPs promote leukocyte chemotaxis. Our data suggest that IL-1 β and IL-17 may play a role in other (neutrophilic) dermatoses, and show epidermal involvement in neutrophilic urticarial dermatosis.

Conflict of interest statement. The authors state no conflict of interest.

Figure legends

Figure 1. Dermal mast cells express IL-1 β in Schnitzler's syndrome. **A.** Immunofluorescence of IL-1 β (red) and tryptase (green, mast cells) in skin sections of healthy controls, Schnitzler's syndrome patients (SchS) with or without myeloid *NLRP3* mosaicism, cold contact urticaria patients (CCU), and cryopyrin-associated periodic syndrome (CAPS) patients. Bar: 100 μ m. **B.** Number of dermal IL-1 β positive cells in healthy controls, SchS (stratified according to the extent of the cellular infiltrate: none, minimal, moderate or extensive), CCU, and CAPS, as counted per field of vision. ** p <0.01. Bars indicate median value.

Figure 2. Neutrophils in SchS and DPU skin lesions are IL-17-positive. Immunofluorescent imaging of neutrophils (MPO, myeloperoxidase, green) and IL-17 (red) of skin sections of Schnitzler's syndrome patients (SchS), cold contact urticaria patients (CCU), and one patient with delayed pressure urticaria (DPU). Bar: 100 μ m.

Figure 3. Dermal mast cells express IL-17. Immunofluorescent staining of mast cells (tryptase, green) and IL-17 (red) of skin sections of Schnitzler's syndrome patients (SchS) and cold contact urticaria patients (CCU). Bar: 100 μ m.

Figure 4. Enhanced epidermal antimicrobial protein expression in lesional skin of Schnitzler's syndrome patients. A. In purified epidermal samples from healthy controls (normal skin, NS; N=5), uninvolved and involved skin from Schnitzler's syndrome (SchS, N=8) patients, *DEFB4* (hBD-2), *PI3* (elafin), *S100A7* (psoriasin), *S100A8* (MRP8), *S100A9* (MRP14) and *CAMP* (LL-37) mRNA levels correlated with the extent of the cellular infiltrate. (The extent of infiltrating immune cells was rated mild (<10 cells in field of view (magnification 200x)), moderate (10-50) or extensive (>50). Delta-Ct method; for graphic representation of mRNA data, all data are plotted relative to one normal skin sample of *DEFB4*. * P <0.05, ** P <0.01, *** P <0.001 compared to normal skin.

B. Staining of skin biopsies from healthy controls, uninvolved and involved skin from Schnitzler's syndrome (SchS) patients revealed induction of psoriasin, elafin and human beta-defensin 2 (hBD-2) in keratinocytes, whereas myeloid-related protein 8 (MRP8) was only detected in neutrophils. True non-lesional skin (without infiltrate) resembled normal skin of healthy volunteers. Bar: 100 μ m.

Figure 5. Relative mRNA expression in primary human keratinocytes upon stimulation with inflammatory mediators. A. *DEFB4*, B. *S100A7*, and C. *IL1B* mRNA expression. Primary human keratinocytes from 7 patients and 6 healthy controls were stimulated with poly:IC 5ng/ml, IL-1 β 50ng/ml, IL-17 30ng/ml, interferon gamma (IFN- γ) 500 U/ml, 10% control serum, or 10% serum from a symptomatic patient. Delta-Ct method; relative expression compared to one control sample. * P <0.05, ** P <0.01 compared to unstimulated control. Error bars: standard error of the mean.

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