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Inflammatory cytokine expression in the skin of patients with postherpetic neuralgia

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Abstract

Objective: To assess the expression of inflammatory cytokines in the affected and normal skin of postherpetic neuralgia (PHN) patients.

Methods: Affected skin and normal skin samples were collected from PHN patients. Inflammatory cell infiltration in the dermis were evaluated by hematoxylin-eosin (HE) staining. A human inflammatory protein array containing 40 cytokines was used to assess expression differences between PHN and control skin. Enzyme linked immunosorbent assay (ELISA) kits were used to confirm cytokine expression in 10 PHN patients.

Results: HE staining showed that the epidermis of PHN skin was thicker than that of contralateral normal skin. Compared with normal skin, there was more infiltration of inflammatory cells into the dermis of PHN skin. The cytokine array detected the presence of 21/40 cytokines; however, only interleukin (IL)-1α showed differential expression between PHN skin and normal skin. ELISA results for IL-1α, IL-16, intercellular adhesion molecule-1, and monocyte chemoattractant protein-1 were consistent with those of cytokine arrays.

Conclusions: Expression of inflammatory cytokines in PHN skin was not significantly altered compared with normal skin. Chronic refractory pain in PHN is not necessarily associated with increased inflammation in the affected skin.

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Introduction

Postherpetic neuralgia (PHN), the most common and refractory sequela of herpes zoster virus infection, lasts for more than 1 month after the eruption of shingles[1] and profoundly affects quality of life.[2] The mechanisms underlying PHN are largely unknown although peripheral pathologies[3–5] and plastic changes in the central nervous system[4–8] are involved. Elucidating molecular abnormalities in patients with neuropathic pain is necessary for the development of mechanism-oriented treatments.[9] Schwann cells and nerve endings in the skin form part of a sensory organ of mechanical pain,[10] the physiological function of which may be influenced by molecular changes in the skin (e.g., expression levels of cytokines and chemokines). Studies focusing on the molecular mechanisms underlying PHN in skin may help in the development of skin-targeting topical medications or treatments.

Inflammation has been recognized as one of the characteristics of chronic pain.[11–13] However, the role of inflammation in PHN skin has not been well studied. We detected abnormal expression of microRNAs (miRNAs) and circular RNAs in PHN skin, and found that the target genes of differentially expressed miRNAs were involved in the FoxO, AMPK and MAPK pathways;[14] these pathways are associated with inflammation under pathological conditions.[15–18] To the best of our knowledge, only one study has evaluated inflammatory factors in PHN skin[19] by assessing expression of selected pro- and anti-inflammatory cytokines (tumor necrosis factor-α, interleukin [IL]-1β, IL-2, and IL-8) using PCR. The majority (11/13) of PHN patients showed no differential cytokine expression in affected skin compared with control skin.[19]

In this study, we observed increased inflammatory cell infiltration in PHN skin. Therefore, we hypothesized that higher levels of proinflammatory cytokines would be found in PHN skin compared with normal skin. A cytokine protein array was used to assess cytokine expression in PHN and normal skin.

Patients and Methods

This study was approved by the Medical Ethics Committee of the Affiliated Hospital of Zunyi Medical University ([2018]10) and registered at the Chinese Clinical Trial Registry (ChiCTR1800017821). All patients provided written informed consent.

Subjects

Patients were recruited from the Department of Pain Medicine in the Affiliated Hospital of Zunyi Medical University. Patients were diagnosed with PHN by two or more chief physicians. Pain intensity was evaluated using the numeric rating scale (NRS, range 0–10). Only PHN patients with intense pain (NRS scores $\geq$5) and pain durations of more than 1 month following healing of herpes zoster lesions were enrolled. To ensure that skin locations were identical

and comparable, only patients with PHN in the thoracic skin (T1–T8) were included.

Skin punch biopsy

Skin was sampled as previously reported.[14] Patients lay prone on an operating table. Following local anesthesia with 1% lidocaine at the lesion site and the mirror site (symmetrical site to the affected skin), skin samples were collected with biopsy punchers (OD = 2 mm). The skin samples contained the epidermis and part of the dermis, with a thickness of 1 to 2 mm.

Hematoxylin-eosin (HE) staining

Skin samples were fixed with 4% paraformaldehyde at room temperature for 24 hours. The samples were embedded in paraffin, dehydrated, then sectioned at a 5-μm thickness. The sections were stained at room temperature with hematoxylin and 0.5% eosin for 5 and 3 minutes, respectively. Tissue sections were observed under a light microscope. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to draw a fixed region of interest (ROI, 500×300 pixels) beneath the epidermis on the $100\times$ HE-stained images. To avoid counting HE-stained blue endothelial cells, inflammatory cells in the ROI were counted manually. The pathologists who quantified the images were blinded to sample information.

Human cytokine protein array

Skin samples were collected, snap frozen in liquid nitrogen and stored at −80°C before use. Samples were homogenized using a grinding rod and a Bio-Plex Cell Lysis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The homogenate was centrifuged at 16,000 ×g to remove precipitates and the supernatant containing the protein of interest was collected. The concentrations of proteins in the supernatants were determined using the bicinchoninic acid assay.[20] Proteins were stored at −80°C.

Inflammatory cytokine expression levels were evaluated using the Quantibody Human Inflammation Array-3 (RayBiotech, Peachtree Corners, GA, USA) according to the manufacturer's protocol (https://www.raybiotech.com/quantibody-human-inflammation-array-3-1-slide/). This chip permitted simultaneous detection of 40 different cytokines. Fluorescent signals were detected using a laser scanner (GenePix 4000B, Axon Instruments, San Jose, CA, USA) set at 555-nm excitation and 565-nm emission. The densities of individual spots were measured using GenePix 6.0 software (Axon Instruments) to determine the relative concentrations of cytokines in PHN skin.

Cytokine expression confirmation by enzyme-linked immunosorbent assay (ELISA)

Human IL-1α (PI565), intercellular adhesion molecule (ICAM)-1 (PI948), and monocyte chemoattractant protein (MCP)-1 (PC130) ELISA kits were purchased from Beyotime (Shanghai, China). The IL-16 ELISA kit was obtained from R&D Systems (DY316, Minneapolis, MN, USA). Cytokine concentrations were assessed according to the manufacturers' protocols using a microplate reader (VARIOSKAN FLASH, Thermo Scientific, Waltham, MA, USA). Optical density values were measured at 450 nm.

Statistical analysis

Data were expressed as means ± standard deviations. Differences between PHN and control skin were evaluated using paired t tests in GraphPad Prism v.7.0 (GraphPad Software, La Jolla, CA, USA). Values of

$P < 0.05$ were considered statistically significant.

Results

Inflammatory cell infiltration in PHN skin

Twenty-one PHN patients were enrolled in the study. Patient demographic information is shown in Table 1. Skin samples from six PHN patients were subjected to HE staining (Figure 1). The thickness (Figure 1b, d, indicated with two-way arrows) was measured and the epidermis of PHN skin was greater than that of control skin at the mirror site (Figure 1e). In addition, there were many more inflammatory cells in PHN skin compared with control skin (Figure 1f).

Analysis of cytokine expression using protein arrays in PHN and healthy skin

The cytokine chip detected expression of 21/40 (Figure 2b–g) cytokines in PHN and normal skin. Only IL-1α showed differential expression between the two types of skin (Figure 2b–g).

ELISA confirmation of cytokine expression in PHN skin

ELISA measurement of IL-α, IL-16, ICAM-1, and MCP-1 showed expression trends that were consistent with the cytokine arrays. Among these four cytokines, only IL-α showed differential expression between the two types of skin. Expression

Table 1. Demographic and clinical variables of 20 patients with PHN.

Patient No.	Age (year)	Gender	Location of lesion	Pain duration (months)	NRS score
HE staining					
1	65	M	Right dorsal T5–7	2.0	7
2	74	M	Right dorsal T5–7	1.5	8
3	54	M	Left dorsal T6–8	1.5	6
4	71	F	Right dorsal T4–6	5.0	7
5	65	M	Right dorsal T6–8	3.0	7
6	67	F	Right dorsal T3–5	6.0	6
Cytokine Array					
7	71	F	Right ventral T5–7	1.5	9
8	62	M	Left ventral 6–8	12.0	6
9	67	F	Right dorsal T4–6	4.0	7
10	68	M	Right side T5–7	2.0	7
11	74	M	Right ventral T5–7	3.0	8
ELISA Verification					
12	71	F	Right dorsal T4–6	8.0	7
13	69	M	Right side T2–4	12.0	7
14	74	M	Right ventral T5–7	4.0	7
15	64	M	Left ventral T6–8	1.0	6
16	72	F	Right side T5–7	1.0	7
17	65	M	Right ventral T5–7	24.0	7
18	74	F	Right side T5–7	1.0	9
19	64	M	Left ventral T6–8	5.0	6
20	63	F	Right paravertebral T4–6	6.0	7
21	61	F	Left side T5–7	12.0	8

PHN, postherpetic neuralgia; HE, hematoxylin-eosin; ELISA, enzyme linked immunosorbent assay; M, male; F, female; T, thoracic; NRS, numerical rating scale.

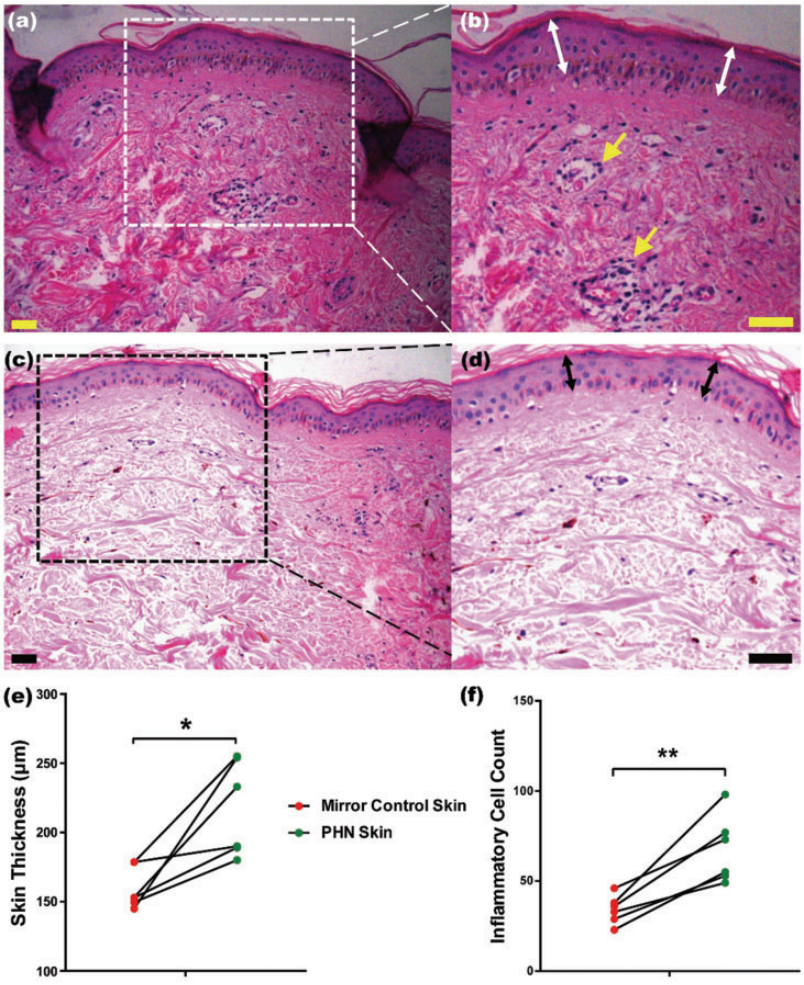

Figure 1. Evaluation of inflammation in PHN and normal skin by HE staining. Sections of PHN affected skin (a–b) and normal mirror site skin (c–d) from patient #1 in Table 1 are shown. On the affected side, the thickness of the epidermis (indicated with two-way arrows in b and d) was greater for PHN skin than for control skin (e), and there were more inflammatory cells (indicated with arrows in b) (e) in PHN skin (f). Scale bar = 200 μm. n = 6, *, $P < 0.05$; **, $P < 0.01$ by paired t tests; PHN, postherpetic neuralgia.

of IL-1α was decreased in PHN skin by ELISA, consistent with the cytokine array.

Discussion

Skin is the lesion site and the location where hyperalgesia and allodynia appear in PHN patients.[21] In this study, we assessed the expression of cytokines in PHN skin using a cytokine protein array for the first time. Twenty-one of the 40 cytokines were detected in PHN and normal skin, indicating that both PHN and healthy skin contain many cytokines. Among these 21 cytokines, only IL-1α showed differential expression in PHN skin compared with normal skin obtained from a mirror site.

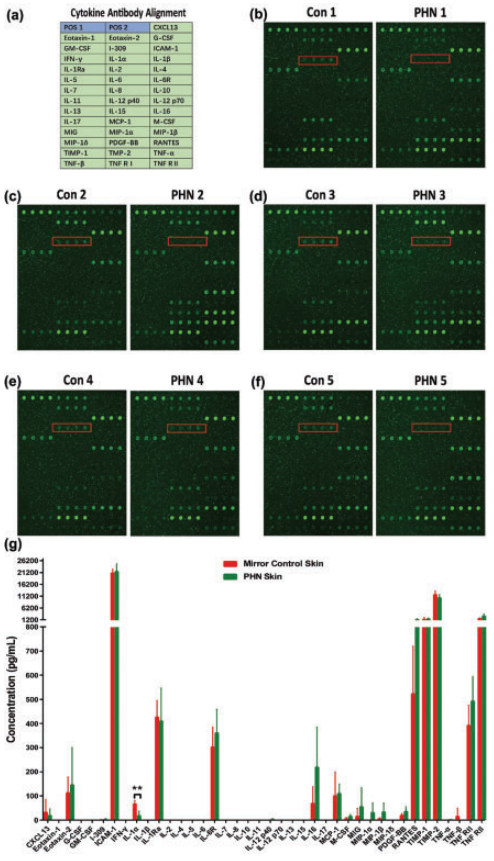

Figure 2. Cytokine expression analysis with protein arrays in PHN and control skin. (a–f) The cytokine array contained antibodies against 40 cytokines on a glass chip. (b–g) Cytokine protein arrays detected the presence of 21/40 cytokines in PHN and normal skin. However, only IL-1α (indicated with red frames in b–f) showed differential expression between the two skin types (g). n = 5, **, $P < 0.01$ by paired t test; PHN, postherpetic neuralgia; IL, interleukin.

Although HE staining showed a marked increase in inflammatory cell infiltration in PHN skin, the protein array results showed no significant changes in cytokine expression in PHN skin. The explanation for this contradiction remains to be further explored. Marked inflammatory cell infiltration in PHN skin may be related to the duration of PHN. The PHN durations of the six patients assessed in the HE staining experiment were within 6 months of one another However, in some PHN patients, neuralgia can last for many years,[5] and skin samples from patients with long PHN histories may show fewer inflammatory cells. The epidermis of PHN skin was thicker than that of normal skin. This finding could result from healing of skin lesions caused by herpes zoster, with consequent formation of scar tissue or keratinized tissue.

Interestingly, IL-1α expression was decreased in PHN skin, and this expression trend was reproduced in ELISA experiments for 10 PHN patients (Figures 2 and 3). This phenomenon is difficult to explain without further experiments. We speculate that there may be structural or molecular changes in PHN skin. For example, immunohistochemistry data showed a decreased density of nerve endings in the epidermis of PHN skin.[22–26] Considering that a variety of cells can express IL-1α,[27] and that barrier cells, such as epithelial cells, express substantial amounts of IL-1α in the steady state,[28] decreased IL-1α expression may have resulted from cellular changes (i.e., cell loss) in PHN skin. In addition, pathological molecular changes in the affected skin of PHN patients could be a reason for a decrease in IL-1α expression because mRNA and circular RNA expression was abnormal in PHN skin.[14] A mutual influence exists among these non-coding RNAs[29] and they potently regulate gene expression.[30–32] If IL-1α is one of their target genes, its expression could be regulated by differentially expressed regulatory RNAs.

Although the expression of many inflammatory factors did not change significantly (< two-fold), this does not necessarily mean that modest changes in expression (e.g. 1.5-fold) do not affect the pathophysiology of PHN. In addition, the expression of receptors for inflammatory factors may have

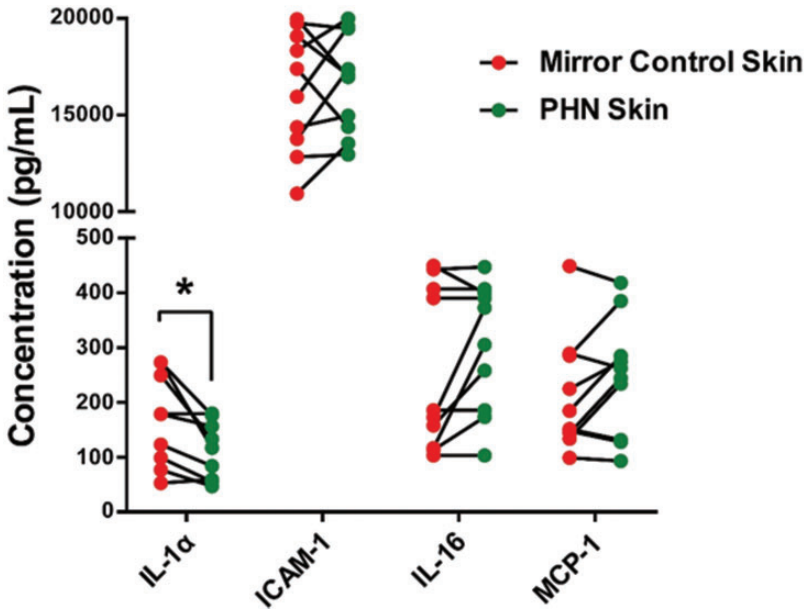

Figure 3. Cytokine expression confirmation by ELISA. IL-α, IL-16, ICAM-1, and MCP-1 showed the same expression trends as the cytokine arrays shown in Figure 2. n = 10, *, $P < 0.05$ by paired t test; ELISA, enzyme linked immunosorbent assay; IL, interleukin; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein.

changed, and eventually minor alterations of inflammatory factor expression may result in amplified effects. In the present study, we did not perform a sample size calculation because we the means and standard deviations of relevant parameters were not available. The limited number of samples may have affected the statistical significance of the results.

Overall, the results of this study showed that the expression of inflammatory cytokines in PHN skin was not significantly different than in normal skin. Chronic refractory pain in PHN is thus not necessarily associated with cytokine expression changes in affected skin.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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