ORIGINAL ARTICLE

Expression of human neutrophil proteins in acne vulgaris

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Abstract

Background In acne vulgaris patients, the presence of a dysregulation of the production of innate and specific antimicrobial peptides has been postulated.

Objective This study aims to determine whether human neutrophil proteins (HNP) 1–3 are expressed in acne patients.

Materials and methods HNP 1–3 expression was investigated in 35 acne patients treated with isotretinoin and in 25 healthy subjects. At the beginning of the study, two skin biopsies were taken from acne patients; one biopsy was taken from an established pustule and one from uninvolved skin, and the biopsies were repeated after treatment. Only one biopsy was obtained from controls.

Results The statistical analysis showed that pustular lesions of acne patients had significantly higher levels of perivascular and interstitial HNP 1–3 expression when compared with the biopsy of uninvolved skin of these patients ($P = 0.003$, $P = 0.001$, respectively) and with that of healthy controls ($P = 0.007$, $P = 0.014$, respectively). Isotretinoin treatment achieved a decrease in the perivascular and interstitial HNP 1–3 expression of pustular lesions ($P = 0.01$, $P = 0.001$, respectively).

Conclusion Our current study demonstrates the novel observation that a recently identified antimicrobial peptide, HNP 1–3, is expressed in neutrophils of acne inflammation but not in uninvolved skin of these patients. These results suggest that HNP 1–3 may contribute to the development of inflammatory lesions of acne.

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Keywords

acne inflammation, acne vulgaris, antimicrobial peptides, isotretinoin, neutrophils

Conflicts of interest

None declared.

Introduction

Acne vulgaris is one of the most common skin conditions encountered by physicians and affects approximately 85% of individuals between the ages of 12–24 years. It is a chronic inflammatory disease of the pilosebaceous unit, and although the disease has been the subject of a great deal of research, its etiopathogenesis is not completely understood. The most widely accepted etiologic factors include ductal epidermal hyperproliferation, excess sebum, inflammation, and the abnormalities of the microbial flora.[1–4] Variation in the microenvironment of the duct could also be important and is believed to influence the production and activity of inflammatory mediators.[1–3] It was also postulated that acne vulgaris patients suffer from a dysregulation of the production of innate and specific antimicrobial peptides.[5]

Defensins, a group of antimicrobial peptides of the innate mammalian immune system, protect the human mucosal epithelia and skin against microbial infections, and are produced in large amounts by neutrophils.[6–9] Defensins contain six cystein residues which form three disulfide bonds. At present, six different α-defensins and two β-defensins have been identified in humans: human neutrophil proteins 1–4 (HNP 1–4) and human defensins 5 and 6 (HD-5 and -6), and human beta defensin 1 and 2 (hBD1 and hBD2).[10] Recent studies indicate a possible role for these antimicrobial peptides' contribution to the disease evolution of the disorders of human pilosebaceous unit.[5,7,11,12] High expression of beta defensins have been found in acne lesions and these are thought to play a key role in protecting the pilosebaceous unit from microbial invasion.[5,11,12] Much less investigation have focused on the HNP expression of the skin; recently, HNP expression was detected in superficial folliculitis and T-cell lymphoma of the skin.[13,14] However, we are not aware of any study evaluating HNP expression in acne. To investigate the possible role of HNP 1–3 in acne, we examined HNP expression in inflammatory acne lesions and uninvolved skin of acne patients.

Materials and methods

Patients

Patients who visited the Acne Polyclinic of Gazi University Department of Dermatology between 2003 and 2005 with a clinical severity grade ranging from 4 to 8 according to Allen-Smith scale[15] were recruited to participate in the study. The study included 35 patients (30 female, 5 male) with acne vulgaris and a control group of 25 healthy subjects (23 female, 2 male) with no known inflammatory skin disease or any other systemic disease. The ages of acne patients ranged from 18 to 40 years in the patients' group and 19 to 40 years in the control group. Exclusion criteria were: patients receiving topical or oral antibiotics or any other anti-acne medications during the 4 weeks prior to the study; lactating patients or those with known or suspicion of pregnancy; patients with hyperlipidemia, cardiac diseases or any other systemic or cutaneous diseases; and those with history of hypersensitivity to isotretinoin.

The treatment regimen consisted of isotretinoin (Ro-accutane®, Roche Pharmaceuticals, Istanbul, Turkey), 0.5–1 mg/kg per day, according to each patient's clinical severity of acne. Isotretinoin was continued until a cumulative dose of 120–150 mg/kg/day was reached. Liver function tests and fasting lipids were evaluated at the baseline, and afterwards, monthly during the treatment. The patients were clinically examined every month until the complete clearance of all lesions.

The study was approved by the Gazi University Hospital Local Ethics Committee. The study and its method were explained verbally and in writing to each prospective patient, and informed written consent was obtained from each subject.

Skin biopsies

At the beginning of the study, two punch (3-mm) skin biopsies in acne areas (back or shoulder region) were obtained from each patient, one from an established pustular lesion and one from uninvolved skin of the same patient. After the complete clearance of the lesions, repeated biopsies were taken from the identical region of the skin that was biopsied for the original lesion. Only one biopsy from the upper back region was obtained from healthy controls. These specimens were reserved for immunohistochemical investigation.

Immunohistochemical staining

Sections (4 μm) were cut from the specimens and placed on poly-L-lysine coated slides. The sections were dewaxed in a microwave oven (56 °C) for 12 h. These were then dewaxed in xylene for 30 min, and rehydrated in graded alcohol. The endogenous peroxidase was consumed by immersing the sections for 3 min in hydrogen peroxide (3%) in absolute methanol, and then incubated in phosphate-buffered saline (PBS). The sections were again incubated in nonimmune blocking solution. The primary antibody solutions (HNP 1–3, clone: D21, Hycult Biotechnology) were applied for 2 h at room temperature. The sections were then incubated with a secondary biotinylated antirabbit antibody solution (secondary antibody, DAKO, High Wycombe, UK) for 2 h and with the avidin-biotin complex for 30 min. The colour was developed with amino ethyl carbazol (AEC), and the slides were lightly counterstained with haematoxylin.

The immunohistochemical assessment was performed by counting HNP-positive cells in periadnexial, interstitial, and perivascular regions in randomly selected, four consecutive epidermal field (magnification ×400), and the results were evaluated for the number of positive cells as the percentage of the total number of cells. All histological slides were reviewed and neutrophil counts were also assessed with the same method in haematoxylin- and eosin-stained sections by the same pathologist, without knowledge of the patient groups and HNP results.

Statistical analysis

Statistical Programme for Social Sciences 11.0 (SPSS 11.0) was used for statistical analysis. The expression of HNP positivity was expressed as mean ± standard deviation (SD). Sex distribution of two groups was compared using chi-squared test. The amount of HNP expression of the three groups was compared using Kruskal–Wallis test; Mann–Whitney *U*-test was used to compare groups two by two. Wilcoxon test was used for comparison of pre-treatment and post-treatment results for case group. Statistical significance was defined as $P < 0.05$. Kendall's correlation coefficient was calculated to evaluate the relation between the neutrophil count and HNP expression.

Results

Age and sex

The study included 35 patients (30 female, 5 male) with acne vulgaris and a control group of 25 healthy subjects (23 female, 2 male). The ages of acne patients ranged from 18 to 40 years (mean ± SD: 26.8 ± 5.4) in the patients' group and 19 to 40 years (mean ± SD: 27.2 ± 5.7) in the control group. There was no difference between the groups according to age and sex analysis ($P > 0.05$ each).

Isotretinoin treatment response

All patients completed the study. Baseline evaluation revealed that 10 (28.6%) acne patients had grade 4; 12 (37.1%) had grade 6 and 13 (34.3%) had grade 8 acne. Patients have used 0.5 to 1 mg/kg/day of isotretinoin with a mean cumulative isotretinoin dose of 129.2 ± 5.8 mg/kg (120–143 mg/kg). This therapy produced complete resolution of skin lesions in 94.2% [33 (94.2%) patients had grade 0, 1 (2.8%) patient had grade 2 and 1 (2.8%) patient had grade 4 acne] of the patients. Four of 35 (11.4%) patients experienced mild elevation of fasting lipids and another two (5.7%) experienced mild elevations in liver transaminases, but these factors did not necessitate withdrawal from the therapy and

Table 1 Comparison of the amount of HNP positivity (%) in pustular lesions and uninvolved skin of acne vulgaris patients with those of control group

HNP expression (%) (mean ± SD)	Acne patients ($n = 35$) Pustular lesion	Uninvolved skin	*P*	Control group ($n = 25$)	*P*
Perivascular	7.6 ± 1.2	–	0.003‡	–	0.007†
Interstitial	5.4 ± 4.3	–	0.001‡	–	0.014†

†Kruskal–Wallis test. ‡Mann–Whitney *U*-test.

levels returned to normal during the follow-up. Most of the patients complained of mucosal dryness during the therapy.

HNP 1–3 expression

Baseline: As shown in the Table 1 and Fig. 1, immunohistochemical staining of the sections showed the presence of perivascular and interstitial expression of HNP 1–3 in 43.3% of pustular acne lesions. In pustular acne lesions, the mean of percentage of immunopositive neutrophils was 7.6 ± 1.2% for perivascular region and was 5.4 ± 4.3% for the interstitial area. This staining was not observed in the uninvolved skin of acne patients or in the healthy control subjects (Fig. 2).

Neutrophils were found in 45% of the pustular acne lesions and their numbers were 8.2 ± 2.3% for perivascular region and 6.8 ± 4.8% for interstitial area. Kendall's correlation coefficient between the amount of neutrophils and the immunoreactivity of HNP were 0.924 ($P < 0.0001$) and 0.946 ($P < 0.0001$), respectively. Since this is on the standard scale for correlation, the observed value shows very high agreement among the amount of neutrophils and the expression of HNP. The mean of percentage of neutrophils in the uninvolved skin of acne patients was 0.1 ± 0.4% for perivascular and was 0 ± 0.2% for interstitial areas. Neutrophils were not found in the healthy control subjects.

The statistical analysis showed that pustular lesions of acne patients had significantly higher levels of perivascular and interstitial HNP 1–3 expression when compared with that of uninvolved skin of these patients ($P = 0.003$, $P = 0.001$, respectively) or with that of healthy controls ($P = 0.007$, $P = 0.014$, respectively). However, regarding HNP 1–3 expression, uninvolved skin of acne patients had no difference from that of healthy controls.

Post-treatment: Post-treatment measurements demonstrated that the amounts of HNP 1–3 expression of neutrophils of the acne inflammation in perivascular and interstitial areas (0.5 ± 0.2%, 0.3 ± 0.6%, respectively) were significantly lower than those of pre-treatment measurements [7.6 ± 1.2% and 5.4 ± 4.3%, respectively ($P = 0.01$, $P = 0.001$, respectively)]. This difference was not observed when we compared post-treatment HNP 1–3 expression levels in pustular lesions to the skin of healthy controls (Table 2).

The mean of percentage of neutrophils of the acne inflammation in perivascular and interstitial areas in treated acne patients were 0.8 ± 0.5%, 0.6 ± 0.4%, respectively. Kendall's correlation coefficient between the amount of neutrophils and the immunoreactivity of HNP were 0.879 ($P < 0.0001$) and 0.919 ($P < 0.0001$), respectively.

Table 2 Effect of isotretinoin treatment on HNP positivity (%) in pustular lesions and in uninvolved skin of acne vulgaris patients

	HNP expression (%) (mean ± SD) Baseline	Post-treatment	*P*
Pustular lesion			
Perivascular	7.6 ± 1.2	0.5 ± 0.2	0.01†
Interstitial	5.4 ± 4.3	0.3 ± 0.6	0.001†
Uninvolved skin			
Perivascular	–	–	‡
Interstitial	–	–	‡

†Wilcoxon test.
‡Statistical analysis was not performed since HNP expression was not observed in any of the groups.

Discussion

Our current study demonstrates the novel observation that a recently identified antimicrobial peptide, HNP 1–3, which has antimicrobial activity and immunological properties, is expressed in perivascular and interstitial neutrophils of acne inflammation, but not in the uninvolved skin of these patients.

HNP 1–3 compromise 30% to 50% of the total protein in azurophilic granules of human neutrophils.[6–8,16] Although the relationship between neutrophils and their products and the development of acne inflammation has repeatedly been documented over the past years,[17–22] the understanding of their role in lesion evolution has been hindered by their presence in lower numbers in the acne inflammation. The sequence of events in the pathogenesis of acne vulgaris has become an important focus of both clinical and basic research. Kligman[17] proposed that neutrophils were the initial cells that infiltrated acne lesions. However, many studies have documented the immunohistochemical evaluation of evolving inflammation in lesions of acne vulgaris and some of the details of acne inflammation have been defined. Norris and Cunliffe[18] and Layton *et al.*[19] have shown that the initial infiltration of the developing inflammatory lesions consisted of mononuclear cells that were predominantly CD4 + T cells and that

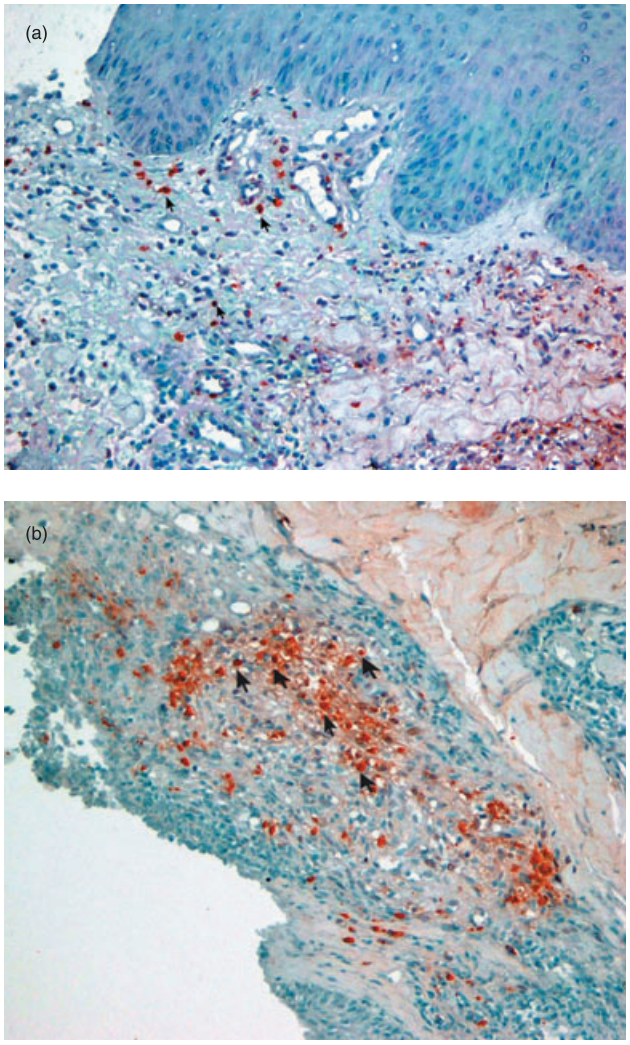

Figure 1 Immunohistochemistry of skin biopsies from acne patients with pustular lesions. Immunoreactivity of human neutrophilic peptides (HNP)-1–3 was observed in the polymorphonuclear (PMN) leucocytes that infiltrated the superficial dermis and pustules (AEC, ×400). HNP positive neutrophils (as shown by arrows) in (a) perivascular and interstitial sites (AEC, ×400) and in (b) folliculitis region (AEC, ×400).

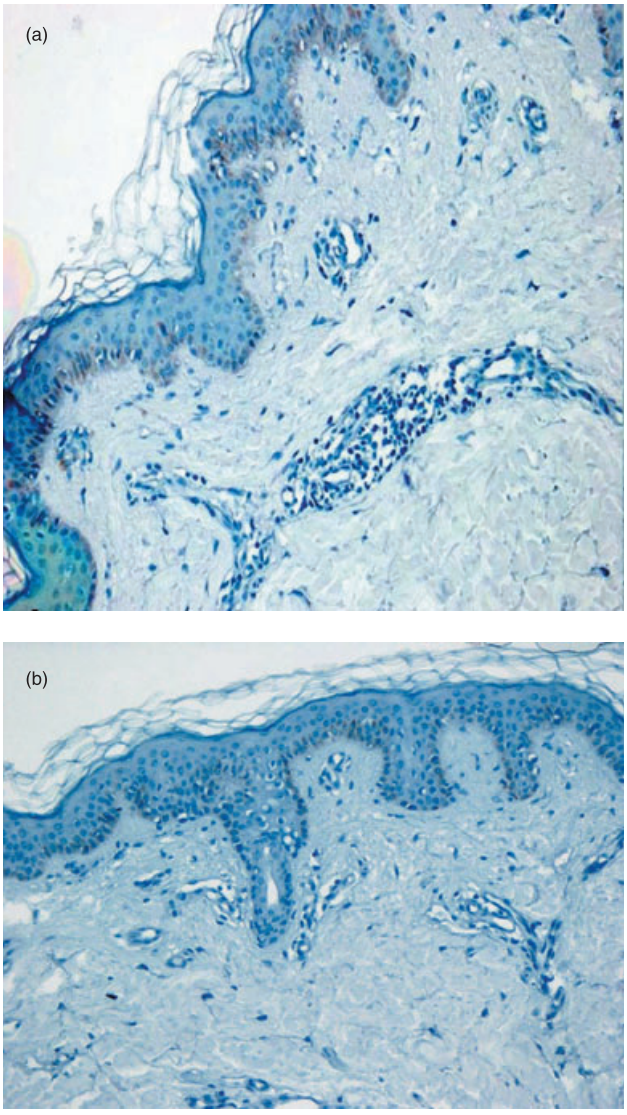

Figure 2 Immunohistochemistry of skin biopsies from (a) control patients (AEC, ×200) (b) uninvolved skin of acne patients (AEC, ×200).

neutrophils appeared later in the course of inflammation. Norris and Cunliffe[18] found neutrophils in 33% of lesions of 72 h duration. The current study also confirmed that neutrophils are found in lower amounts in inflammatory acne lesions.

Our results provide evidence that human neutrophils in inflammatory acne are expressing HNP 1–3. In the augmentation of inflammation of acne, activated monocytes stimulate neutrophils to produce various cytokines.[1] Interleukin (IL)-8, a potent T-cell chemoattractant and activator of neutrophils, is produced in many cell types including monocytes and endothelial cells and is thought to have a role in acne inflammation.[1] It was also shown that the release of proteins including HNP 1–3 from neutrophils can be triggered by proinflammatory mediators, such as IL-8.[1,20–23] Taken together, these findings suggest that HNP 1–3 expression may also occur during this process, and that HNP may have a role in acne pathogenesis.

Although the mechanisms involved have yet to be fully elucidated, *Propionibacterium acnes* appears to play an important role in the pathogenesis of acne inflammation.[1–3] In the augmentation of inflammation of acne, *P. acnes* induces the production of chemotactic factors and reactive oxygen species by human neutrophils[20–26] and

stimulates cells of the non-specific immune system to produce proinflammatory cytokines such as tumor necrosis factor-α, IL-1β, and IL-8.[1] It is interesting to note that among various kinds of these cytokines, at least IL-8 has the ability to trigger proteins including HNP 1–3 from neutrophils.[1,20–23] In addition, recent studies have shown that *P. acnes* triggers antimicrobial peptide and cytokine secretion of keratinocytes via Toll-like receptors (TLR) 2 and 4,[27] and the treatment of SZ95 sebocytes with *P. acnes* induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines.[14] Although it is not known if this holds true for acne patients, a similar interaction may exist between *P. acnes* and neutrophils.

Taken together, we propose that the reason for antimicrobial molecules such as HNP 1–3 being expressed in acne vulgaris lesion may be due to the induction of neutrophils either by monocytes or *P. acnes* production during the initial inflammatory response. At present, however, it is difficult to predict the exact reason of HNP expression in acne vulgaris without further experimentation. The detailed mechanisms of the sequence of these events will be clarified when the functions and the effects of these peptides are totally understood. On the other hand, control subjects have not shown a positive staining of HNP 1–3, and treatment of acne lesions with isotretinoin provided a significant decrease in the expression of HNP 1–3. Therefore, our findings provide strong evidence that HNP 1–3 may contribute to the development of the inflammatory lesions of acne.

Current knowledge of immunopathogenesis of acne vulgaris fails to provide clues as to the underlying mechanisms for the expression of HNP in acne lesions. At present, with the findings of our study, we can only speculate the mechanism by which these antimicrobial peptides may be involved in the pathogenesis of acne vulgaris. The relationship between antimicrobial peptides and skin diseases has been investigated in many studies,[13,16,28] and their role in linking innate and adaptive immunity has been proposed.[29,30] In addition to their antimicrobial effects, neutrophil α-defensins induce selective chemotaxis of CD45RA/CD4 T cells, CD8 T cells, and immature dendritic cells at nano-molar concentrations.[13,24,31,32] By this way, as postulated previously by Chertov *et al*.[23] HNP may contribute or provide acute inflammatory reactions to develop into immunologically mediated chronic inflammatory responses dominated by mononuclear cells. HNP may also contribute to the development of specific immune response by inducing the migration of immature dendritic cells toward the inflammatory sites or facilitating the recruitment of T cells to the inflammatory sites or regulating the activation of the classical complementary pathway.[31] This issue deserves further attention because the findings of many studies achieved a significant amount of evidence that acne develops as a consequence of specific immune responses, particularly a type IV delayed hypersensitivity reaction.[3,30] Altogether, these data plus our findings suggest that HNP may have a role in the development of specific immune response in acne inflammation.

In summary, this is the first study to demonstrate the presence of HNP 1–3 in the neutrophils of acne vulgaris patients. HNP expression was not observed in uninvolved skin of acne patients and healthy controls in the current study. In addition, treated acne patients showed a significant decrease in the expression of HNP 1–3 in accordance with a decrease in the inflammatory activity of the acne lesions. From this point of view, as neutrophils took a later part in inflammation of acne lesions, it may be possible that regulation of HNP expression may also participate in the resolution of the inflammation in acne lesions. Isotretinoin remains the most effective drug for acne treatment. Although the mechanism has not been completely elucidated, isotretinoin decreases inflammation in acne. Isotretinoin inhibits *P. acnes* growth by changing the follicular milieu,[33] and possibly has the ability to regulate TLR2 expression and activation which may contribute to its anti-inflammatory effect;[34] however, in the absence of further studies, it would not be rational to build a link between anti-inflammatory effect of isotretinoin and HNP expression. In our study, there was an almost complete concordance between the neutrophil counts and the amount of HNP expression; therefore, it is currently unknown whether HNP1–3 is a specific marker involved in acne inflammation, mediating a specific immune response or merely reflects the presence of neutrophils. Additional research on acne population will likely shed more light on these issues, as will studies in other neutrophilic disorders once the exact functions of these antimicrobial peptides are identified.

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