Th17-Associated Cytokine Profile in Cutaneous Lesion of Behçet's Disease

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This certifies that the Master's Thesis of **Kyu-Yeop Lee** is approved.

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<ABSTRACT>

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Behçet's disease (BD) is a multisystemic chronic inflammatory disease with various cutaneous lesions. Studies on the cytokine profiles in BD have been performed extensively to clarify the pathogenesis of BD. Interleukin (IL)-17 is exclusively produced by a newly identified CD4+ T-helper subset that was recently named Th17. The IL-17 plays a key role in the regulation of immune and inflammatory response and the progression of autoimmune diseases. Th17 cells essentially require both TGF-β and IL-6 for differentiation from naive CD4+ T cells. IL-17 and Th17 are recently suggested as a new alternative proposal in the pathogenesis of the diseases with immunologic background which is not prejudiced to Th1 or Th2. Therefore, we checked the level of IL-17 and cytokines related to Th17 differentiation in the skin lesions of BD to study the role of IL-17 and Th17 in cutaneous lesions of BD.

In this study, immunohistochemical staining of skin sections revealed increased IL-6 expression and TGF-β1 expression in dermis and subcutaneous layer of BD. However, no statistically significant difference was observed in IL-17 expression between skin lesions of BD and normal control skins. Therefore, this study suggests that localized pathogenic mechanism in the erythema nodosum-like lesions of active BD may be related with IL-6 related immune process rather than IL-17 secreted from Th17-associated immune reaction.

Key words: Behçet's disease, IL-6, IL-17, TGF-β, Th17

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I. INTRODUCTION

The histopathogenesis of Behçet's disease (BD) is regarded as chronic recurrent inflammation which involves multiple organs with various combinations of mucocutaneous, ocular, musculoskeletal, cardiovascular, respiratory, gastrointestinal, and neurologic involvements[1-4]. Dermatologic symptoms of BD can be appeared as erythema nodosum-like skin lesions, folliculitis, pyoderma gangrenosum, erythema multriforme and thrombophlebitis[5].

CD4+ effector T cell have been categorized into two subsets: T helper type 1 (Th1) and Th2. Recently, another subset of T cells that produce interleukin (IL)-17 has been identified and termed Th17 that is highly proinflammatory and induces severe autoimmunity. Interleukin (IL)-17 is a 30- to 35-kDa homodimeric polypeptide cytokine cloned in 1993 and originally named

cytotoxic T lymphocyte-associated antigen-8 (CTLA-8) and IL-17 acts *in vitro* and *in vivo* as a potent inflammatory cytokine[6]. It has pleiotropic activities, one of which is to coordinate tissue infiltration by inducing the expression of pro-inflammatory cytokines (such as IL-6 and TNF), chemokines and matrix metalloprotease, which mediate tissue destruction[6]. IL-17 is also involved in the proliferation, maturation and chemotaxis of neutrophils[7]. Th17 cells which produce IL-17 and induce inflammatory reaction require both TGF-β and IL-6 for differentiation from naive CD4+ T cells[8,9]. IL-23 is dispensable for this function, but necessary for previously differentiated Th17 cells' expansion and survival[8,9]. Expression of IL-17 has been detected in sera and target tissues of patients with various diseases, including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematous, Kawasaki disease, familial Meditteriean fever and asthma[10-15].

The analysis of cytokine mRNA expression within BD mucocutaneous lesions showed a high increase in the expression of Th1 cytokines and the absence of Th2 cytokines, suggesting that BD skin lesions are probably related to Th1-mediated immunity[16]. However, the cytokine profile studies of serum of BD patients had showed no consistency, although changes of the cytokine levels in the serum of BD patients were extensively investigated by different research groups with different methods[16-30] (Table 1). For example, Frasasnito et al. suggested that Th1-type immune response has major pathogenetic role in BD with investigation of serum cytokine levels and

cytokine production by peripheral blood lymphocytes[18], however, divergent cytokine production profile of peripheral blood mononuclear cells from BD patients of increased secretion of IL-4, IL-10, and IL-13 (Th2 cell response), almost normal secretion of IL-2, but minimal or deficient secretion of interferon(IFN)-γ and IL-12 (Th1 cell response) was also reported[19]. Increased IL-17 was also reported in the serum of the active BD patients[17], however, IL-17 concentration of the skin lesions of BD has never been measured in the study until now.

IL-17 and Th17 are recently suggested as a key role in the regulation of immune and inflammatory response, especially the diseases of which clinical course shows spontaneous remissions and relapses similar to those of various autoimmune disease and the indicators of immune response is not prejudiced to Th1 or Th2. Therefore, we checked the changes of IL-17, IL-6, TGF-β in BD skin lesions with immunohistochemical staining to study cytokine profile of Th17 in cutaneous lesions of BD and compared them with psoriasis and normal skin tissues.

Table 1. Reported cytokine change in Behçet's disease

Cytokine	Skin Lesion	Serum	Reference
Th1 cytokine			
IL-2		+	[18,24,26]
IL-12	+*	+, ±, -	[17,18,23,24,25,28]
IFN-γ	+	+, -	[16,17,18,24,25,27]
TNF-α	+	+	[19,22,23,26,27]
Th2 cytokine			
IL-4	±	+	[16,17,18,24,25]
IL-10	+	+	[16,17,24,25]
IL-13	±	+	[16,24,25]
IL-6		+	[17,19,23,26]
IL-8	+	+	[16,19,20,21]
IL-17		+	[17]
IL-18		+	[17,24]
IL-23	+¶	±	[28]
TGF-β		±	[29,30]

+, increased in Behçet's disease (BD) or active BD; ±, no change; -, decreased

*, IL-12 p40

¶, IL-23 p19

II. MATERIALS

For immunohistochemical staining of skin lesions, we studied 5 patients with BD fulfilling the criteria of the International Study Group for BD[28]. Two patients were male, and three patients were female. All patients had clinically active disease at time of biopsy (more than two clinical manifestations of BD) and presented with erythema nodosum-like skin lesions without treatment for at least for four weeks. Biopsy specimens were obtained from erythema nodosum-like skin lesions 2 to 5 days after their appearance, with a 4-mm skin biopsy punch after administration of local anesthesia (lidocaine with epinephrine). Specimens were immediately fixed in 10% buffered formalin, dehydrated in ethanol, and embedded in parffin. Biopsy specimens of control groups were obtained from five healthy volunteers and five psoriasis patients with same procedure.

III. METHODS

1. Immunohistochemical staining

Sections were cut from 15 paraffin blocks from 5 BD patients, 5 psoriasis patients, and 5 normal controls for immunohistochemistry. Commercially available purified monoclonal anti-human IL-6 antibody (R&D systems, Minneapolis, Minnesota, U.S.A.), purified monoclonal anti-human TGF-β1 antibody (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.), purified rabbit polyclonal anti-human IL-17 (p19) antibody (Santa Cruz Biotechnology), and Histostain-DS kit (Zymed Laboratories, South San Francisco, California, U.S.A.) were used in immunohistochemical staining. The detailed procedures are as follows. Prior to staining, tissue slides were de-paraffinized with xylene and re-hydrated in a graded series of ethanol to remove embedding media. Then tissue sections were pre-treated by boiling in citrate buffer (pH 6.1) in a microwave (1 minute with high power and 9 minutes with medium power) and washed and cooled in a phosphate buffered saline (PBS) bath for 10 minute. After washing, non-specific binding was blocked with serum blocking solution of Histostain-DS kit for 10 minutes. Then, tissue sections were incubated with anti-human IL-6 antibodies for 12 hours at 4℃ in a humidified chamber. After incubation with the primary antibody, sections were rinsed with PBS containing 0.05% Tween-20 for 2 minutes, 3 times, and treated with biotin conjugated secondary antibody with

10 minutes incubation. After incubation with secondary antibody, sections were rinsed PBS containing 0.05% Tween-20 for 2 minutes, 3 times, reacted with alkaline phosphate conjugate with 10 minutes incubation and rinsed in the same way. Then we applied substrate-chromogen mixture to each section, incubated 10 minutes and rinsed with distilled water containing 0.05% Tween-20 and stained with hematoxylin for counter-staining. Finally specimens were dehydrated with gradient alcohol and xylene and mounted with permanent mounting medium.

Immunohistochemical staining with anti-human TGF-β1 antibody and anti-human IL-17 antibody were performed in the same way.

2. Scoring of staining intensity

Two independent investigators independently reviewed all of the sections without knowledge of which antibody they were scoring. Discrepancies in estimations were reconciled by a concurrent review using a multi-headed microscope. The expression of the IL-6, TGF-β1, and IL-17 were blindly assessed and scored separately in the basal and suprabasal epidermis, papillary and reticular dermis and subcutaneous fat tissue. The quantity of staining was evaluated using the following arbitrary units: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The score was adjusted for each part of the skin, adding scores of each investigator, so that score 6 referred to the maximum within all specimens. Epidermal expression was

defined as the sum of basal and suprabasal scores, whereas dermal expression was the sum of papillary and reticular dermal scores. Score of the subcutaneous expression was doubled instead of division to two portion.

3. Statistical analysis

Semiquantitative measures of IL-6, IL-17, and TGF-β1 immunohiscochmical stainings were evaluated statistically. Differences in arbitrary scores among BD group, psoriasis group and normal group were compared with ANOVA test.

IV. RESULTS

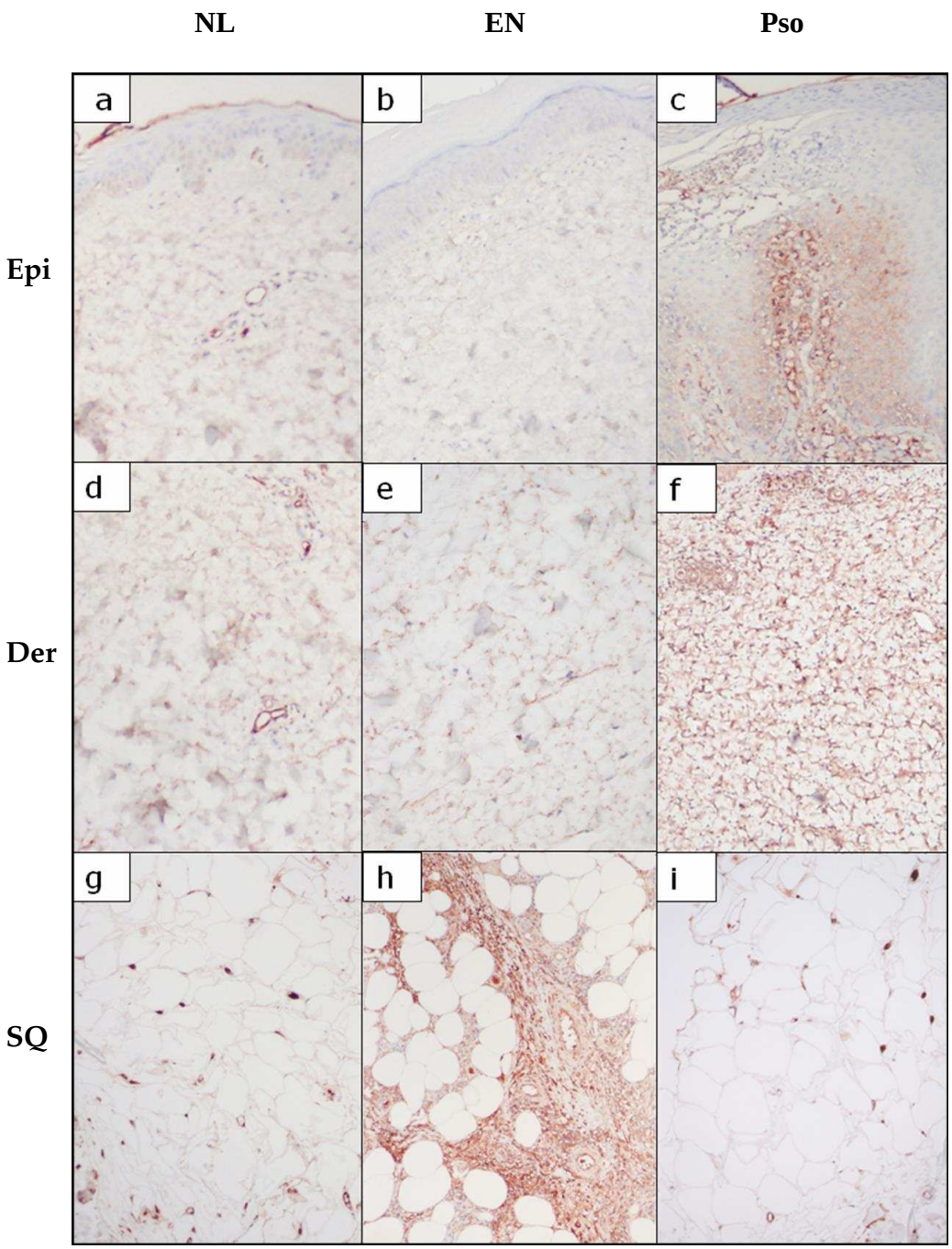
1. Immunohistochemical staining with anti-IL-6 antibody

To confirm the expression and localization of IL-6, we stained normal (n=5), erythema nodosum-like BD lesional (n=5), and psoriatic lesional (n=5) skin paraffin embedded sections with the anti-human IL-6 antibody. The expression of IL-6 in the epidermis of skin was strong in psoriasis, especially lower portion. However, epidermis of erythema nodosum-like BD lesional skin did not show significant difference from normal human skin (Fig. 1A. a, b, c). In papillary dermis, perivascular cells and inflammatory cells expressed this molecule in psoriatic lesional skin (Fig. 1A. c, f). In contrast, in normal human skin and in erythema nodosum-like BD lesional skin, IL-6 expression in the dermis was present in only a limited number of cells (Fig. 1A. d, e). In the subcutaneous tissue, septal infiltrating inflammatory cells showed strong IL-6 expression in erythema nodosum-like lesion (BD) (Fig. 1A. h). However, normal and psoriatic human skin showed no IL-6 expression in the subcutaneous tissue with scant inflammatory cellular infiltration (Fig. 1A. g, i).

The expression of IL-6 in normal, erythema nodosum-like BD lesion, and psoriatic lesion was scored in arbitrary units and was summarized in Fig. 1B. The expression of IL-6 in the dermis did not differ significantly among three

groups. However, the expression of IL-6 in the epidermis was significantly stronger in psoriatic lesional skin compared with normal controls and erythema nodosum-like lesion of BD ($p<0.01$). The expression of IL-6 in the subcutaneous tissue was stronger in erythema nodosum-like lesion of BD compared with normal controls and psoriatic lesion ($p<0.01$).

(A)



(B)

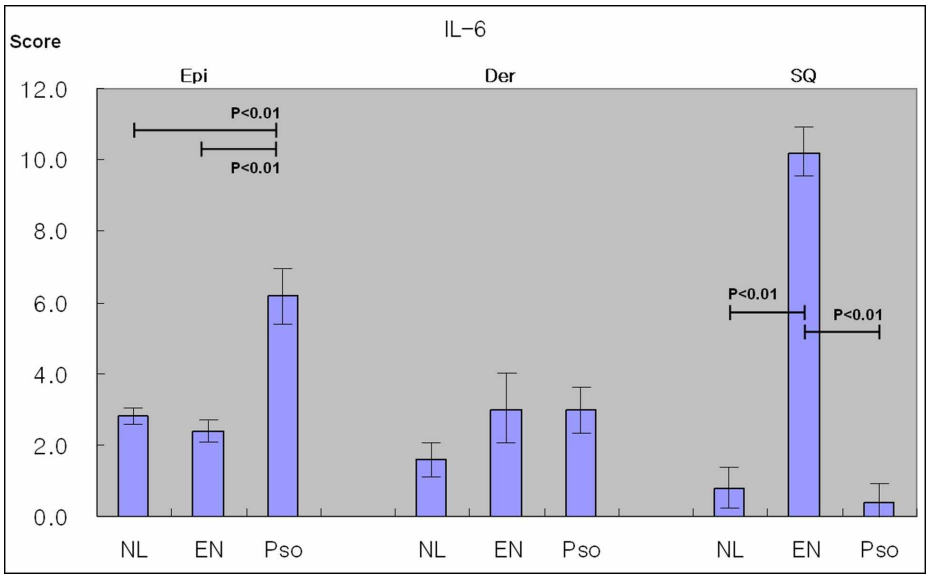


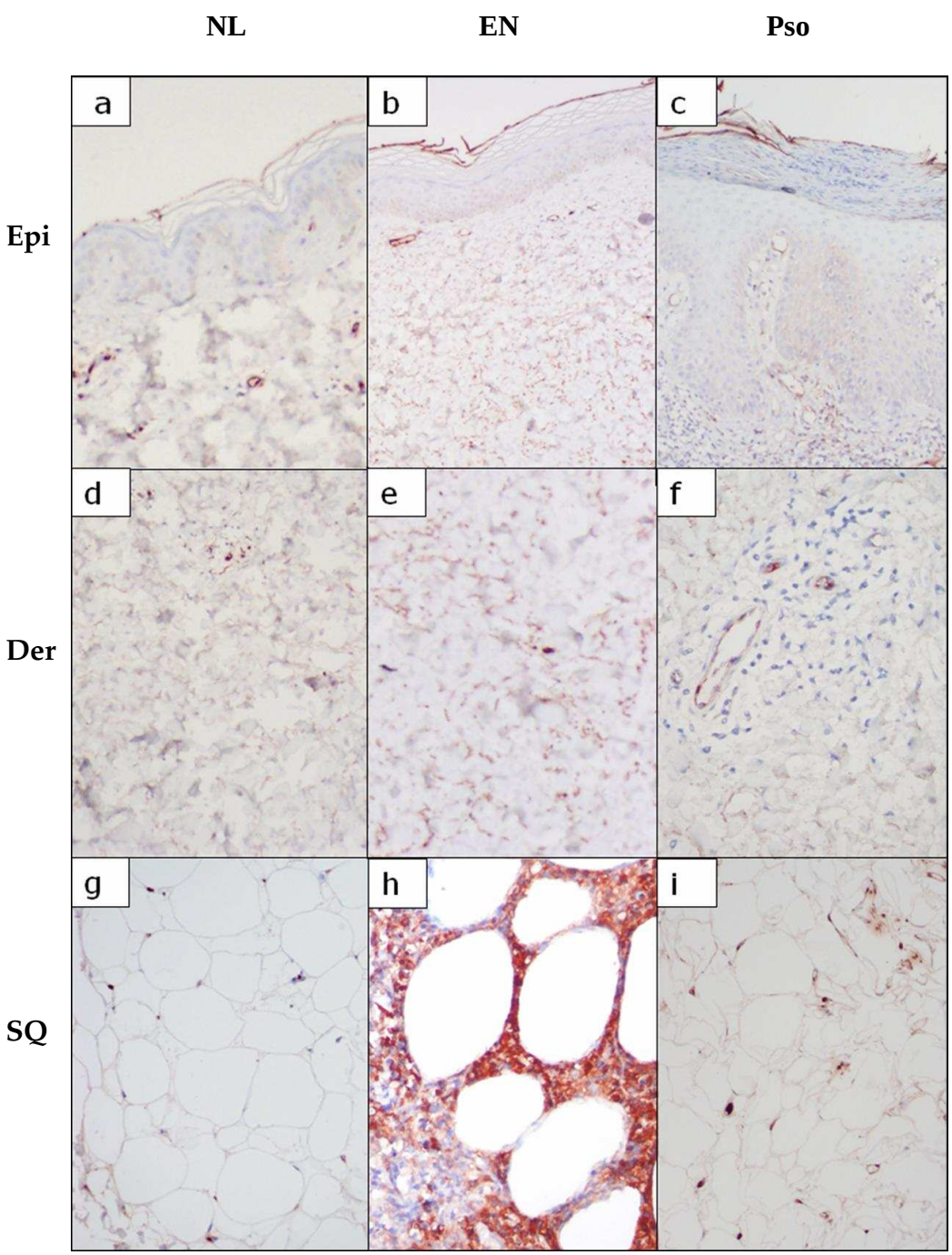
Figure 1. (A) Immunohistochemical staining with anti-human IL-6 antibody. (original magnification, ×100). **(B)** Statistical analysis of immunohistochemical staining shows significantly higher expression of epidermis of psoriatic lesional skin and subcutaneous layer of erythema nodosum-like skin lesion of Behçet's disease. Values are mean ± SD. Abbreviations: NL, normal skin; EN, erythema nodosum-like lesion BD; Pso, psoriatic lesion; Epi, epidermis; Der, dermis; SQ, subcutaneous fat.

2. Immunohistochemical staining with anti-TGF-β1 antibody

To confirm the expression and localization of TGF-β1, we stained normal (n=5), erythema nodosum-like BD lesional (n=5), and psoriatic lesional (n=5) skin paraffin embedded sections with the anti-human TGF-β1 antibody. TGF-β1 expression in the epidermis of the psoriatic lesional skin was slightly strong compared with other two groups (Fig. 2A. a, b, c). TGF-β1 expression in the dermis of all three groups was scant (Fig. 2A. d, e, f). In the subcutaneous tissue, septal infiltrating inflammatory cells showed strong TGF-β1 expression in erythema nodosum-like BD lesion (Fig. 2A. h). However, normal and psoriatic human skin in which there were scant inflammatory cellular infiltration showed no TGF-β1 expression in the subcutaneous tissue (Fig. 2A. g, i).

The expression of TGF-β1 in normal, erythema nodosum-like BD lesion, and psoriatic lesion was scored in arbitrary unit and was summarized in Fig. 2B. The expression of TGF-β1 in the epidermis and in the dermis of psoriatic lesional skin is slightly stronger than normal human skin and erythema nodosum-like BD lesion, however, there is no statistical significance. The expression of TGF-β1 in the subcutaneous fat layer was stronger in erythema nodosum-like BD lesion compared with normal control and psoriatic lesion ($p<0.01$).

(A)



(B)

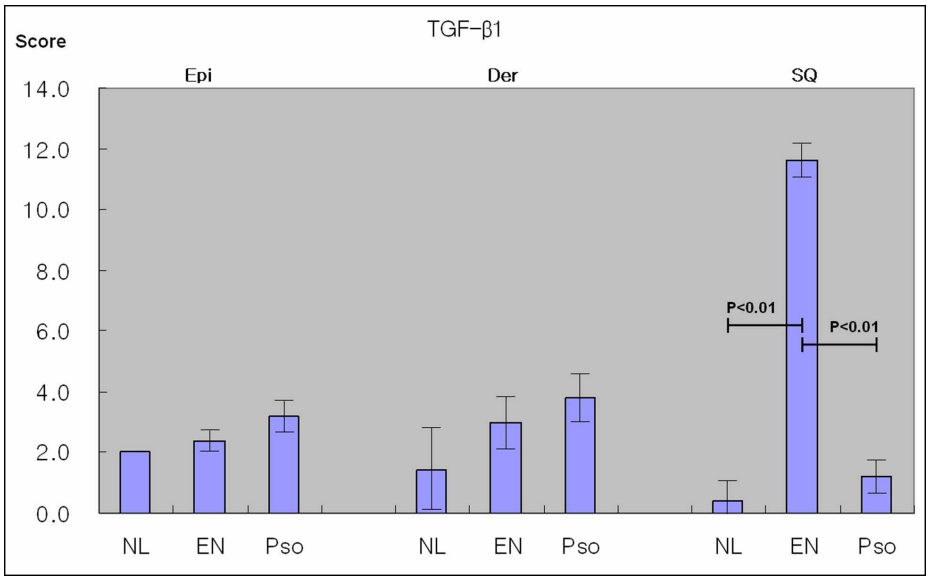


Figure 2. (A) Immunohistochemical staining with anti-human TGF-β1 antibody (original magnification, ×100). (B) Statistical analysis of immunohistochemical staining shows significantly higher expression of subcutaneous layer of erythema nodosum-like skin lesion of Behçet's disease. Values are mean ± SD. Abbreviations: NL, normal skin; EN, erythema nodosum-like lesion BD; Pso, psoriatic lesion; Epi, epidermis; Der, dermis; SQ, subcutaneous fat.

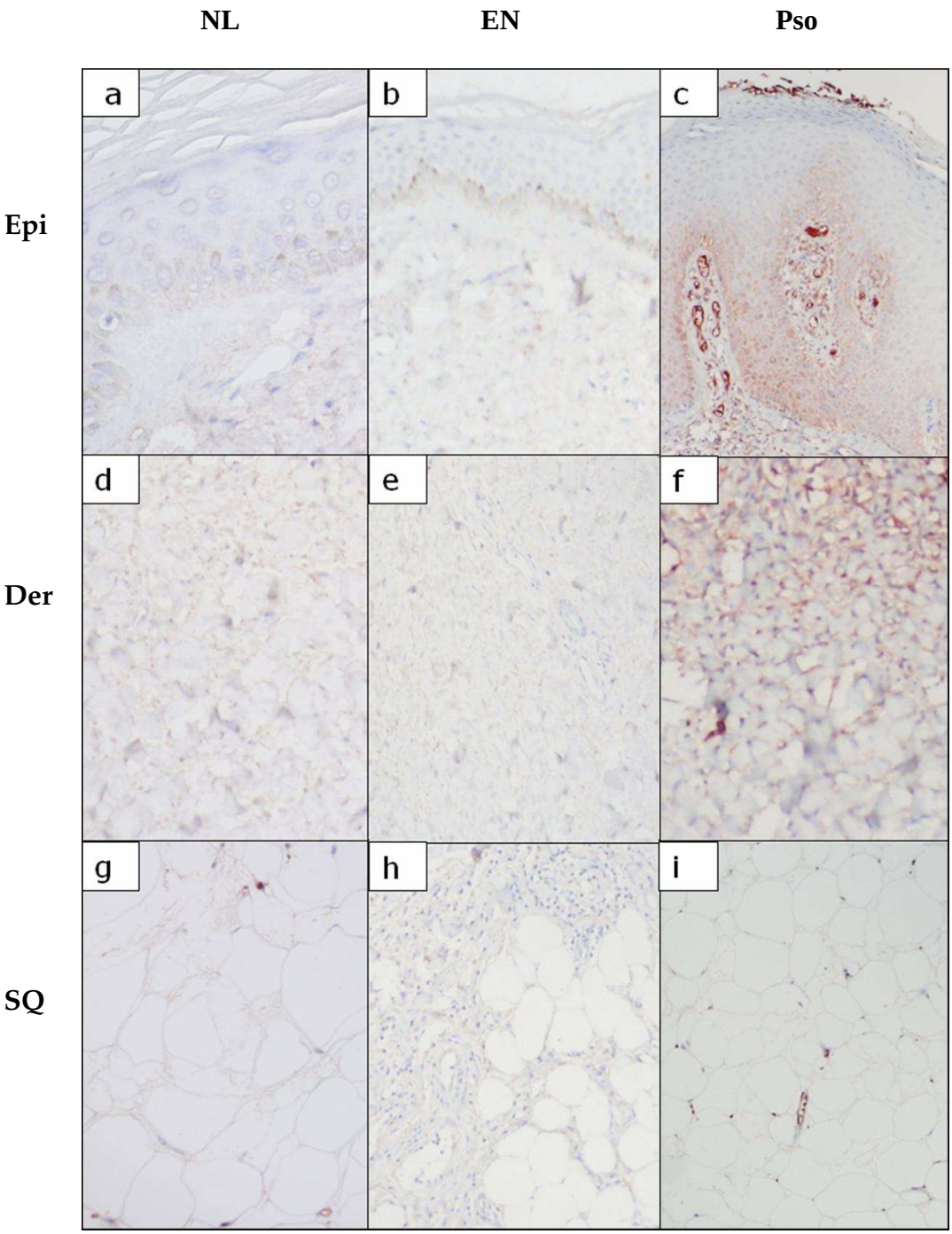
3. Immunohistochemical staining with anti-IL-17 antibody

To confirm the expression and localization of IL-17, we stained normal (n=5), erythema nodosum-like BD lesional (n=5), and psoriatic lesional (n=5) skin paraffin embedded sections with the anti-human IL-17 antibody. The expression of IL-17 in the epidermis of skin was strong in psoriasis, especially lower portion. But epidermis of erythema nodosum-like BD lesional skin did not show significant difference from normal human skin (Fig. 3A. a, b, c). In papillary dermis, perivascular cells and inflammatory cells expressed this molecule in psoriatic lesional skin (Fig. 3A. c, f). In contrast, in normal human skin and in erythema nodosum-like BD lesional skin, IL-17 expression in the dermis was present in only a limited number of cells (Fig. 3A. d, e). In the subcutaneous tissue, septal infiltrating inflammatory cells did not show IL-17 expression in erythema nodosum-like BD lesion (Fig. 3A. h) and normal and psoriatic human skin in which there were scant inflammatory cellular infiltration also showed no IL-17 expression in the subcutaneous tissue (Fig. 3A. g, i).

The expression of IL-17 in normal, erythema nodosum-like BD lesion, and psoriatic lesion was scored in arbitrary unit and was summarized in Fig. 6. The expression of IL-17 in the dermis and subcutaneous fat layer did not differ significantly among three groups. However, the expression of IL-17 in

the epidermis was stronger in psoriatic lesion compared with normal control and erythema nodosum-like BD lesion ($p<0.01$).

(A)



(B)

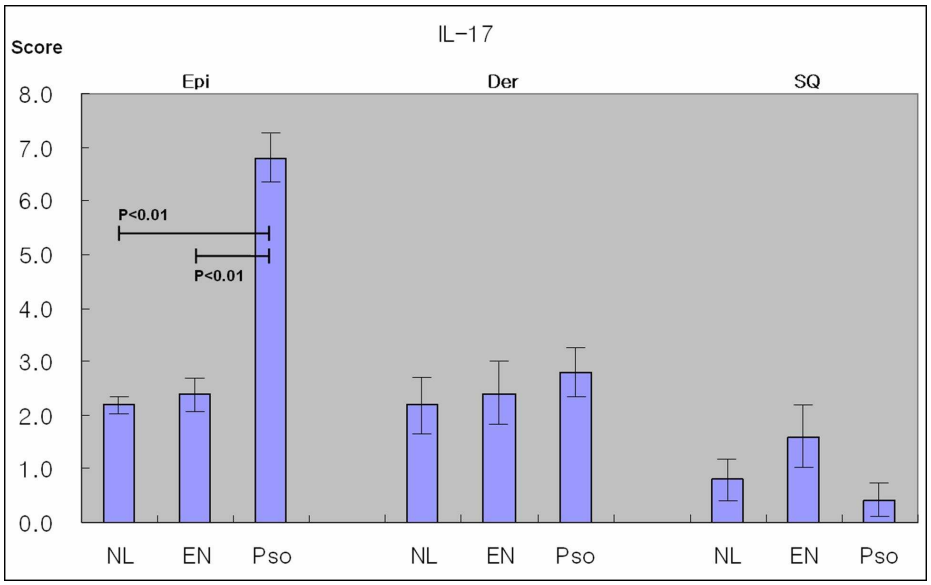


Figure 3. (A) Immunohistochemical staining with anti-human IL-17 antibody (original magnification, ×100). **(B)** Statistical analysis of immunohistochemical staining shows significantly higher expression of epidermis of psoriatic lesional skin. Erythema nodosum-like skin lesion of Behçet's disease does not show statistically significance. Values are mean ± SD. Abbreviations: NL, normal skin; EN, erythema nodosum-like BD lesion; Pso, psoriatic lesion; Epi, epidermis; Der, dermis; SQ, subcutaneous fat.

V. DISCUSSION

Cytokines play significant role in the immune response and inflammation. Recently helper T cells may differentiate into three functional subsets: Th1 cells, which secrete interferon-gamma (IFN-γ), IL-2, tumor necrosis factor (TNF) and promote cell-medicated immunity, Th2 cells, which produce IL-4, IL-5, IL-10, and IL-13 and promote antibody-mediated immunity, and Th17 cells, recently added group, which produce IL-17, TNF, IL-6, and IL-22 and induce tissue inflammation and autoimmunity[32]. During the past decade, cytokines were mainly investigated in sera, biologic fluids (e.g., plasma, synovial fluid, cerebrospinal fluid), or supernatants of cultured peripheral blood mononuclear cells (PBMCs) from patients with BD. Ahmed et al reported that increased expression of interferon IFN-γ, TNF-α and IL-12 in the mucocutaneous lesions of BD, indicating Th1 skewed response and Th2 cytokines (IL-4 , IL-13) were not increased[16]. However, increased expression of IL-10, a Th2-related cytokine, was observed and this result is hard to explain with Th1 associated cell-medicated immunity and increased IL-23p19 expression was observed in the cutaneous lesions of BD patients[28]. Because increased IL-6, IL-17 and TGF-β in sera of BD patients and increased IL-23p19 expression in the BD skin lesions had been reported, we predict that IL-17 and Th17 may play a role in the BD skin lesions.

IL-6, which was originally identified as a B cell differentiation factor, is now known to be a multifunctional cytokine that regulates the immune response, hematopoiesis, acute phase response and inflammation including differentiation of Th17 cells[33]. The overproduction of IL-6 might lead to abnormal B cell differentiation and antibody production in various autoimmune disease and chronic inflammatory disease[33,34]. Increased IL-6 also had been reported in the serum of BD patients and psoriasis patients, in the psoriatic lesional skin[17,35-39], and in the bronchoalveolar lavage and in the cerebrospinal fluid from BD patients[40,41]. Our data, significantly stronger IL-6 expression in the epidermis of psoriatic lesion and subcutaneous fat layer of BD skin lesion, is consistent with previous reports.

TGF-β induces not only accumulation of fibroblasts and matrix but also modulation of immune response and inhibition of epithelial cellular proliferation. Variable results had been report of TGF-β expression in the epidermis of psoriasis and some authors argued that the expression of TGF-β is different according to their isoforms[42-44]. However, TGF-β receptors were decreased in the epidermis of psoriatic lesions, and it was suggested that the down-regulation of TGF-β actions might contribute to the pathogenesis of psoriasis[42-45]. In BD, no difference in the serum TGF-β concentration and increased TGF-β activity in the synovial fluid compared with normal control were reported[29,30,46], but there have been no report of cutaneous lesions. Our data showed strongly increased expression of TGF-β in the erythema

nodosum-like BD lesion. There are possibilities of immunomodulatory function of TGF-β in chronic inflammatory process and also fibrosis following active inflammation[47].

IL-17 did not increase in BD skin lesions in our study, despite of lesional elevation of cytokines (IL-6, TGF-β, IL-23p19) which are known as essential factors for differentiation, expansion and survival of Th17 cells[8,28]. Because increased IL-17 expression in the epidermis of psoriatic lesions was observed without non-specific staining, this results is consistent with previous reports[9], it is hard to assume that materials and methods of immunohistochemical staining of this study are not proper. Concentration of IL-23p40 in BD skin lesion was not increased although increased expression of IL-23p19 was observed [28]. It is possible that IL-17 concentration in BD skin lesion is related IL-23p40 concentration rather than IL-23p19 concentration.

Considering our results and known mechanism of IL-6 mediated immune response[33], our data suggests that IL-6 may have a role in the immune complex mediated pathology rather than Th17 mediated immune response in the erythema nodosum-like lesion of BD. In fact, there are also controversy whether the serum concentration of IL-17 in BD patients increase or not compared with controls[17,48].

In summary, we observed elevated expression of IL-6 and TGF-β in erythema nodosum-like lesions of active BD patients compared with normal human skin using immunohistochemical staining with monoclonal antibody.

However, we could not observe statistically significant differences of IL-17 expression between BD patients and normal controls. On the basis of the results of this study, IL-6 may play a role in the localized pathogenic mechanism through immunologic process in the erythema nodosum-like lesions of active BD patients. Further studies of larger samples may be helpful for the clarification of the role of IL-17 and Th17 in the cutaneous lesions of BD.

VI. CONCLUSION

In this study, we performed immunohistochemical stains for IL-6, TGF-β, and IL-17 on normal skins, erythema nodosum-like BD skin lesions and psoriatic skin lesions. The results were scored by investigators and analyzed. The results are summarized as follows:

1. Increased IL-6 expression was observed in the erythema nodosum-like skin lesions of BD patients and the skin lesions of psoriasis patients in comparison with normal control skins.
2. Increased TGF-β expression was observed in the erythema nodosum-like skin lesions of BD patients in comparison with normal control skins and the skin lesions of psoriasis patients.
3. No difference of IL-17 expression was observed in the erythema nodosum-like skin lesions of BD patients in comparison with normal control skins. Increased IL-17 expression in the epidermis of the skin lesions of psoriatic patients was observed compared with normal control skins and erythema nodosum-like skin lesions of BD patients.

Therefore, this study suggests that IL-6 may play a role in the localized pathogenic mechanism in the erythema nodosum-like lesions of active BD patients. However, further studies of larger samples may be helpful for the clarification of the role of IL-17 and Th17 in the cutaneous lesions of BD.

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국 문 요 약

베체트병의 피부 병변에서 Th17과 연관된 싸이토카인의 발현

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베체트병은 아직 병인이 밝혀지지 않고 신체 여러 장기를 침범하는 만성 염증성 질환으로, 베체트병에서 싸이토카인의 변화는 다양하게 연구되어 왔다. 인터루킨(IL)-17은 대부분이 Th17이라 일컬어지는, 최근에 규명된 CD4+ T세포에서 생산되는 싸이토카인으로, 면역 및 염증 반응과 자가면역 질환에서 중요한 역할을 한다. 미분화된 CD4+ T세포에서 Th17로의 분화에서는 TGF-β와 IL-6이 필수적이다. Th17은 Th1이나 Th2 중 어느 한쪽으로 규명하기 어려운 면역 기전에서 새로운 기전이 될 수 있을 것으로 생각되어, 저자들은 베체트 환자의 피부 병변에서 IL-17과 Th17의 역할을 알아보기 위해 IL-17과 Th17의 분화와 연관된 싸이토카인 변화를 조사하였다.

피부조직의 면역조직화학염색을 시행하였을 때, 정상인과 비교시 베체트병 환자의 결절홍반양 피부 병변에서 IL-6와 TGF-β의 발현

증가가 관찰되었다. 건선 환자의 피부 병변에서는 IL-6와 IL-17의 발현 증가가 관찰되었으나 베체트병 환자의 피부 병변은 정상인과 비교시 IL-17의 발현에 차이가 없었다.

그러므로 본 연구는 활동성 베체트병 환자의 결절홍반성 병변의 국소적 병리 기전에서 Th17에서 분비하는 IL-17 보다는 IL-6와 TGF-β와 연관된 면역 반응이 관여될 수 있다는 점을 시사한다.

핵심되는 말: 베체트병, IL-17, IL-6, TGF-β