Quantification of the local inflammatory cytokines of erythema-nodosum like skin lesions in Behçet's disease using laser capture microdissection

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Directed by Professor Dongsik Bang

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Min Ju Choi

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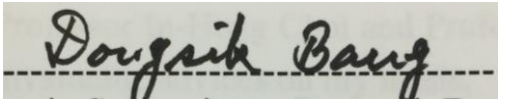
This certifies that the Master's Thesis of
Min Ju Choi is approved.



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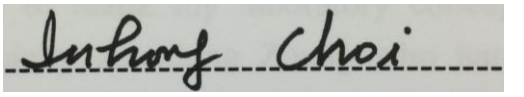

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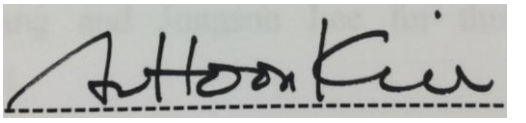

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ABSTRACT

Quantification of the local inflammatory cytokines of erythema-nodosum like skin lesions in Behçet's disease using laser capture microdissection

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Introduction: Behçet's disease (BD) is a chronic multisystemic inflammatory disease characterized by oral ulcer, genital ulcer, ocular lesions and skin involvements. Cutaneous manifestations are common in BD, and previous studies reported that erythema nodosum (EN)-like lesions in BD are histologically different from classical EN. Immunologically, both Th1 and Th17 mediated cytokines are important in skin lesions of BD, but specific cytokine profiles at panniculus in EN-like lesions have not been studied. Laser capture microdissection (LCM) is a recently developed technique which enables delicate analysis of specific cell populations within tissue samples. In this study, we tried to identify the dominant cytokine milieu at the inflammatory foci of EN-like skin lesion in BD using LCM.

Methods: A total of 12 BD patients fulfilling the International Study Group for BD criteria were enrolled, and 18 skin samples including non-lesional skin tissues were taken. Inflammatory foci within panniculus were excised using LCM, and the levels of mRNA expression of cytokines were measured by quantitative RT-PCR.

Results: Inflammatory foci of EN-like lesion of BD showed increased mRNA expression of IFN-γ, IL-17A and IL-10 compared with non-lesional skin. The overall mRNA levels of IFN-γ and IL-17 in the EN-like skin lesions from patients who were taking systemic medication

were significantly lower than those from patients who were not treated. When the EN-like lesions were further divided into acute and resolving lesions, there was significantly increased expression of IFN-γ and IL-17A in the acute EN-like lesions.

Conclusion: Microdissection of the inflammatory foci within EN-like BD skin lesions showed Th1- and Th17-biased cytokine milieu. IFN-γ and IL-17A were significantly elevated in active EN-like skin lesions. Cytokine levels of IL-10 and IL-4 were also slightly elevated, although it did not reach statistical significance.

Key words : Behçet's disease, Erythema nodosum-like skin lesion, Laser capture microdissection

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I. INTRODUCTION

Behçet's disease (BD) is a chronic multisystemic inflammatory disease with oral ulcer, genital ulcer, ocular lesion and skin lesions.[1,2] Other minor features of BD include arthritis, vascular, gastrointestinal, neurological, and epididymitis.[3] BD occurs worldwide with highest prevalence in the Far East, Middle East, and the Mediterranean areas.[2] The pathogenesis of BD remains unknown, but genetic susceptibility such as HLA-B51 is thought to play a role.[4] Also, recent genome-wide association studies have revealed that variants in the MHC class I, IL-10, and IL23R-IL12RB2 are associated with the pathogenesis of BD.[5,6] Infection such as herpes simplex virus and *Streptococcus sanguinis* is thought to trigger BD in genetically susceptible patients.[7] Autoimmune and autoinflammatory conditions, heat shock protein, endothelial cell dysfunction, and various cytokines and chemokines are also postulated to be associated in the pathogenesis of BD.[8,9]

Various cutaneous manifestations are the hallmark of BD and occurs in approximately 38 to 99% of BD patients.[2] Skin manifestations range from erythema nodosum (EN)-like lesion, papulopustular lesion, erythema multiforme-like lesion, Sweet's syndrome-like lesion to less common lesions such as pyoderma gangrenosum, thrombophilebitis, and purpura.[10] Since BD is a systemic vasculitis which can affect all sizes and types of vessels, vasculitis is commonly encountered in skin biopsy samples of BD, including EN-like lesions. Some studies revealed that EN-like skin lesions in BD is characterized by panniculitis with numerous neutrophil infiltration as well as necrotic adipocytes, suggesting that different mechanisms may be involved in EN-like skin lesions in BD apart from classical EN.[11]

In 2004, Ben, *et al.*[12] reported increased IL-8, monocyte chemoattractant protein 1, IFN-γ, and IL-12 mRNA in skin lesions of BD with almost absent levels of IL-4 and IL-13, suggesting that Th1 pathway may play a major role in the skin manifestations of BD. Lew, *et al.*[13] reported increased levels of IL-23 p19 mRNA in EN-like skin lesions of BD patients, proposing Th17 pathway maybe also involved in the pathogenesis of BD. There have been other reports of immunohistochemical staining of BD skin lesions with impression that Th2 pathway may also play a role in the pathogenesis of BD.[14]

Skin lesions in BD usually improve with time. Therefore, it is important that we understand the natural course of the skin manifestation, which can aid in developing effective treatment based on the pathogenesis. Yamaguchi, *et al.*[15]

discovered that natural killer cells control a Th1 response in BD patients, and the change to natural killer type 2 was associated with disease remission, suggesting a role for natural killer cells in the disease activity of BD patients. Cho, *et al.*[14] reported that IL-4 expression was higher than IFN-γ in skin lesions of BD, suggesting that Th2 pathway mediated cytokines may also contribute to pathogenesis of BD. Jenkins, *et al.*[16] postulated that Th2 pathway inflammation is mediated by IL-4, resulting in local macrophage proliferation, emphasizing the local proliferation of inflammatory cells.

Previous studies have used various methods including immunohistochemistry and RT-PCR in defining which cytokines and chemokines are dominant in the BD skin lesions and serum.[8-14] However, serum levels do not necessarily correlate with the level of inflammation at the specific site of inflammation, in this case, the skin. Furthermore, previous studies have utilized skin tissue samples as a whole, and there have been no reports using only the active inflammatory foci of the tissue samples.

Laser capture microdissection (LCM) is a powerful tool which enables delicate molecular analysis by dissection of specific cell populations within tissue samples.[17] In this study, we spotlighted on the inflammatory foci of panniculitis within the EN-like skin lesions in BD, and aimed to examine the immunologic mechanism of the cutaneous skin lesions of BD. The aim of this study was to investigate the local cytokine milieu of EN-like skin lesions in BD

patients using LCM technique, further analyzing the difference between active and resolving EN-like skin lesions of BD patients.

II. MATERIALS AND METHODS

1. Patient selection and skin sample preparation

This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. (Project Number 4-2012-0890) All the patients fulfilled the diagnostic criteria of the International Study Group for BD[1] with EN-like skin lesions. A less than three-day duration of EN-like skin lesion with erythema and tenderness was considered an active lesion, whereas duration of more than three-days, dusky colored, EN-like skin lesions were considered a resolving lesion. Skin biopsy was performed on either lesional or non-lesional skin lesions of BD patients. Non-lesional skin lesions of BD were prepared from opposite lower extremity where there was no evidence of EN-like skin lesions.

2. Tissue preparation using laser capture microdissection (LCM)

A 4mm biopsy was performed on either lesional or non-lesional skin lesions of BD patients and was immediately immersed in OCT compound (Sakura Finetek USA Inc., Torrance, California, USA) and kept in -80℃. Frozen samples were sectioned at 10μm thickness and mounted on polyethylene naphthalate (PEN)-membrane slide glass (Leica Microsystems GmbH, Wetzlar,

Germany). Laser Microdissection (Leica, LMD 6500, Seoul, Leica Korea) was used to separate subcutaneous adipose tissue with perivascular infiltrating inflammatory cells from the epidermis and dermis. (Fig. 1) The microdissected tissue samples were transferred to a 0.5mL aseptic PCR tube (Greiner Bio-one, Washington, D.C, USA) by gravity.

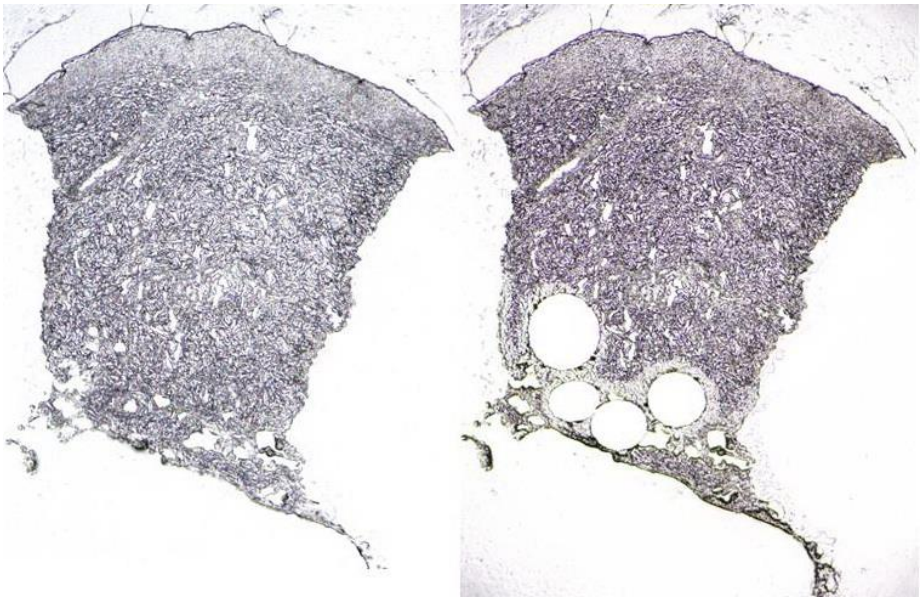

Figure 1. Laser capture microdissection (LCM) of EN-like skin lesions. Frozen section of the EN-like lesion before (A) and after (B) laser microdissection. (B) Panniculitis foci and perivascular infiltration is microdissected using LCM. The tissue in the vacant spots are further analyzed for mRNA extraction and amplification. (A, B: x 40)

3. mRNA extraction and real-time PCR

Total RNA was isolated with TriZol reagent (Canadian Life Technologies, Burlington, Ontario, Canada) and the cDNA was synthesized using a Superscript II RNase H reverse transcriptase (Canadian Life Technologies). The primers (Bioneer, Seoul, Korea) used are indicated in Table 1. The gene expression was monitored using an ABI 7500 software (Applied Biosystems, Foster City, CA, USA) according to the standard procedure. The real-time PCR cycles included 40 cycles of general denaturation at 94℃ for 30 seconds, annealing, and elongation at 60℃ for 1 minute, except for the first cycle with a 15-minute denaturation and last cycle with a 7-minute elongation at 72℃. Real-time polymerase quantification of the signals was performed by normalizing the gene signals with β-actin signal.

Table 1. List of primer sequence.

Gene		Sequence
Hu IL-4	F	CTTCCCCCTCTGTTCTTCTT
Hu IL-4	R	CTGCTCTGTGAGGCTGTTCA
Hu IFN-γ	F	GTCCAACGCAAAGCAATACA
Hu IFN-γ	R	CTCTTCGACCTCGAAACAGC
Hu IL-17A	F	CATGAACTCTGTCCCCATCC
Hu IL-17A	R	CCCACGGACACCAGTATCTT
Hu IL-10	F	CCAAGCTGAGAACCAAGACC
Hu IL-10	R	GGGAAGAAATCGATGACAGC
β-actin	F	ATAGCACAGCCTGGATAGCAACGTAC
β-actin	R	CACCTTCTACAATGAGCTGCGTGTG

Abbreviations: F, Forward primer; R, Reverse primer.

4. Data analysis

All analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). IL-4, IL-10, IL-17A, and IFN-γ expressions were evaluated by the Mann-Whitney U-test between different categories. Analysis of three or more groups was done using analysis of variance (ANOVA) Kruskal-Wallis test. P values of <0.05 were considered statistically significant. Graphs were expressed as mean ± SEM using Prism (Graphpad software, San Diego, CA, USA) software.

III. RESULTS

1. Patients and tissue samples

A total of 12 BD patients fulfilling diagnostic criteria of the International Study Group for BD[1] with EN-like skin lesions (2 males, 10 females, M:F = 1:5, median age 41.3 years) were enrolled in the study. Six BD patients underwent lesional and non-lesional biopsy, and other six patients had lesional skin biopsies. A total of 12 EN-like skin lesions of BD and 6 non-lesional BD skin were collected. The characteristics of BD patients in this study are summarized in Table 2. Out of 12 BD patients, 4 patients were on oral medication. HLA-B 51 genotyping was positive in 6 of 9 tested BD patients.

Table 2. Clinical characteristics of BD patients

Patient Number	Sex	Age	Pathergy reaction	OU	GU	Ocular lesions	Skin lesions (active or resolving)	Systemic medication	HLA-B51 genotyping
1	F	52	no	yes	no	yes	resolving	colchicine	negative
2	M	42	no	yes	yes	no	active	none	positive
3	F	28	no	yes	yes	no	active	none	not done
4	F	24	no	yes	yes	yes	resolving	colchicine	positive
5	F	39	no	yes	yes	no	active	colchicine and azathioprine	not done
6	F	50	no	yes	yes	yes	active	none	not done
7	F	53	no	yes	yes	yes	active	none	negative
8	F	62	no	yes	yes	no	active	none	negative
9	F	44	no	yes	yes	no	active	colchicine	positive
10	F	36	no	yes	yes	no	active	none	positive
11	M	25	no	yes	no	yes	active	none	positive
12	F	41	no	yes	yes	no	active	none	positive

Abbreviations; OU, oral ulcer; GU, genital ulcer.

2. mRNA expression of IFN-γ, IL-17A and IL-10 were elevated in EN-like skin lesions of BD patients

The mRNA expression of IFN-γ, IL-17A, IL-4 and IL-10 were measured between lesional and non-lesional EN-like skin lesions of BD patients. Figure 2 shows the illustration of the real-time PCR amplification plot of IFN-γ, IL-17A, IL-10, IL-4. Some cytokine levels of mRNA expression in EN-like skin lesions of BD patients was greater than that of non-lesional BD skin (Fig. 3). Statistically significant difference was noticed in IFN-γ (p=0.023), IL-17A (p=0.0402) and IL-10 (p value 0.013). Although there was a tendency towards elevated mRNA expression of IL-4 in EN-like skin lesions of BD patients compared with non-lesional BD skin, it was not statistically significant (p=0.204).

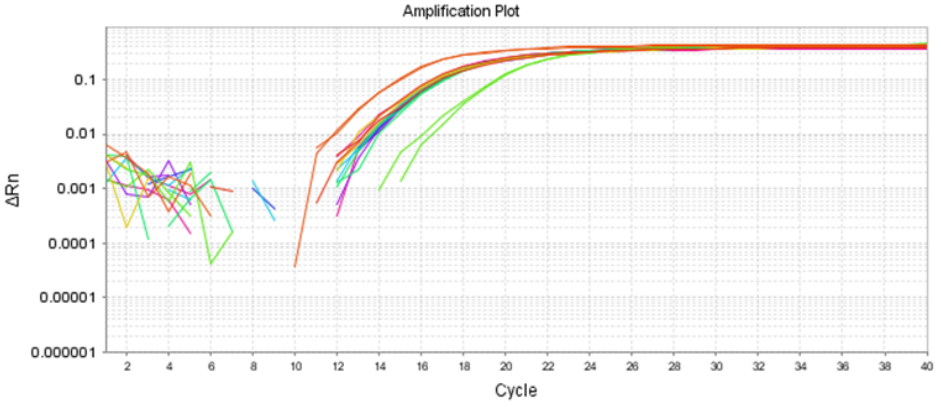

Figure 2. Illustration of the real-time PCR amplification plot of cytokines IFN-γ, IL-17A, IL-10, and IL-4.

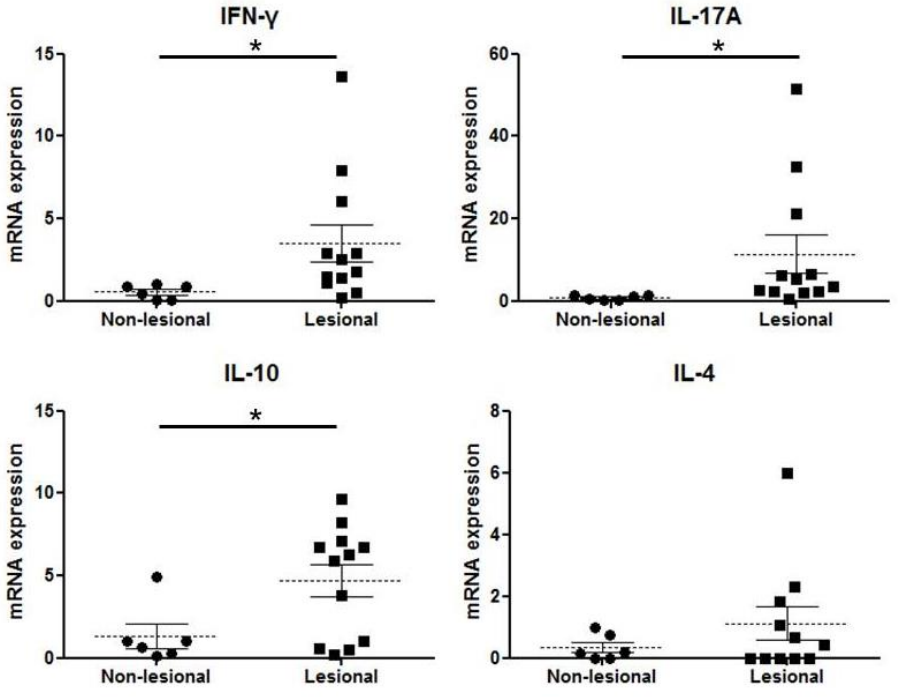

Figure 3. mRNA expression of EN-like skin lesions in BD patients compared with non-lesional skin lesions of BD patients. Statistically significant values are marked with asterisk (*). Vertical dotted lines indicate mean value, and vertical lines indicate SEM. (p=0.023 for IFN-γ, p=0.0402 for IL-17A, and p=0.013 for IL-10, respectively)

3. Effectiveness of medication in EN-like skin lesions of BD patients

Of 12 BD patients, 4 patients were on systemic medication due to BD related symptoms. The comparison of cytokine mRNA levels between non-lesional BD skin, EN-like skin lesion without medication, and EN-like skin lesion with medication revealed statistically significant values in IFN-γ and IL-17A ($p=0.0202$ and 0.0091, respectively) (Fig. 4). In this study, the levels of mRNA of IL-10 and IL-4 were not significantly reduced after oral medication. ($p=0.0949$ and 0.2495, respectively)

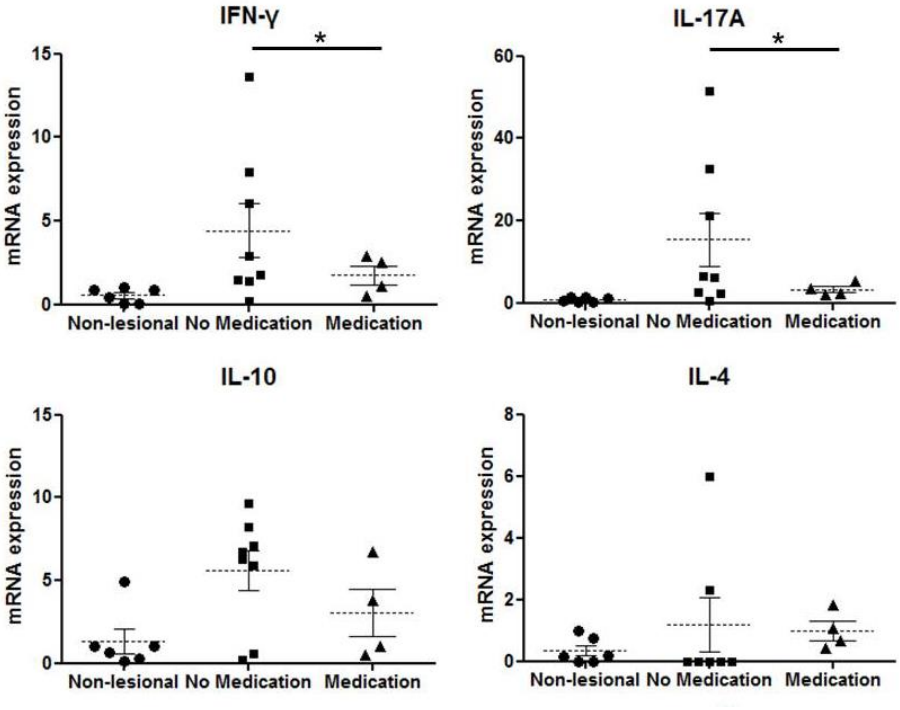

Figure 4. Effects of medication in EN-like skin lesions of BD patients. Statistically significant values are marked with asterisk (*). Vertical dotted lines indicate mean value, and vertical lines indicate SEM. ($p=0.0202$ for IFN- γ and $p=0.0091$ for IL-17A, respectively)

4. Difference in cytokine mRNA levels according to symptom severity

Comparison of the mRNA levels of different cytokines between active and resolving EN-like skin lesions were undertaken. As compared to non-lesional skin, the levels of mRNA of IFN-γ and IL-17A were elevated in active EN-like skin lesions compared to resolving EN-like skin lesions. ($p=0.0208$ and 0.0112, respectively) (Fig. 5). The similar trend was also observed in IL-10 and IL-4 mRNA cytokine levels in active and resolving EN-like BD skin lesions, although not statically significant. ($p=0.1271$ and 0.5527, respectively)

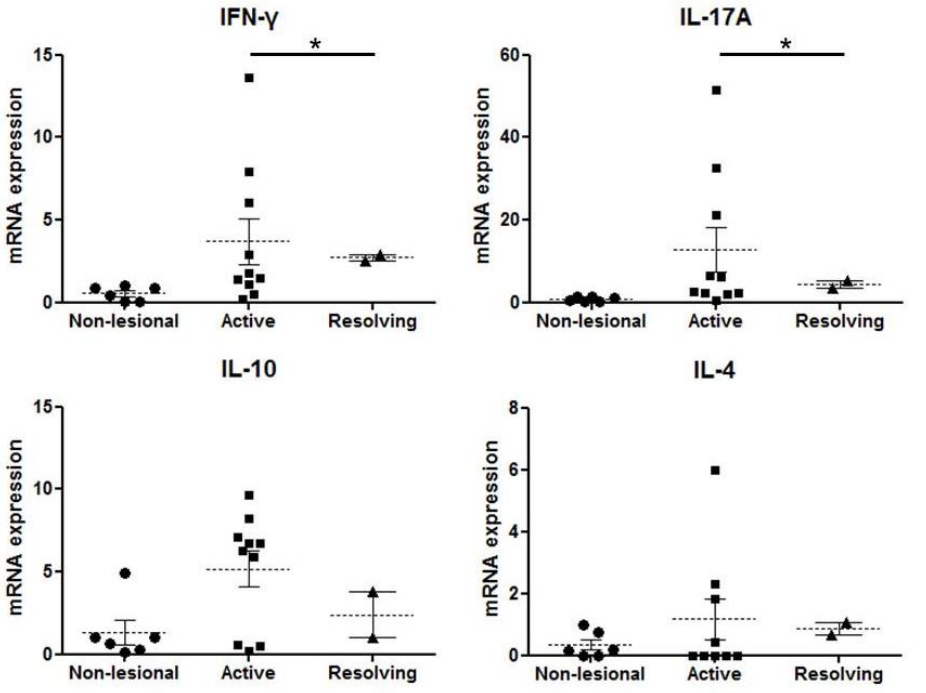

Figure 5. Comparison of the mRNA levels of different cytokines among non-lesional skin lesion, active EN-like skin lesion, and resolving EN-like skin lesions. Statistically significant values are marked with asterisk (*). Vertical dotted lines indicate mean value, and vertical lines indicate SEM. (p=0.0208 for IFN- γ and p=0.0112 for IL-17A, respectively)

IV. Discussion

Behçet's disease (BD) is thought to be triggered by underlying genetic susceptibility, infectious agents, and other environmental factors.[7] Infectious agents such as herpes simplex virus (HSV), varicella zoster virus, human herpes virus 6 and 7, cytomegalovirus and *Streptococcus sanguinis* have been isolated in various cutaneous lesions of BD patients, mostly at oral and genital ulcerations of BD patients.[7,18,19]

Cutaneous manifestations are considered very important in BD. To diagnosis BD, one has to have recurrent oral ulcerations plus two of four criteria which are genital ulcerations, positive pathergy test, skin lesions and eye lesions.[1] Except eye lesions, all other criterias are focused on skin manifestations.

Skin manifestation of BD is diverse, and can be seen as papulopustular lesion, erythema multiforme-like lesions, EN-like lesion, thrombophilebitis, purpura, pyoderma gangrenosum and many more.[10] Among these various lesions, we have chosen the EN-like skin lesion because it is relatively aseptic compared to oral, genital ulcerations or papulopustular lesions.[13] Furthermore, EN-like lesions are usually located in the lower extremities of BD patients, which make it easy for specimen collection.

There have been reports regarding studies including the histopathologic and immunologic characteristics of BD related skin lesions. Histopathologically, BD related skin lesions show features of vasculitis and thrombosis is occasionally seen.[10,11,20] Classical EN lesions show septal dominant panniculitis

with lymphocyte dominant infiltration, where EN-like skin lesion in BD show vasculitis with neutrophil dominant but mixed cellular infiltration in the septal and lobular subcutaneous fat layer.[11,20] Immunologic characteristics of BD skin lesions have been investigated largely under immunohistochemical staining of various markers, but it was in 2004 that the analysis of cytokine expression within BD skin lesions using reverse transcriptase-PCR was introduced.[12] In the study, there was increased level of IFN-γ, IL-8, IL-12. Since then, there have been diverse reports that Th1 pathway is dominant in skin lesions of BD.[12,21] In recent years, Th17 pathway was newly recognized and was demonstrate that it also has a role in skin lesions of BD.[13,14,22,23] Table 3. Shows list of cytokines previously reported to be elevated in EN-like skin lesions, serum, or peripheral blood mononuclear cell (PBMC). [11-14,24-26]

Until now, colchicine is considered to be the mainstay medication for treating symptoms of BD.[7] The 4 BD patients who were on systemic medication were all taking colchicine in this study. EN-like skin lesions of BD patients who were taking oral medication had significantly lower levels of IFN-γ and IL-17A, which indicates that the medication is very effective in reducing the inflammation.

Table 3. List of cytokines reported to have been altered in BD patients

Cytokine	EN-like skin lesion		Others	References
	IHC	RT-PCR	PBMC, serum	
Th1 axis				
IL-12/23 p40		elevated		12
IFN-γ		elevated *	elevated	11, 26
TNF-α				
IL-21			elevated	
IL-12			NS	13
IL-27			elevated	26
Th17 axis				
IL-17(A)		*	elevated	26
IL-23 p19			NS	12
IL-23 p19		elevated		12
Th2 axis				
IL-4	elevated	NS *	elevated	11, 14
IL-10		elevated *	elevated	11
IL-22		elevated	elevated	25
IL-13		NS		12
Others				
IL-8		elevated		11

Abbreviations: EN, erythema nodosum; IHC, immunohistochemical staining; RT-PCR, Reverse transcription polymerase chain reaction; PBMC, Peripheral blood mononuclear cell; NS, non significant. Our data is marked with asterisk (*).

Previous study has demonstrated that BD skin lesions had higher levels CD68 positive macrophage compared with CD4, CD8 positive T cells or FoxP3 positive regulatory T cells in immunohistochemical staining.[14] Furthermore, the cytokine staining of IL-4 was stronger than IFN-γ.[14] This suggested that Th2 pathway may play a role in BD skin lesions and together with the data that Th2 inflammatory pathway is mediated by IL-4,[16] we aimed to see whether was a difference in cytokine levels of EN-like skin lesions in BD patients in acute and resolving lesions. We found out that in accordance to previous reports, there was elevated IFN-γ and IL-17A in EN-like skin lesions in BD patient, which was again confirmed by RT-PCR. We also found significant increase in IL-10 in EN-like skin lesions in BD patients but could not find significant difference in IL-4. We further divided the EN-like skin lesions into acute and resolving skin lesions, and found out that IFN-γ and IL-17A were significantly elevated in acute EN-like skin lesions than resolving EN-like skin lesions. We could not find elevated IL-4 in EN-like skin lesions nor find remarkable difference between active and resolving lesions.

One of the reasons why there was no significant difference may be that most of the resolving EN-like skin lesions had poor cell infiltration and number of cells. The two resolving EN-like skin lesions had scarce cellular infiltration compared to other biopsy specimens. The number of cells, rather than different secreted cytokines may influence the total dominant cytokine profile. Also, there has been a report which suggest that presence of EN-like skin lesions in

BD maybe an indicator of the mildness of BD symptoms.[27] BD patients enrolled in our study all had EN-like skin lesions, which may have lead into no significant difference of IL-4 between active or resolving EN-like BD skin lesions.

Recent reports have suggested that adipokine or adipocytokine may be involved in metabolic syndrome and rheumatic diseases.[28-30] Among various adipokines, resistin is known to induce insulin resistance and upregulate the production of IL-6 and tumor necrosis factor alpha (TNF-α) secretion from macrophages.[31] In a report, there was a positive correlation between serum resistin and TNF-α level, and patients with high serum level resistin had more severe BD symptoms.[28] Other report has discovered that adipokines in obese mice could upregulate the inflammatory process by driving the Th1/Th17 differentiation.[32] Different adipokine levels may have influenced the levels of cytokine in this study.

The strength of this study is that we used laser capture microdissection (LCM) to isolate the pure cell population from EN-like skin lesions of BD patients. It is sometimes difficult to distinguish between primary and secondary changes, so we aimed to take a deeper look at the cardinal cytokine changes at the inflammatory site, in this case the subcutaneous fat layer and adjacent vessels. Also, previous reports have largely depended on immunohistochemical staining and counting the positively stained cells by naked eye to determine the expression of the cytokines.[14,20,23] We have used quantitative PCR instead of

immunohistochemical staining to focus at the mRNA proteins. Lastly, the EN-like skin lesions of BD patients were compared with non-lesional skin lesions of BD patients, not healthy controls, making them a good control specimen.

The limitation of our study is we analyzed only the key cytokines of Th1, Th2 and Th17. It would have been more instructive if we had other cytokines of Th1, Th2 and Th17 pathways.

V. CONCLUSION

In conclusion, we evaluated a more precise cytokine milieu of the panniculitis foci in EN-like skin lesions of BD using laser capture microdissection. We found elevated IFN-γ and IL-17A in EN-like skin lesions compared to non-lesional BD skin. Also, the mRNA levels of IFN-γ and IL-17A in EN-like BD skin lesions correlated with symptom severity. We could not find evidence of elevated IL-4 in resolving EN-like skin lesions compared to acute EN-like skin lesions of BD patients. Further studies should be focused in comparison of the BD skin with normal healthy controls given the fact that there is already a baseline inflammation in normal looking skin of BD patients, and techniques that would segregate even more homogenous cell population are welcomed. It would also be necessary to evaluate the effect of various adipokines in the subcutaneous fat tissue.

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ABSTRACT(IN KOREAN)

베체트병 환자의 결절 홍반양 병변에서의 미세절제를 통한 국소 염증성 사이토카인의 정량적 연구

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최 민 주

배경: 베체트병은 만성적인 다기관을 침범하는 혈관염으로, 구강궤양과 성기궤양, 눈병변과 피부병변을 특징으로 한다. 베체트병의 피부병변은 환자의 대다수에서 나타나며, 면역학적으로는 Th1과 Th17반응이 우세할 것으로 생각되고 있다. Laser capture microdissection (LCM)은 비교적 최근에 도입된 장비로 레이져를 사용하여 조직에서 원하는 부분의 세포만 선택적으로 분리해낼 수 있는 기술이다. 본 연구에서는 LCM을 이용하여 베체트병의 결절홍반양 피부병변의 지방층염 부분에서 일어나는 사이토카인 환경의 변화를 알아보고자 하였다.

방법: 베체트병의 진단기준을 만족하면서 동시에 결절홍반양 피부병변을 가진 베체트병 환자에서 조직을 채취하였고, 이에 대한 사이토카인 정량적 분석을 시행하였다.

결과: 결절홍반양 피부병변에서 시행한 정량적 mRNA분석 결과 IFN-γ와 IL-17A, 그리고 IL-10의 발현이 유의미하게 증가된 것을 확인할 수 있었다. 전신적으로 투약을 한 환자들의 경우 그렇지 않은 환자들보다 IFN-γ와 IL-17A의 발현이 더 낮은 것이 확인되었다. 피부병변을 급성 병변과 그렇지 않은 병변으로 나누어 분석한 결과, 급성 병변에서 IFN-γ과 IL-17A의 발현이 유의미하게 더 높았다.

결론: 베체트병 환자의 결절홍반양 병변에서 병변의 핵심이 되는 지방층과 주변 혈관을 LCM을 이용하여 분리해내어 우세한 사이토카인을 알아본 결과, IFN-γ와 IL-17A, 그리고 IL-10이 통계학적으로 유의미하게 상승되어 있음을 알 수 있었다. 이러한 병변을 활성도가 높은 병변과 그렇지 않은 병변으로 나누어 분석한 결과, IFN-γ와 IL-17A이 활성도가 높은 급성의 병변에서 통계학적으로 유의미하게 높았으며, IL-10과 IL-4는 증가된 경향을 보이는 것을 확인할 수 있었다.

핵심되는 말: 베체트병, 결절홍반양 병변, Laser capture microdissection