The significance of S100A12 in the pathogenesis of Behçet's disease and its value as an activity marker

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ABSTRACT

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Behçet's disease (BD) is a recurrent multisystemic inflammatory disease characterized by neutrophilic vasculitis. Histopathologic findings of skin lesions of BD are mainly neutrophilic vascular reactions. Neutrophil activation is an important characteristic of the active phase of BD. Enhanced superoxide, lysosomal enzymes production and chemotaxis of neutrophils from patients with BD were observed. These observations suggest that the neutrophils are overactive, which leads to tissue injury.

S100A12 is a member of the S100 family of calcium binding proteins. It interacts with the multiligand receptor for advanced glycation end products (RAGE) found on macrophages, endothelium

and lymphocytes. Extracelluar functions of S100A12 include potent chemotactic activity. The aim of the study is to determine tissue expression and serum levels of S100A12 in BD patients. Serum S100A12 was significantly increased in sera from patients in active and inactive BD period compared with controls. Serum S100A12 levels significantly decreased after treatment. Serum S100A12 levels were significantly correlated with disease activity. Immunohistochemical staining of skin sections revealed increased S100A12 expression in the lesions of panniculitis and vasculitis of BD patients. These findings suggest that S100A12 may contribute to BD pathogenesis related to neutrophil hyperactivity. In addition, S100A12 could be used as an activity marker in monitoring disease activity.

Key words: Behçet’s disease, Disease activity, S100A12, IL-8

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I. INTRODUCTION

Behçet's disease (BD) is a recurrent multisystemic inflammatory disease characterized by recurrent oral aphthous and genital ulcers, ocular lesions, skin lesions, occasionally accompanied by articular, urogenital, vascular, gastrointestinal and neurologic involvements[1-3]. Skin manifestations of BD may appear as erythema nodosum-like lesions, folliculitis, erythema multiforme, Sweet-like lesions and thrombophlebitis[1,4]. The etiology of the disease remains unknown, although genetic factors, infectious agents and immunological mechanisms have been implicated and studied[4].

Histopathologic findings of erythema nodosum-like lesions of BD are mainly neutrophilic vascular inflammation with accompanying changes in subcutis[5-7]. Jorizzo et al. suggested that the lymphocyte predominating

reaction might be following in time, a neutrophilic vascular reaction during the evolution of these lesions[7]. Hyperactivity of neutrophils is important in the pathogenesis of BD. Increased chemotaxis, phagocytosis, superoxide and lysosomal enzymes production as well as enhanced expression of CD 11a and CD18 on the cell surface have been reported in the neutrophils of BD patients[8-10]. These observations suggest that the neutrophils are overactive, which leads to tissue injury[11,12].

S100A12 (calgranulin C; extracellular newly identified RAGE binding protein, EN-RAGE) is a member of the S100 family of calcium binding proteins[13]. S100A12 is secreted in inflamed tissues or in the bloodstream by activated neutrophils[13,14]. It interacts with the multiligand receptor for advanced glycation end products (RAGE) as a receptor transducing proinflammatory signals in the endothelium and cells of the immune system. RAGE is found on macrophages, endothelium and lymphocytes[13-15]. Extracelluar functions of S100A12 include potent chemotactic activity comparable with other chemotactic agents[14,15]. S100A12 has been reported to enhance the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the endothelium[13]. Binding of S100A12 to the extracelluar domain of membrane RAGE activates intracellular signal cascade including nuclear factor (NF)-κB and induces

secretion of cytokines, such as tumor necrosis factor-α (TNF-α)[13,15-17]. Thereby S100A12 mediates pro-inflammatory effects on lymphocytes, endothelial cells, neutrophils and mononuclear phagocytes[13,18]. Expression of S100A12 has been reported in various diseases, especially non-infectious inflammatory diseases such as arthritis, vasculitis and chronic inflammatory bowel disease (Table 1).

Table 1. Disease associations of S100 A12

Condition	Species	References
Collagen-induced arthritis	Mouse	19, 20
Colitis	Mouse	15
Hypersensitivity	Mouse	15
Vasculitis	Mouse	21
Rheumatoid arthritis	Human	14, 20, 22, 23
Psoriatic arthritis	Human	22
Juvenile idiopathic arthritis	Human	24
Vasculitis	Human	16, 25
Inflammatory bowel disease	Human	24

S100A12 is overexpressed at local sites of inflammation[13,18]. High concentrations are found in serum, plasma, synovial fluid and stool during inflammation[13,18]. According to these studies, S100A12 proteins are sensitive

parameters for the monitoring of disease activity and response to treatment in individual patients[26]. Anti-S100A12 antibodies and soluble RAGE has been reported to suppress inflammation in murine model of bovine serum albumin-induced hypersensitivity and chronic bowel inflammation[19,20]. Suppression of the ligand-RAGE axis may be a possible target for therapeutic intervention.

Interleukin (IL)-8 is a chemotactic and leukocyte activating cytokine. It increases expression of adhesion molecules and thus causes neutrophils to adhere to endothelial cells. In previous studies, increasing serum IL-8 levels were found in patients with active BD[27-29].

The aim of the study is to determine tissue expression and serum levels of S100A12 in BD patients. Furthermore, we investigated the expression of S100A12 in the sera of patients before and after treatment and evaluated the value of S100A12 as a monitoring tool of the disease activity in BD.

II. PATIENTS AND METHODS

1. Patients

We included 10 patients (mean age 38.6±9.2 years; 2 males and 8 females) with BD fulfilling the criteria for diagnosis of the International Study Group for BD[30]. All patients were in the active period. Disease was defined as active BD if at least two of the following criteria were present within 4 weeks: oral ulcer, genital ulcer, skin lesions, ocular lesions, arthritis, urogenital, vascular, gastrointestinal and neurologic involvements. The activity of BD was calculated by Behçet's Disease Current Activity Form (BDCAF)[31]. The patients were evaluated in both active (before treatment) and inactive (after treatment) periods. The mean activity scores of active period and inactive period were 3.7±1.3 (range 2-6) and 0.5±0.4 (range 0-1), respectively. Blood samples were collected at the time of diagnosis, prior to the initiation of treatments. Two serum samples were obtained from each patient, in active and inactive periods of BD. Sera of ten age- and sex- matched healthy individuals were used as controls. These patients received colchicine (n=10), prednisolone (n=9), minocycline (n=1), azathioprine (n=2), sulfasalazine (n=1), nonsteroidal anti-inflammatory drugs (n=7). Ten patients with active Kawasaki disease (KD, mean age 2.5±1.2 years; 6 males and 4 females) were

disease controls. The study was approved by the institutional review board and informed consent was obtained from each participant.

2. Measurements of S100A12 and IL-8 concentrations

Serum S100A12 and IL-8 levels were evaluated before and after treatment. Peripheral venous blood samples for determination of serum S100A12 and IL-8 were drawn into vacutainer SST tubes (Becton Dickinson, Mountain View, CA, U.S.A.) and allowed to clot at room temperature (20°C to 24°C) for 30 minutes. The tubes were centrifuged at 1000 times g for 10 minutes. The sera were aliquoted and stored at -70°C until they were tested for serum levels. Serum S100A12 and IL-8 levels were determined (in duplicates) using commercial S100A12 enzyme linked immunosorbent assay (ELISA) kits (CycLex Co. Ltd., Nagano, Japan) and IL-8 ELISA kits (R&D Systems, Minneapolis, MN, U.S.A.). These procedures were performed according to the manufacturers' instructions.

3. Immunohistochemical studies

Paraffin embedded sections of skin biopsies from active BD patients were used to detect S100A12 expression. We studied five patients with active BD. Biopsy specimens were taken from erythema nodosum-like skin lesions 2 to 4

days after their appearance, with a 4-mm skin biopsy punch after injection of local anesthesia (lidocaine with epinephrine). Biopsy specimens of control groups were taken from five healthy volunteers with the same procedure. Specimens were immediately fixed in 10% buffered formalin, dehydrated in ethanol, and embedded in paraffin. Commercially available purified mouse monoclonal anti-human S100A12 (calgranulin C) antibody (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.) was 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 10 mM sodium citrate buffer (pH 6.0) in a microwave (95° C for 2 minutes) and washed and cooled in a phosphate buffered saline (PBS) bath for 20 minutes. After washing, non-specific binding was blocked with 5% normal goat serum for 20 minutes. Then, tissue sections were incubated with anti-human S100A12 antibodies for 2 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 30 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 HRP-streptavidin reagent to each section, incubated for 30 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.

4. Statistical analysis

The Mann-Whitney U test and Wilcoxon signed-rank test were performed to determine significant differences between distinct categories. Correlations were analyzed using Spearman's correlation coefficient. *P* values of less than 0.05 were considered significant. Statistical analyses were performed using the Statistical Product and Service Solutions program (SPSS Inc, Chicago, Ill, U.S.A.) for windows (version 15).

III. RESULTS

1. Serum levels of S100A12

Serum S100A12 was significantly increased in the active BD period (median 1134, range 373-1211 ng/ml, $p<0.001$), in the inactive BD period (median 299, range 21-682 ng/ml, $p=0.041$) and in patients with active KD (median 356, range 54-1677 ng/ml, $p=0.028$) compared with controls (median 68, range 18-442 ng/ml) (Figure 1).

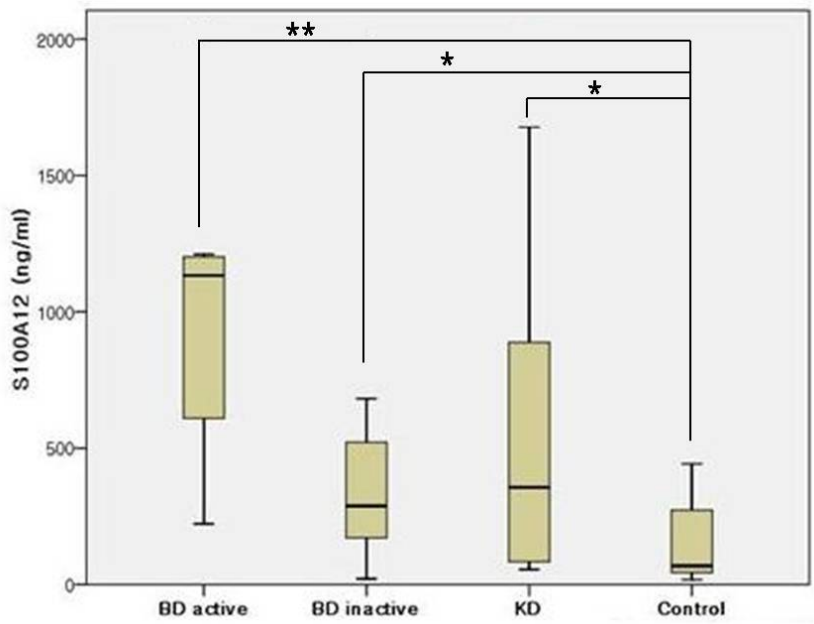

Figure 1. Serum levels of S100A12 in active and inactive period of Behçet's disease, Kawasaki disease and controls. Serum S100A12 was significantly increased in sera from patients in active Behçet's disease period (**$p<0.001$), patients in inactive Behçet's disease period (*$p<0.05$) and patients with Kawasaki disease (*$p<0.05$) compared with controls.

Serum S100A12 levels decreased significantly from baseline (p=0.017). The median serum S100A12 levels were 1134 ng/ml (range 373-1211 ng/ml) before treatment and 299 ng/ml (range 21-682 ng/ml) after treatment.

Although the difference was statistically significant, one patient showed an increase of S100A12 after treatment (Figure 2).

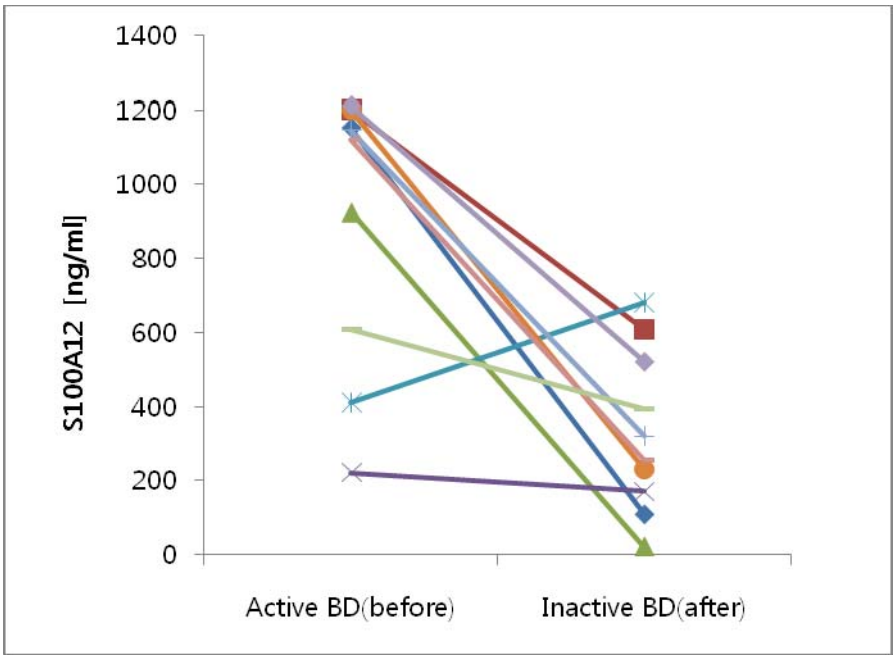

Figure 2. Comparison of serum S100A12 levels between before and after treatment in Behçet's disease patients. Serum S100A12 levels of 10 Behçet's disease patients were evaluated before and after treatment. Serum S100A12 levels significantly decreased after treatment (p=0.017).

2. Serum levels of IL-8

Serum IL-8 was significantly increased in the active BD period (median 932, range 176-2207 pg/ml, $p<0.001$), compared with controls (median 15, range 10-208 pg/ml). Serum IL-8 was not increased in the inactive BD period (median 48, range 11-3322 pg/ml, $p=0.290$) and in patients with KD (median 13, range 9-300 pg/ml, $p=0.436$) compared with controls (median 15, range 10-208 pg/ml) (Figure 3).

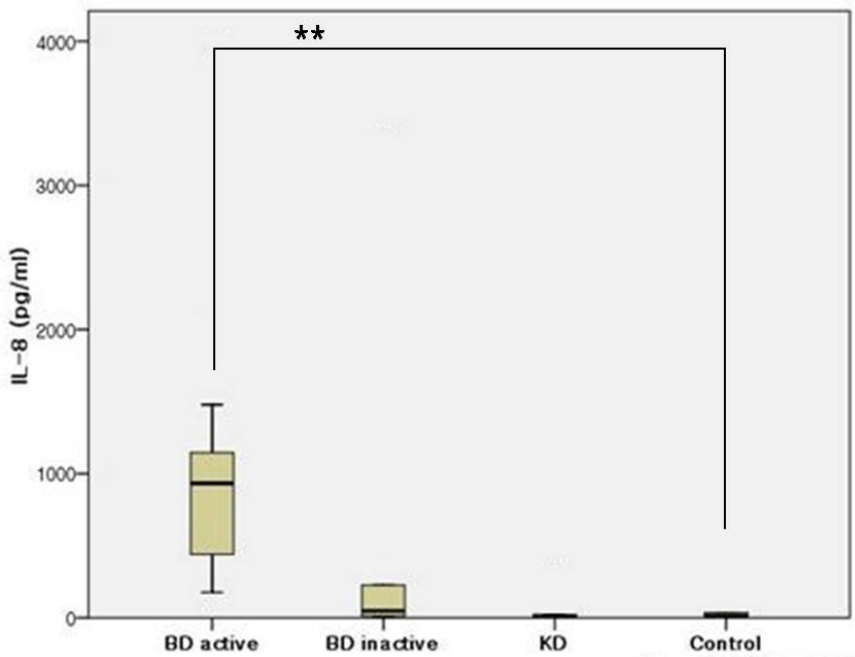

Figure 3. Serum levels of IL-8 in active and inactive period of Behçet's disease, Kawasaki disease and controls. Serum IL-8 was significantly increased in sera from patients in active Behçet's disease period (**$p<0.001$) but not in sera from patients in inactive Behçet's disease period and patients with Kawasaki disease compared with controls.

Serum IL-8 levels decreased from baseline, but it is not statistically significant (p = 0.285). The median serum IL-8 levels were 932 pg/ml (range 176-2207 pg/ml) before treatment and 48 pg/ml (range 11-1132 pg/ml) after treatment. Two patients showed an increase of IL-8 after treatment (Figure 4).

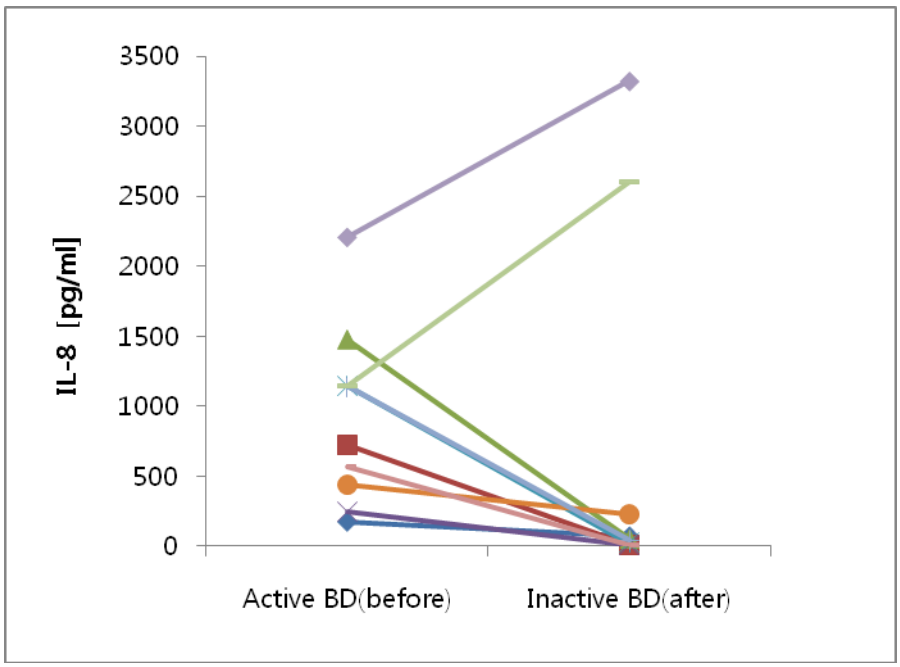

Figure 4. Comparison of serum IL-8 levels between before and after treatment in Behçet's disease patients. Serum IL-8 levels of 10 Behçet's disease patients were evaluated before and after treatment. Serum IL-8 levels decreased from baseline, but it is not statistically significant (p = 0.285).

3. Correlation of serum S100A12 and IL-8 with disease activity in Behçet's disease

Activity score, as a parameter of the severity of BD manifestations present over the previous 4 weeks, was significantly correlated with serum S100A12 levels (Spearman's coefficient=0.464, p=0.039), but not IL-8 (Figure 5).

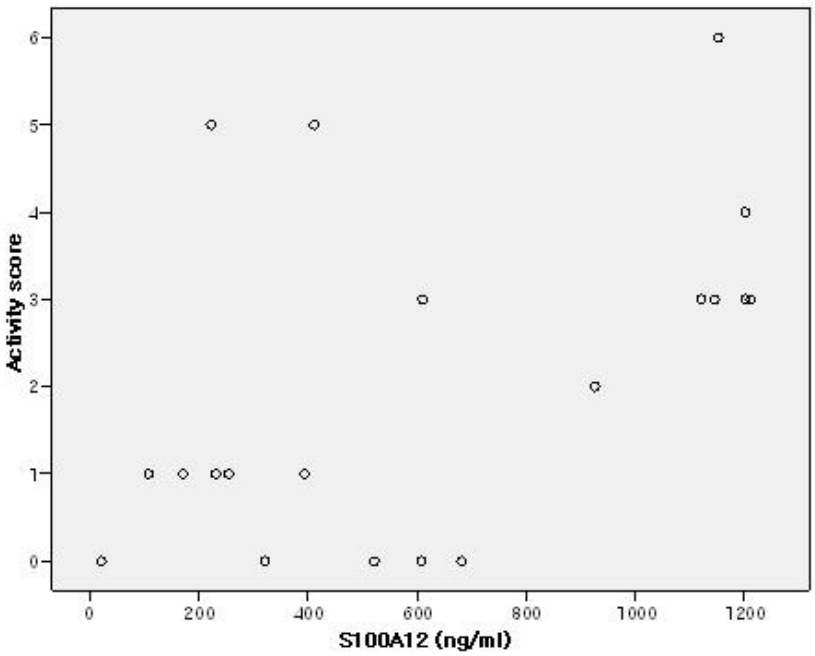

Figure 5. Comparison between activity score and serum S100A12 levels in Behçet's disease patients. Serum S100A12 levels were significantly correlated with disease activity.

4. Immunohistochemical staining with anti-S100A12 antibody

Strong immunoreactivity of S100A12 was seen in the lesions of panniculitis and vasculitis of BD patients. There were S100A12 positive cells in infiltrates.

S100A12 was expressed in an extracelluar distribution surrounding S100A12 positive cells, reflecting secretion of the protein. The extent of reactivity differed between cases, but staining pattern was similar in all biopsies examined. There was only sparse S100A12 expression in control skin (Figure 6).

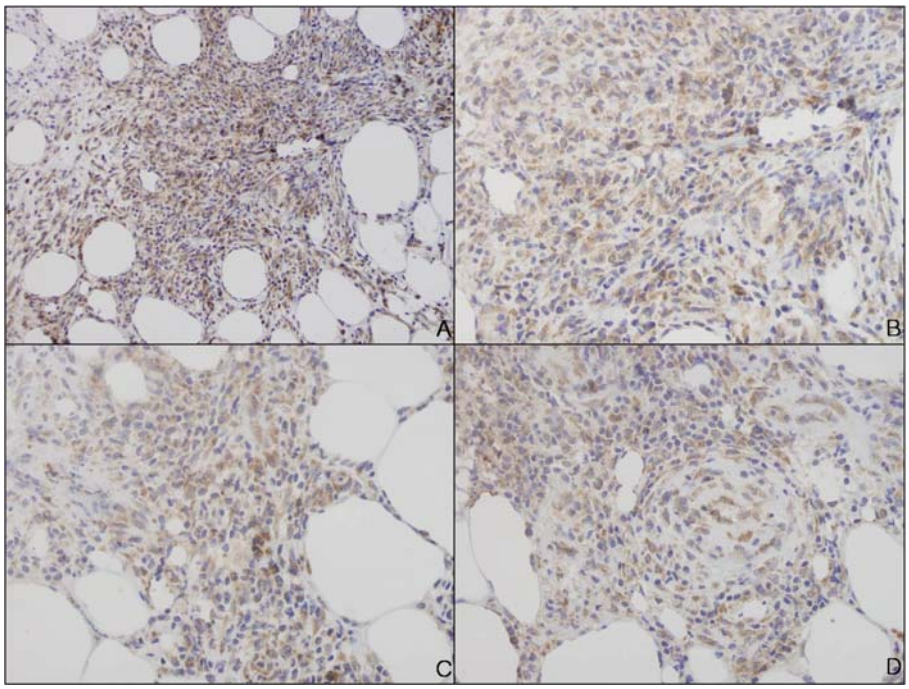

Figure 6. Immunohistochemical staining with anti-S100A12 antibody. Strong expressions of S100A12 throughout polymorphonuclear cells infiltrated area of panniculitis lesion (A,B,C) and vasculitis lesion (D) (original magnification, A: ×200, B,C,D: ×400).

IV. DISCUSSION

BD is a multisystemic inflammatory disorder characterized mainly by vasculitis. Neutrophil infiltration is an important characteristic of the active phase of BD. It has been demonstrated that neutrophils are overactive in BD. Enhanced superoxide, lysosomal enzymes production and chemotaxis of neutrophils from patients with BD were observed[8,9,11]. The mechanism underlying the hyperactivity of neutrophils in BD is not elucidated completely. Th1 type cytokines and chemokines such as IL-8, IL-17, TNF-α and interferon-ɣ (IFN-ɣ) have been suggested to be the cause of neutrophil hyperactivity[32,33]. IL-18, which is produced by antigen presenting cells, may enhance the neutrophil functions[33]. Secretion of S100A12 by activated granulocytes has been demonstrated[17,24,34]. In this study, analysis of S100A12 in erythema nodosum-like skin lesions and sera indicated that this protein is expressed and secreted at local site of inflammation. S100A12 has been reported to be strongly chemotactic for other leukocytes[13,18]. Infiltration of S100A12 positive polymorphonuclear cells may be an early process prior to invasion of mononuclear cells. Ligation of RAGE by S100A12 activates NF-κB and NF-κB activation may protects BD T cells against apoptosis via expression of antiapoptotic genes[13,35]. NF-κB is also induced by TNF-α,

which is found in high amounts in skin lesions and sera of BD patients[36-39]. Activation of RAGE by binding of S100A12 to RAGE results in upregulated expression of proinflammatory cytokines, such as TNF-α, IL-2 and IL-6[13,18]. It has been demonstrated that TNF-α stimulated S100A12 secretion in peripheral neutrophils[24,34]. Released TNF-α may stimulate granulocytes to secrete S100A12. Therefore, TNF-α and S100A12 may constitute an inflammatory positive feedback loop[18]. S100A12 antagonists used in murine model of bovine type collagen induced arthritis and decreased expression in affected tissues[19,20]. Thus, S100A12 is a target for novel anti-inflammatory therapies in autoimmune disorders.

Some studies have shown that expression of adhesion molecules such as ICAM-1 and VCAM-1 of the endothelium is increased in BD patients[40,41]. Endothelial adhesion properties of mononuclear cells and neutrophils are enhanced due to the increased expression of ICAM-1 and VCAM-1 on the endothelium[40]. S100A12 has been reported to enhance the expression of ICAM-1 and VCAM-1 on endothelium[17,18].

In the present study, it was observed that S100A12 serum levels are elevated in BD and these elevations are associated with the clinical disease activity. Serum S100A12 levels decreased significantly after systemic treatment. These results suggest that S100A12 could be used as an activity marker for BD.

Patients with active KD had significantly higher S100A12 levels than healthy controls in this study. In previous reports, elevated serum concentrations of S100A12 have been detected in KD, which is correlated with the inflammatory disease activity[34,42].

The role of IL-8 in BD has been demonstrated in several investigations. IL-8 is a potent chemoattractant and activator of neutrophils[27]. Some studies showed that serum levels of IL-8 in BD patients were increased and correlated with disease activity[27-29]. However, there were contradictory results that IL-8 levels were not related to disease activity in BD patients[43,44]. In this study, serum IL-8 was significantly increased in the active BD, but not increased in the inactive BD period compared with controls. Serum IL-8 levels decreased after systemic treatment, but it is not statistically significant. Serum IL-8 concentrations were not correlated with activity score. Based on this finding, it is thought that S100A12 is a better indicator of BD activity than IL-8 in this study population.

KD is a systemic vasculitis syndrome mainly affecting small and medium sized arteries, particularly the coronary artery[34,42]. Histopathologically, transient infiltration by neutrophils has been observed in the very early stage of acute KD, before infiltration of mononuclear cells, suggesting that neutrophils acts as a trigger in the pathogenesis of coronary artery lesions[45]. It

is usually responsive to treatment with high doses of intravenous immunoglobulin (IVIG) [34,42]. Although neutrophils may be early effector cells for vascular endothelial damage in the acute phase of KD, there are few reports on the role of IL-8 in KD. Lin et al. reported that elevated serum IL-8 levels during the first week of illness may be associated with a higher risk of coronary aneurysm formation[46]. However, Suzuki et al. found that serum IL-8 levels before and after IVIG showed no significant differences and Asano et al. revealed that plasma levels of IL-8 were elevated in the acute phase of KD compared with healthy controls, but the difference was not statistically significant[47,48]. In this study, serum IL-8 was not increased in patients with KD compared with controls. Neutrophilic vasculitis is a common finding in BD and KD. A significant increase of serum S100A12 was observed in both diseases. However, serum IL-8 was significantly increased in the active BD, but not in the active KD. These findings suggest that there are differences in neutrophil activation between BD and KD. It has remained to be elusive.

V. CONCLUSION

An increase in serum S100A12 levels in BD was found in this study. Immunohistochemical studies on erythema nodosum-like skin lesions from patients with active BD showed S100A12 expression in the lesions of panniculitis and vasculitis. These suggest that S100A12 may contribute to BD pathogenesis related to neutrophil hyperactivity. In addition, S100A12 could be used as an activity marker in monitoring the disease activity. However, further studies of larger samples may be helpful for clarification of the role of S100A12 in the pathogenesis of BD.

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ABSTRACT (IN KOREAN)

베체트병의 병인에서 S100A12의 의의와 질병 활성도 지표로서의 효용성

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한은천

베체트병은 여러 장기를 침범하는 만성 염증성 질환으로서 아직 병인이 밝혀지지 않고 있다. 병변의 주요 조직병리학적 소견은 혈관염으로 중성구 또는 단핵구가 침윤되어 있다. 특히 급성기에서 염증 발생과 관련된 증상은 중성구의 과다 활성이 연관되어 있을 것이라고 제시되어 왔다. 중성구에서의 여러 라이소자임 효소의 증가, 과산화물의 생성 증가 및 화학 주성능의 증가를 보인 실험 결과들은 베체트병에서 중성구의 과활성화가 조직 손상을 유발할 수 있음을 뒷받침 해준다.

S100A12는 칼슘 결합 단백으로 S100 단백군에 속하며 중성구에서 특이적으로 발현되고 대식세포, 내피세포, 림프구에서 표현되는 receptor for advanced glycation end products (RAGE)에 결합함으로써 여러 염증 반응들이 유도된다. 특히 세포외 단백으로 존재할 경우 강력한 화학 주성을 나타낸다.

베체트병의 병인에 있어 중성구의 이동과 침윤이 중요한 역

할을 하므로 베체트병 환자의 혈청에서 S100A12을 측정하고 피부병변에서 면역화학염색을 통해 이의 발현 양상을 관찰하여 임상 증상과의 연관성을 알아보고자 본 연구를 시행하였다.

실험 결과에서 혈청 S100A12의 농도는 베체트병 환자의 활성기와 비활성기에서 정상 대조군과 비교하여 유의하게 증가하였으며, 치료 후에 농도가 유의하게 감소하였다. 혈청 S100A12 농도는 질병의 임상적 중증도와 양의 상관 관계를 보였다.

피부의 결절홍반양 병변에서 시행한 면역조직화학염색에서는 S100A12의 현저한 발현을 관찰할 수 있었다.

이러한 결과들은, 베체트병의 병인에 S100A12가 관여하며 이를 베체트병의 활성도를 평가하는 표지자로 이용할 수 있음을 시사해준다.

핵심되는 말: 베체트병, 질병활성도, S100A12, IL-8