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審査学位論文
（博士）
Serial changes of serum cytokines in Crohn’s disease following treatment with adalimumab

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Key words: Crohn's disease, adalimumab, cytokine levels
ABSTRACT

Background/Aims: We examined serum cytokine levels in Crohn’s disease (CD) patients before and after Adalimumab (ADA) treatment. Methodology: A total of 24 patients with CD were enrolled (4 colonic type, 6 ileal type, 14 ileo-colonic type). Patients were divided into two groups according to disease duration. Patients were given ADA (160 mg at week 0 and 80 mg at week 2), followed by maintenance therapy (40 mg every other week). Serum levels of 17 cytokines were simultaneously determined using a Bio-Plex suspension array system before, 4 and 8 weeks after ADA treatment. Serum CRP levels were also measured before, 4 and 8 weeks after treatment. Results: IL-6 and MCP-1 levels were significantly decreased in all CD patients and in the ileo-colonic type 8 weeks after ADA treatment compared to before treatment ($P < 0.05$). MCP-1 levels were significantly decreased 8 weeks after treatment compared to pre-treatment samples if disease onset occurred longer than 8 years. A significant correlation was noted between CRP and IL-6 levels. Conclusions: The reduction of IL-6 and MCP-1 would be an important role for the improvement of inflammation after ADA treatment in CD which might be associated with disease types and disease duration.
INTRODUCTION

Crohn’s disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract that is characterized by recurrent inflammation at any location along its length. Crohn’s disease typically manifests itself by the formation of bowel strictures, ileus, and fistulas. Development of this condition appears to be controlled by type-1 T-helper cells (Th1) and is associated with elevated concentrations of Th1-polarizing cytokines.

CD is associated with a Th-1 immune response, in which Th-1 cells secrete tumor necrosis factor α (TNF-α). TNF-α, a proinflammatory cytokine that plays an important role in CD pathogenesis, can be upregulated by IL-1 and granulocyte macrophage colony-stimulating factor (GM-CSF).

The chimeric anti-TNF-α monoclonal antibody infliximab (IFX) is reportedly useful in induction therapy and maintenance therapy for CD. It binds to TNF-α with high affinity, thereby neutralizing its biologic activity. IFX treatment reduces serum levels of IL-6, IL-7, IL-8, IL-12, and MIP-1β. This reduction appears to mediate the decrease of inflammation in CD. Serum IL-6 and IL-1β concentrations have been shown to be decreased in rheumatoid arthritis after IFX treatment, and IFX decreases serum IL-18 levels in CD patients.

In rheumatoid arthritis (RA) patients after ADA treatment, serum cytokine

Serial changes of serum cytokines in CD following treatment with ADA
(IL-1β, IL-6 and TNF-α) levels were decreased\textsuperscript{13,14}, and matrix metalloproteinase-3 (MMP-3) and CD 68 also showed significant decreases. Because serum levels of IL-6, IL-21, IL-23, and TNF-α were significantly decreased after ADA treatment in responders, the beneficial effect of ADA therapy might involve a decrease in Th17-related cytokines in responders in RA\textsuperscript{15}. ADA and methotrexate (MTX) treatments downregulated peripheral Th17 cells and serum IL-6 levels in RA\textsuperscript{16}. In CD patients, plasma levels of TNF-α and IL-6 were reduced after ADA therapy\textsuperscript{17}, and complete endoscopic healing was associated with a significant reduction of mucosal cytokines IL-17, IL-23, IFN-γ and TNF-α\textsuperscript{18}.

However, the influence of ADA on serial changes in cytokine levels has not been well characterized in CD, especially it has not been well known the association between clinical types or disease durations and cytokine levels after ADA treatment in CD. Therefore, in the present study, we examined cytokine levels in CD patients before and after ADA administration.

**METHODS**

A total of 24 patients with active CD were enrolled (15 men, 9 women; mean age, 35.8 years; 4 colonic type, 6 ileal type, 14 ileo-colonic type; underlying disease
duration, 7.6 years).

Patients were divided into two groups by disease duration. The first group included patients whose disease onset occurred less than 8 years. This group included nine patients who had initiated ADA treatment (6 men, 3 women; mean age, 32.0 years; underlying disease duration, 3.7 years; 2 colonic type, 2 ileal type, 5 ileo-colonic type). The second group included those who had had disease longer than 8 years. This group included 15 patients who had initiated ADA treatment (8 men, 7 women, mean age, 37.6 years; underlying disease duration, 9.9 years; 2 colonic type, 4 ileal type, 9 ileo-colonic type). The demographics of CD patients are shown in Table 1. There were no statistical differences in gender, age, disease duration, or Crohn’s disease activity index (CDAI) between colonic, ileal and ileo-colonic types of CD.

Patients were given ADA (160 mg at week 0 and 80 mg at week 2), followed by scheduled maintenance therapy (40 mg every other week). Treatments were followed by physical examination and CDAI scoring before, 4 and 8 weeks after treatment. Serum levels of CRP were also measured before, 4 and 8 weeks after ADA treatment.

We determined the levels of 17 serum cytokines (including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNF-α, INF-γ, granulocyte colony-stimulating factor (G-CSF), GM-CSF, macrophage inflammatory protein-1β) Serial changes of serum cytokines in CD following treatment with ADA
Serial changes of serum cytokines in CD following treatment with ADA (MIP-1β), and macrophage chemoattractant protein-1 (MCP-1) before, 4 and 8 weeks after treatment using a Bio-Plex human cytokine 17-plex panel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a Bio-Plex suspension array system (Bio-Rad Laboratories, Inc.). The range of detection for all targets was 2 - 32,000 pg/mL. Serum samples (minimum volume, 12 µL) were diluted (1:4) using Bio-Plex human serum diluent kits (Bio-Rad Laboratories, Inc.). Assays were performed according to the manufacturer’s instructions. Briefly, 50 µL of each serum sample, diluted 1:4, were added to a suspension of beads coated with 17 primary antibodies in each well of an assay plate and incubated for 30 min at room temperature with shaking at 300 rpm. After incubation, the beads were washed with washing buffer three times and subsequently reacted with a mixture of 17 types of biotin-conjugated secondary antibody. After a 30 min reaction, the beads were again washed and resuspended in assay buffer containing streptavidin-phycoerythrin (Str-PE). After 10 min of agitation at room temperature, the beads were washed and resuspended in assay buffer without Str-PE. Cytokine concentrations were measured using a Bio-Plex Array Reader (Bio-Rad Laboratories, Inc.). Cytokine types were identified on the basis of the luminescence of the beads, and cytokine concentrations captured by primary antibody on the beads were determined using PE luminescence. Standard curves for cytokines
were generated using the reference cytokine concentrations supplied by the manufacturer. Duplicate examination was performed for each sample.

Further, all patients provided informed consent after they were informed of the study purpose and the nature of the procedures involved. Adherence was made to the Principle of Good Clinical Practice and the Declaration of Helsinki at all times.

STATISTICAL ANALYSIS

Data were expressed as means (SD) or as median (25th-75th percentiles) if appropriate. Within-group comparisons were analyzed by paired Student’s t-test or a non-parametric approach using the Mann Whitney U test. Baseline comparisons between groups were performed using one-way analysis of variance. Correlations were expressed using Spearman’s rank correlation coefficient. A P value < 0.05 was considered to be statistically significant. Data were analyzed using SPSS II for Windows (version 11.0 J; SPSS, Inc., Chicago, IL, USA).

RESULTS

CDAI Score

CDAI scores for all CD patients averaged 230.8 ± 140.5 before treatment,

Serial changes of serum cytokines in CD following treatment with ADA
164.7 ± 83.4 after 4 weeks of treatment, and 144.3 ± 91.0 after 8 weeks of treatment. CDAI scores 4 and 8 weeks after treatment were significantly decreased compared to those prior to treatment ($P < 0.05$) (Figure 1). No significant differences in CDAI scores were observed before, 4 and 8 weeks after treatment in colonic, ileal or ileo-colonic types. No significant differences in CDAI scores were also observed before and after treatment in patients treated less than 8 years of disease onset or in patients who had had the disease for longer than 8 years.

**Serum CRP levels**

Serum CRP levels in the total CD patient population averaged 1.84 ± 2.32 prior to treatment, 0.74 ± 1.12 after 4 weeks, and 0.71 ± 1.45 after 8 weeks of treatment. CRP levels 4 and 8 weeks after treatment were significantly decreased compared to those before treatment ($P < 0.05$). CRP levels with ileo-colonic type of CD patients 4 weeks after treatment were significantly decreased compared to those before treatment. No significant differences in serum CRP levels were observed 4 and 8 weeks after treatment in colonic or ileal types, and 8 weeks after treatment in the ileo-colonic type. No significant differences in serum CRP levels were observed 4 and 8 weeks after treatment in patients treated less than 8 years of disease onset or in patients who had had the
Cytokine levels

Among (serum) 17 cytokines, serum IL-6 and MCP-1 levels were significantly decreased 8 weeks after treatment compared to those before treatment in all CD patients ($P < 0.05$) (Table 2). Especially in the ileo-colonic type, serum IL-6 levels were significantly decreased both 4 and 8 weeks after treatment compared to those before treatment. Moreover, MCP-1 levels were significantly decreased 8 weeks after treatment compared to those before treatment (Figure 2). No significant differences in serum levels of any of the 17 cytokines were observed before, 4 and 8 weeks after treatment in either ileal or colonic types. Serum MCP-1 levels were significantly decreased 8 weeks after treatment compared to those before treatment in patients who had had the disease for longer than 8 years (Figure 3). In contrast, no significant differences in serum levels of any of the 17 cytokines were observed before and after treatment in patients treated less than 8 years of disease onset.

Relationship between CDAI scores and cytokine levels

No significant correlations were observed between the 17 serum cytokines and...
Relationship between CRP levels and cytokine levels

A significant correlation was noted between CRP levels score and serum IL-6 levels before and after treatment in CD patients ($r=0.54$, $P < 0.001$) (Figure 4). No significant correlations were observed between other cytokines and CRP levels.

DISCUSSION

Recent therapies have targeted pro-inflammatory cytokines for CD management, as exemplified by the administration of anti-TNF-a-depleting antibodies\(^{19}\).

Chemotactic cytokines (chemokines) are likely involved in the upregulation, perpetuation, and exacerbation of inflammatory and tissue-destructive processes involved in the immunopathogenesis of inflammatory bowel disease (IBD). The expression of MCP-1 was markedly increased in inflamed intestinal specimens from patients with ulcerative colitis (UC) and CD. In the present study, we simultaneously analyzed 17 serum cytokines using a multiplex assay system to elucidate the serial changes in their levels that are clinically and statistically associated with the efficacy of ADA therapy in patients with active CD. We demonstrated that IL-6 and MCP-1 levels
were decreased following ADA administration.

The macrophage is a pivotal mediator of innate immunity and appears to play a key role in stimulating the subsequent development of adaptive immunity in CD\textsuperscript{20}). During inflammation, chemokines, and various peptide and nonpeptide mediators of inflammation are generated locally and stimulate monocytes to migrate into the site of inflammation where they differentiate into macrophages. One mechanism underlying monocyte infiltration during acute inflammatory processes is the increased expression of MCP-1\textsuperscript{21}).

The ability of epithelial cells to produce chemokines could play an important role in initiating the immunopathophysiologic events associated with the upregulation and perpetuation of inflammation in UC and CD\textsuperscript{22}). MCP-1 levels were upregulated in both UC and CD\textsuperscript{22,23}), and the cells responsible for MCP-1 production lie deep in the lamina propria adjacent to the muscularis mucosa. Immunohistochemistry showed that MCP-1 was expressed by macrophages, smooth muscle cells, and endothelial cells in IBD\textsuperscript{24}). Moreover, constitutive MCP-1 expression by epithelial cells was detected, and this may contribute to the mucosal immune response by attracting monocytes and T lymphocytes\textsuperscript{22}). Though MCP-1 is expressed constitutively in the intestinal colonic mucosa by surface epithelium, during active CD it is upregulated in many different cells.
within the lamina propria, including mononuclear cells resembling lymphocytes and macrophages. Cytokines, such as IL-1 and TNF-α, are known to induce MCP-1 secretion by the activation of transcription factor NF-κB.

IL-6 is a pleiotropic cytokine responsible for T-cell stimulation and proliferation and is primarily secreted by macrophages and monocytes promoting granulocyte and macrophage colony formation. IL-6 has also been associated with CD because of its capability to induce synthesis of acute phase proteins and inflammatory chemical pathways. Previous studies suggested that adult patients with CD have higher serum or plasma levels of IL-6 compared with controls. The predominant sources of IL-6 in patients with IBD during inflammation are activated monocytes, macrophages, and, to a lesser extent, epithelial cells. Serum IL-6 is a sensitive marker of immune activation and inflammatory states in IBD, elevated serum IL-6 levels have been detected in both acute and chronic inflammation, and altered IL-6 production has been observed in various inflammatory conditions, including CD. In fact, the IL-6 signaling pathway is crucial to the pathogenesis and physiopathology of CD. Anti-IL-6 was proposed as a useful anti-inflammatory agent in IBD and other autoimmune diseases and some success in the treatment of RA with this agent has been reported.

Serial changes of serum cytokines in CD following treatment with ADA
In this study, decreases in IL-6 and MCP-1 levels after ADA treatment were observed in the ileo-colonic type. Furthermore, reduced MCP-1 levels after ADA treatment were observed in patients who had the disease for longer than 8 years. The reasons for this result may be attributed to the number of patients in the ileal and colonic types because the number of patients who had the disease for less than 8 years was low. Further study is required to clarify the relationship between the effect of ADA and changes in cytokine levels in patients with CD of different types and durations. Additional analyses of the Th17 subset should be included in future studies.

Our results showed that following ADA treatment, changes of serum IL-6 levels showed a positive and significant correlation with CRP levels, although similar relationships were not identified for the other cytokines. Therefore, at present, serum IL-6 level is considered to be a potentially useful marker of the efficacy of ADA treatment in CD patients.

In conclusion, the reduction of IL-6 and MCP-1 would be an important role for the improvement of inflammation after ADA treatment in CD which might be associated with disease types and disease durations.

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Serial changes of serum cytokines in CD following treatment with ADA
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CONFLICT OF INTEREST

The authors have no conflict of interest in connection with the publication of this manuscript.
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manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest

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Serial changes of serum cytokines in CD following treatment with ADA


Serial changes of serum cytokines in CD following treatment with ADA
Figure Legends

Figure 1: CDAI scores 4 and 8 weeks after treatment were significantly decreased compared to those prior to treatment (P < 0.05).

Figure 2: In the ileo-colonic type, serum IL-6 levels were significantly decreased both 4 and 8 weeks after treatment compared to those before treatment. MCP-1 levels were significantly decreased 8 weeks after treatment compared to those before treatment.

Figure 3: Serum MCP-1 levels were significantly decreased 8 weeks after treatment compared to those before treatment in patients who had had the disease for more than 8 years.

Figure 4. Correlation between changes in CRP levels and serum IL-6 levels before and after treatment in CD patients. A significant correlation was noted between CRP levels and serum IL-6 levels before and after treatment in CD patients (r=0.54, P < 0.001)
Table 1. Characteristics of CD patients

<table>
<thead>
<tr>
<th>CD</th>
<th>Colonic</th>
<th>ileal</th>
<th>ileo-colonic</th>
<th>Total</th>
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<tbody>
<tr>
<td>Number</td>
<td>4</td>
<td>6</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td>Gender (n)</td>
<td>Male / Female</td>
<td>3 / 1</td>
<td>3 / 3</td>
<td>8 / 6</td>
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<tr>
<td>Age (years)</td>
<td>Mean ± SD</td>
<td>32.8 ± 15.1</td>
<td>42.3 ± 7.8</td>
<td>33.6 ± 8.7</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>8.0</td>
<td>7.0</td>
<td>8.3</td>
<td>7.6</td>
</tr>
<tr>
<td>8 years&gt; (n)</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>8 years&lt;= (n)</td>
<td>2</td>
<td>4</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>CDAI</td>
<td>Mean ± SD</td>
<td>240.1 ± 228.0</td>
<td>155.3 ± 68.0</td>
<td>260.5 ± 132.5</td>
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Table 2: Cytokine levels before and after ADA: All patients

<table>
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<th>(pg / mL)</th>
<th>Before</th>
<th>4 weeks after</th>
<th>8 weeks after</th>
</tr>
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<td>IL-1β</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
</tr>
<tr>
<td>IL-2</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
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<tr>
<td>IL-4</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.0 [2.0 – 15.2]</td>
<td>2.0 [2.0 – 8.2]</td>
<td>2.0 [2.0 – 3.4] *</td>
</tr>
<tr>
<td>IL-7</td>
<td>8.0 [6.4 – 10.4]</td>
<td>7.7 [5.7 – 11.3]</td>
<td>7.7 [5.5 – 10.8]</td>
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<tr>
<td>IL-8</td>
<td>17.4 [9.8 – 33.6]</td>
<td>13.4 [11.5 – 39.5]</td>
<td>12.4 [8.6 – 33.3]</td>
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<tr>
<td>IL-10</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
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<tr>
<td>IL-12 (p70)</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 3.3]</td>
<td>2.0 [2.0 – 2.0]</td>
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<tr>
<td>IL-13</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
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<td>IL-17</td>
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<td>2.0 [2.0 – 2.0]</td>
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<tr>
<td>G-CSF</td>
<td>4.8 [2.0 – 9.5]</td>
<td>3.4 [2.0 – 7.3]</td>
<td>3.4 [2.0 – 7.8]</td>
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<tr>
<td>GM-CSF</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
<td>2.0 [2.0 – 2.0]</td>
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<tr>
<td>IFN-γ</td>
<td>2.0 [2.0 – 3.6]</td>
<td>2.0 [2.0 – 7.2]</td>
<td>2.0 [2.0 – 6.0]</td>
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<tr>
<td>MCP-1</td>
<td>21.8 [14.7 – 32.6]</td>
<td>20.5 [12.1 – 29.8]</td>
<td>16.3 [7.5 – 23.0] *</td>
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<td>MIP-1β</td>
<td>135.0 [66.3 – 200.8]</td>
<td>119.5 [50.0 – 172.5]</td>
<td>99.2 [65.1 – 169.3]</td>
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<td>TNF-α</td>
<td>2.0 [2.0 – 3.0]</td>
<td>2.0 [2.0 – 2.8]</td>
<td>2.0 [2.0 – 4.3]</td>
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</table>

*P<0.05 vs. before
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