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Original Article

Changes in cytokine profile may predict therapeutic efficacy of infliximab in patients with ulcerative colitis

Running title: Cytokines and infliximab in UC

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Abstract

Background and Aim: Infliximab is an established therapy for ulcerative colitis (UC). The aim of this study was to examine various serum cytokine levels and to identify possible markers predictive of therapeutic efficacy of infliximab for UC patients.

Methods: Twenty-one patients with moderately active UC were given intravenous infliximab (5 mg/kg) at 0, 2 and 6 weeks as induction therapy. The serum levels of 17 cytokines were determined using a Bio-Plex suspension array system before and 8 weeks after induction therapy. Partial Mayo score and serum C-reactive protein (CRP) levels were used for the determination of clinical activities at 0, 8 weeks after the treatment. The overall therapeutic effect was determined at 26 weeks according to the partial Mayo score.

Results: The median value of the partial Mayo score decreased significantly 8 weeks after the treatment (from 6 to 1.5, $P < 0.05$). However, CRP levels did not change significantly. Levels of serum interleukin (IL)-8 ($P < 0.05$) and macrophage inflammatory protein (MIP)-1β ($P < 0.005$) significantly decreased 8 weeks after the induction. Serum levels of the other 15 cytokines did not change significantly. At 26 weeks, 13 of 20 patients (65%) were responders while 7 patients were non-responders. Levels of serum IL-6 at 8 week were significantly lower in responders than in non-responders ($P < 0.05$).

Conclusions: Serum IL-8 and MIP-1β seem to be sensitive markers for UC patients treated with infliximab, while IL-6 at 8 week after induction therapy may be predictive of subsequent response to infliximab.

Key words: ulcerative colitis, infliximab, IL-6, IL-8, MIP-1β

Introduction

Ulcerative colitis (UC) is a chronic inflammatory disorder of obscure origin.[1-3] Whereas the disease is intractable to medical therapies, it has been shown that infliximab, a chimeric monoclonal antibody to tumor-necrosis factor alpha (TNF-α), is efficacious for the induction and the maintenance of remission for patients with UC.[4,5] Cellular effects of TNF-α are diverse and include cell proliferation, differentiation and apoptosis.[6,7] Infliximab not only neutralizes soluble TNF-α but also damages cells by the antibody-dependent cell death pathway.[4.5] It has also been assumed that alterations of the mucosal cytokine profile may be an important action of infliximab for UC. Rismo et al[8] reported that high expression of Th1- and Th17-related cytokines in the mucosa of UC patients were predictive of a favorable outcome of infliximab induction therapy. However, changes in various circulating cytokine profiles prior to and after infliximab treatment have not been extensively examined.

In the present study, we investigated patients with UC who were treated with infliximab. We assessed serial changes in serum cytokine profiles in order to elucidate whether there were any specific cytokines, that could serve as biomarkers of the disease and that predicted the efficacy of infliximab.

Methods

Patients and study protocol. During the period between August 2010 and December 2013, we enrolled 21 Japanese patients with moderately active UC with a Mayo score of 5-10 points (partial Mayo score of 3-9 points).[4,9] The diagnosis of UC was based on clinical, radiological, endoscopic, and pathological findings consistent with the disease. None of the patients had undergone biological treatment (i.e., infliximab or adalimumab). Table 1 indicates demographic data describing the study patients. There were 9 men and 12 women with a median age of 34 years. Eleven patients had total colitis and 10 patients had left-sided colitis. The duration of the disease ranged

from 17 to 225 months with a median of 96 months. Thirteen patients were steroid dependent, and 7 patients were steroid naïve. All patients were given intravenous infliximab (5mg/kg) at 0, 2 and 6 weeks as induction therapy. Subsequently, infliximab was administered every 8 weeks for maintenance therapy. Blood samples for cytokine analyses and serum levels of C-reactive protein (CRP) were obtained before and 8 weeks after the administration of infliximab. Serum CRP measurements were performed by immunonephelometry with the Behring Nephelometer Analyzer II using the N High Sensitivity kit (Dade Behring, Marburg, Germany) according to the manufacturer's instructions.[10] This study was approved by Human Ethics Review Committee of Iwate Medical University. Informed consent was obtained from each patient prior to the enrollment.

Assessment of disease activity and therapeutic effect of infliximab. The partial Mayo score (PMS; from 0 to 9 points)[9] was used for the determination of clinical activities at 0, 8 and 26 weeks after the treatment. Clinical response was defined as a decrease in the PMS of 2 points or more from the baseline value and a decrease of 30 % or more from baseline.[11] Response to infliximab treatment was evaluated at weeks 8 and 26 according to PMS. The responder was defined as a patient with a clinical response 26 weeks after the start of infliximab treatment, while the non-responder was defined as a patient without clinical response 26 weeks after treatment.

Assessment of cytokine profile. Serum aliquots for cytokine measurement were stored at -80°C until the assay. Serum levels of 17 cytokines were determined using a Bio-Plex human cytokine 17-plex panel and a Bio-plex suspension array system (Bio-Rad Laboratories, Inc. Hercules, CA, USA).[12,13] We assessed the levels of interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ,

TNF-α, granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1β and monocyte chemotactic protein (MCP)-1. 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.[12,13] Briefly, 50 μl of each diluted serum sample 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 the incubation, the beads were washed 3 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 re-suspended in assay buffer containing streptavidin-phycoerythrin (Str-PE). After 10 min of agitation at room temperature, the beads were washed and re-suspended 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. For statistical analysis, cytokine concentrations of less than the lower limit of detection were given a value of half of the limit of detection.[14]

Statistical analysis. Numerical data are expressed as the median (range). Comparisons of the serum cytokine levels between groups were assessed by the Mann-Whitney *U* test. For comparisons within the same subjects, the Wilcoxon signed-rank test was applied. Correlations between PMS and serum parameters, such as CRP or cytokine levels, were evaluated using Spearman's rank correlation coefficient test. A receiver operating characteristic (ROC) curve was generated by plotting the sensitivity against one minus specificity, and the area under the curve (AUC) was calculated. All the statistical analyses were performed using the JMP7 software package

(SAS Institute, Cary, NC, USA). In each test, a *P* value less than 0.05 was considered statistically significant.

Results

Efficacy of infliximab. The median value of PMS for 21 patients was 6 (range, 3 - 9) at baseline and it significantly decreased to 1.5 (0-6) after 8 weeks of treatment ($P < 0.001$; Fig. 1a). At 8 weeks, a clinical response was achieved in 16 patients (76%), but not in the other 5 (24%). Serum CRP was 0.35 (0.1 - 0.9) mg/dL at baseline and 0.1 (0.1 - 6.12) mg/dL 8 weeks after the initiation of treatment ($P = 0.208$; Fig. 1b). The CRP values did not decrease significantly in patients with total colitis or in those with left-sided colitis.

Changes in cytokine profiles. Table 2 shows serum cytokine concentrations and disease activities at baseline and 8 weeks after induction therapy with infliximab. Serum IL-8 and MIP-1β levels significantly decreased 8 weeks after the initiation of treatment when compared to those at baseline ($P = 0.024$ and 0.002, respectively; Fig. 2 a, b). Whereas there was a trend towards a decrease in IL-6 levels 8 weeks after the treatment, the difference did not reach statistical significance ($P = 0.057$). No significant differences were observed in the other 14 cytokines between baseline and 8 weeks after treatment. No significant correlation was observed between PMS and any of the cytokines, including IL-8 and MIP-1β, at baseline (data not shown). At 8 weeks, there was a statistically significant correlation between IL-6 and PMS ($r = 0.529$, $P = 0.049$).

Comparison of clinical features between responders and non-responders. One patient was excluded from the analysis because the patient refused scheduled maintenance infliximab administration. At 26 weeks, 13 of the 20 patients (65%) were

responders and 7 patients (35%) were non-responders. Table 3 compares the clinical characteristics of patients between responders and non-responders. Whereas the value of PMS at baseline was not different between the two groups, the score was significantly lower in responders than in non-responders at 8 weeks and 26 weeks ($P < 0.001$). In responders, the value of PMS at 8 weeks (1 [0 - 4]) had significantly decreased compared to that at baseline (8 [3 - 9], $P < 0.001$). In non-responders, the value of PMS did not change significantly. In responders, the value of PMS at 26 weeks (0 [0-3]) significantly decreased compared to that before treatment (8 [3-9], $P < 0.001$). No significant differences were observed between the two groups with regard to age, gender, disease duration, disease extent, concomitant medications, or CRP levels.

Comparison of cytokine levels between responders and non-responders. Table 4 compares serum cytokine levels between responders and non-responders, at baseline and 8 weeks after the induction of infliximab, respectively. None of the 17 cytokine levels at baseline differed between responders and non-responders. At 8 weeks, however, serum levels of IL-6 were significantly lower in responders than in non-responders ($P = 0.030$, Fig. 3a). Whereas serum levels of IL-1β and TNF-α at 8 weeks seemed to be lower in responders than non-responders, the differences were not statistically significant ($P =0.063$ and 0.096, respectively). There was not any difference in the other cytokines, including IL-8 ($P = 0.410$), between responders and non-responders. The AUC from ROC analysis for IL-6 levels 8 weeks after the induction of infliximab was 0.8022 when the cut-off value was set at 0.71 pg/mL; the sensitivity and specificity for predicting a response were 0.9231 and 0.7143, respectively (Fig. 3b).

Discussion

While infliximab has been established as a useful therapeutic strategy for patients with moderate-to-severe UC, predicting the efficacy of the treatment has been difficult. In the present study, we examined serum cytokine profiles in patients with active UC under induction therapy with infliximab in order to elucidate whether any cytokine could predict long-term efficacy of the medication. Consequently, we found that serum IL-8 and MIP-1β decreased significantly during the induction, and that IL-6 at 8 weeks may be predictive of subsequent response to infliximab in UC.

The pathogenesis of inflammatory bowel disease (IBD), that is, UC and Crohn's disease (CD), is based on complicated cytokine-mediated signaling pathways. Recent investigations have shown that most of the pathways are induced by intestinal T-cell activation via inflammatory mediators. The inflammatory mediators include a number of cytokines such as TNF-α, IL-1β, IL-6, IL-12, IL-23, and IL-10.[15-18] In our present study, IL-8 and MIP-1β were remarkable parameters showing significant decrease after induction therapy with infliximab in patients with UC.

IL-8 is a chemokine that recruits and activates neutrophils, that are abundant in the intestinal lesions of UC.[19,20] IL-8 expression increased significantly in active UC mucosa and is therefore thought to play an important role in the perpetuation of inflammation in UC. Elevated levels of IL-8 mRNA and protein have been widely demonstrated in UC when compared to histologically normal colonic mucosa, and both active UC and CD patients express increased levels of IL-8. The majority of previous studies on IL-8 expression in the mucosa of UC and CD have demonstrated that IL-8 was more likely to be predominant in active than in inactive disease.[19,20] In our present study, serum IL-8 levels decreased significantly at 8 weeks, whereas such a decrease was not found in CRP (Fig.1). These results suggest that serum IL-8 is a more reliable biomarker than CRP for the assessment of disease activity in UC under induction therapy with infliximab.

In general, serum CRP is believed to be a reliable marker for early response to

infliximab in patients with CD and UC.[21-23] However, CRP values did not change significantly after the induction of infliximab in our subjects (Fig.1b). While the reason for the insignificant change in CRP remains obscure, medications prior to the administration of infliximab, such as 5-aminosalicylic acid, corticosteroid and/or immunomodulators might have influenced the CRP levels at baseline.

The frequency of MIP-1α and MIP-1β expressing cells has been shown to be significantly greater in severely inflamed gut mucosa than in controls.[24,25] These studies showed that both MIP-1α and MIP-1β levels were significantly increased in both CD and UC patients compared with controls. MIP-1β production has never been localized to specific cell types in IBD mucosa, but rather epithelial expression was positive at cell-to-cell junctions, especially in the more severely inflamed tissue. In addition to MIP-1β, MCP-1 was also reported to be expressed in severely inflamed mucosa of patients with active IBD.[24-26] In our present study, serum MIP-1β decreased significantly after induction therapy with infliximab, whereas we did not find such a drastic change in MCP-1. It has been shown that medications for UC such as hydrocortisone, 5-aminosalicylic acid and cyclosporine reduce expression and production of MCP-1.[26] Our subjects had been treated by such medications prior to enrollment for this investigation. It thus seems possible that infliximab may have directly or indirectly affected production of MIP-1β, thereby resulting in therapeutic efficacy.

We also examined whether the production of any of the 17 cytokines (including IL-8 and MIP-1β) differed before or 8 weeks after infliximab infusion between responders and non-responders as defined at 26 weeks. We found that prior to treatment, the serum levels of the cytokines examined here did not differ between the responders and non-responders (Table 4). At 8 weeks after the first infusion, however, we found that serum level of IL-6 was significantly lower in responders than in non-responders. In addition, only IL-6 levels at 8 weeks correlated with PMS

significantly. The AUC from ROC analysis using IL-6 (0.8022) was sufficient to establish the predictive factors for a long-term therapeutic response to infliximab. Because the number of patients in this study was small, the validity including the cut-off value (0.71 pg/dL) should be validated in future studies with a larger number of patients.

It has been demonstrated that production of IL-6 by mucosal macrophages and CD4+ T cells is increased both in experimental colitis and in human IBD.[15-17] IL-6 can exert pro-inflammatory functions by activating antigen presenting cells and T cells. In addition, the IL-6-soluble IL-6 receptor complex prevents apoptosis of mucosal T cells and activates pro-inflammatory cytokine production.[15,17] Furthermore, blockade of IL-6 signaling by monoclonal antibodies is effective in suppressing intestinal inflammation in mouse models, which suggests IL-6 as a potential therapeutic target in IBD.[15,16]

The present study has several limitations. First, we could not examine circulating infliximab concentration or antibody to infliximab, which is inevitably associated with the long-term clinical response to infliximab. In addition, the number of patients was small, which might cause a type 2 error for the comparison. Thus, our results need to be validated for the clinical application of the identified cytokines. Finally, we did not examine endoscopic severity in our study population. Therefore, our data may not be applicable to mucosal healing of the disease. However, we believe that this study is important since this is the first prospective study focusing on circulating cytokines including IL-6, IL-8 and MIP-1β in UC patients treated by induction therapy with infliximab.

In conclusion, our investigation indicated that among 17 cytokines measured, IL-8 and MIP-1β may be sensitive markers for the assessment of patients with UC under induction therapy with infliximab. Furthermore, the response of circulating IL-6 during the induction therapy may be predictive of subsequent therapeutic efficacy

of infliximab, and presumably, predictive of loss of response to infliximab.

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Conflicts of interest

None.

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27. **Table 1** Baseline characteristics of patients with UC

Age at entry (years, range)	35 (16-62)
Gender (male / female)	9 / 12 (43% / 57%)
Duration of UC (months, range)	96 (17-225)
Extent of disease	
Total colitis	11 (52%)
Left-sided colitis	10 (48%)
Concomitant medications	
5-Aminosalicylates	21 (100%)
Corticosteroids	14 (67%)
Azathioprine/6-mercaptopurine	8 (38%)
Mayo score (range)	8 (5-10)
Partial Mayo score (range)	6 (3-9)
CRP (mg/dL, range)	0.3 (0.10-3.90)
Current smoker	0 (0%)

Table 2 Changes of serum cytokine levels and disease activities at baseline and 8 weeks after induction therapy with infliximab

Cytokines	Baseline (pg/mL)	8 weeks (pg/mL)	*P* value
IL-1β	0.6 (0.2-1.7)	0.5 (0.2-54.6)	0.490
IL-2	0.3 (0.3-2.3)	0.3(0.3-443.2)	0.280
IL-4	0.3 (0.3-3.7)	0.3 (0.3-12.2)	0.440
IL-5	0.1 (0.1-0.3)	0.1 (0.1-6.6)	0.620
IL-6	4.4 (0.2-47.1)	0.2 (0.2-582.9)	0.057
IL-7	9.6 (0.2-12.7)	8.0 (0.2-186.3)	0.230
IL-8	15.0 (0.1-110.2)	7.5 (0.1-425.6)	0.024
IL-10	0.7 (0.3-45.9)	0.4 (0.1-95.8)	0.360
IL-12	5.7 (0.2-78.7)	3.7 (0.2-1838.4)	0.980
IL-13	2.2 (0.1-23.1)	2.3 (0.1-17.8)	0.590
IL-17	0.5 (0.4-132.6)	0.5 (0.5-104.4)	0.470
G-CSF	3.5 (1-89.3)	1.0 (1.0-205.8)	0.290
GM-CSF	6.6 (6.6-275.2)	6.6 (6.6-1418.8)	1.000
IFN-γ	20.0 (5.6-56.6)	20 (20.0-6276.4)	0.110
MCP-1	15.1 (1.7-193.1)	19.4 (3.8-405.3)	1.000
MIP-1β	388.5 (0.6-1126.9)	271.8 (0.6-766.3)	0.003
TNF-α	0.4 (0.2-20.7)	0.4 (0.2-377.6)	0.550
PMS	6 (3-9)	1.5 (0-6)	<0.001
CRP (mg/dL)	0.3 (0.10-3.90)	0.1 (0.10-6.12)	0.208

Table 3 Comparison of responders and non-responders at 26 weeks after the infliximab induction therapy

	Responders (n=13)	Non-responders (n=7)	*P* value
Age (years)	37 (16-62)	28 (21-50)	0.320
Gender (male / female)	5 / 8	3 / 4	0.560
Duration of UC (months)	124.4 (17-225)	71 (22-192)	0.080
Extent of disease			
Total colitis	8 (62%)	3 (43%)	0.500
Left side colitis	5 (38%)	4 (57%)	
Concomitant medications			
5-Aminosalicylates	13 (100%)	7 (100%)	1.000
Corticosteroids	10 (77%)	4 (57%)	0.480
Azathioprine/6-mercaptopurine	6 (46%)	2 (29%)	0.530
Partial Mayo score			
Baseline	8 (3-9)	6 (4-8)	0.610
8 weeks	1 (0-4)*	6 (4-6)	<0.001
26 weeks	0 (0-3)*	5 (4-7)	<0.001
CRP (mg/dL)			
Baseline	0.2 (0.10-3.90)	0.5 (0.10-2.65)	0.970
8 weeks	0.1 (0.10-6.12)	0.2 (0.10-2.40)	0.150
26 weeks	0.1 (0.10-6.21)	0.3 (0.1-2.98)	0.032

* $P < 0.001$ compared to baseline.

Table 4 Comparison of cytokine levels at baseline and 8 weeks after induction therapy between responders and non-responders

	Baseline (pg/mL)			8 weeks (pg/mL)		
	Responder (n=13)	Non-responder (n=7)	*P* value	Responder (n=13)	Non-responder (n=7)	*P* value
IL-1β	0.5 (0.2-19.6)	0.7 (0.4-1.7)	0.300	0.4 (0.2-1.3)	0.7 (0.3-54.6)	0.063
IL-2	0.3 (0.3-4.0)	0.3 (0.3-0.3)	0.580	0.3 (0.3-1.2)	0.3 (0.3-443.2)	0.780
IL-4	0.3 (0.3-4.3)	0.3 (0.3-1.7)	0.750	0.3 (0.3-2.6)	0.3 (0.3-12.2)	0.170
IL-5	0.1 (0.1-6.6)	0.1 (0.1-0.2)	0.940	0.1 (0.1-0.3)	0.1 (0.1-0.1)	0.250
IL-6	5.0 (0.2-47.1)	5.0 (0.2-31.1)	0.940	0.2 (0.2-30.4)	2.6 (0.2-582.9)	0.030
IL-7	9.1 (0.2-12.7)	10.1 (7.1-12.4)	0.720	7.1 (1.3-186.3)	8.2 (6.5-17.5)	0.580
IL-8	14.9 (0.1-79.1)	18.3 (5.4-110.2)	0.220	7.1 (0.1-425.6)	9.8 (4.7-17.9)	0.410
IL-10	1.0 (0.3-45.9)	0.3 (0.3-7.0)	0.480	0.3 (0.3-95.8)	0.3 (0.1-53.4)	0.970
IL-12	5.0 (0.2-78.7)	9.8 (0.2-22.2)	0.400	1.4 (0.1-88.5)	6.4 (0.2-1838.4)	0.130
IL-13	1.8 (0.1-23.1)	2.5 (1.6-10.3)	0.210	0.6 (0.1-17.8)	3.4 (1.4-6.2)	0.360
IL-17	0.4 (0.4-132.6)	0.4 (0.4-0.4)	0.250	0.4 (0.4-104.4)	0.4 (0.4-0.4)	0.770
G-CSF	1.0 (1.0-89.3)	4.4 (1.0-11.1)	0.830	1.0 (1.0-205.8)	1.0 (1.0-34.5)	0.970
GM-CSF	6.6 (6.6-275.2)	6.6 (6.6-6.6)	0.560	6.6 (6.6-105.0)	6.6 (6.6-1418.8)	0.970
IFN-γ	2.0 (2.0-56.6)	2.0 (2.0-2.0)	1.000	2.0 (2.0-514.0)	2.0 (2.0-6275.4)	0.971
MCP-1	14.0 (1.7-193.1)	21.7 (5.7-127.2)	0.350	16.9 (6.1-278.9)	22.9 (3.8-405.3)	0.450
MIP-1β	386.6 (0.6-1126.9)	394.9 (177.0-603.5)	0.510	234.6 (0.6-766.3)	308.4 (145.0-378.6)	0.410
TNF-α	0.4 (0.2-20.7)	1.1 (0.2-2.6)	0.880	0.2 (0.2-9.8)	1.2 (0.2-377.6)	0.096

Figure Legends

Figure 1. Partial Mayo score (PMS) and serum CRP levels before and 8 weeks after induction treatment with infliximab. **(a)** PMS after 8 weeks (1.5 [0-6]) was significantly decreased compared to baseline (6 [3-9], $P < 0.05$). **(b)** Initial median CRP levels (0.3 mg/dL [0.10-2.65]) did not change significantly before and 8 weeks after the treatment (0.1 mg/dL [0.10-6.12], $P = 0.208$).

Figure 2. Changes in serum IL-6, IL-8 and MIP-1β 8 weeks after treatment. **(a, b)** Serum IL-8 (a) and MIP-1β (b) levels significantly decreased 8 weeks after treatment compared to those before treatment ($P = 0.024$ and 0.003, respectively).

Figure 3. **(a)** Comparison of serum levels of IL-6 at 8 weeks between patients who respond (responders) and did not (non-responders) at 26 weeks. Serum IL-6 levels were significantly lower in responders than in non-responders ($P = 0.030$). Horizontal bars indicate median values.

(b) ROC curve for IL-6 levels at 8 weeks after the induction therapy for predicting clinical response at 26 weeks. AUC was 0.8022; the sensitivity and specificity were 0.9231 and 0.7143, respectively, when the cut-off value of IL-6 was set at 0.71 pg/mL.