Decrease of reactive-oxygen-producing granulocytes and release of IL-10 into the peripheral blood following leukocytapheresis in patients with active ulcerative colitis

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AIM: To investigate the clinical efficacy of leukocytapheresis (LCAP) in patients with active ulcerative colitis (UC), and to elucidate the mechanisms by determining the changes in the cytokine levels in the peripheral blood and of the functions of the peripheral blood leukocytes in these patients.

METHODS: The subjects were 19 patients with active UC, with a mean clinical activity index (CAI) of 9.2. The LCAP was conducted using Cellsorba E. In each session of LCAP, 2-3 L of blood at the flow rate of 30-50 mL/min was processed. The treatment was carried out in approximately 1-h sessions, once a week, for 5-10 wk. Blood samples for determination of the cytokine levels were collected from the inflow side of the column (site of dehematization; at the start of LCAP) and outflow side of the column (at the end of LCAP). Blood samples for the determination of reactive-oxygen-producing cells were collected from the peripheral blood before and after LCAP.

RESULTS: LCAP resulted in clinical improvement in all the 19 patients of UC recruited for this study. Remission (CAI: ≤4) was noted in 15 (79%) of the 19 patients. The blood level of the pro-inflammatory cytokine IL-6 was found to be decreased following treatment by LCAP, and the level of the anti-inflammatory cytokine IL-10 at the outflow side of the LCAP column was found to be significantly elevated as compared to that at the inflow side of the column. The reactive-oxygen-producing granulocytes in the peripheral blood of UC patients was increased as compared to that in healthy persons and the increase was found to be decreased following treatment by LCAP.

CONCLUSION: LCAP exerted a high therapeutic efficacy in patients with active UC. Our findings suggest that LCAP is associated with enhanced production of the inhibitory cytokine IL-10 to indirectly inhibit the functions of the inflammatory leukocytes, and that inflammation is also considerably attenuated by the direct removal of reactive-oxygen-producing neutrophils from the peripheral blood.

Key words: Ulcerative colitis; Reactive-oxygen; Leukocytapheresis; Interleukin-10

INTRODUCTION

Ulcerative colitis (UC), as well as Crohn’s disease (CD), is one of the prototype nonspecific inflammatory bowel diseases (IBDs) of unknown cause. Drugs such as salazosulfapyridine[1], 5-aminosalicylic acid[2], corticosteroids[3,4], and immunosuppressants[5] have been used for the treatment of UC; these drugs attenuate the inflammation in the colonic mucosa by their anti-inflammatory and immunosuppressive actions, to cause disease remission. However, some patients with UC are even refractory to the strong anti-inflammatory and immunosuppressive actions of steroids, and surgical treatment often have been considered as the ultimate treatment modality for such patients.

Extracorporeal circulation treatment methods that have been shown to be highly efficacious for the treatment of UC patients refractory to steroids have recently been developed in Japan. One such is the so-called granulocyte and monocyte adsorption apheresis (GMA)[6-8], in which mainly granulocytes and monocytes are removed from the blood, while another is leukocytapheresis (LCAP)[9], in which granulocytes, monocytes, as well as lymphocytes, are...
removed from the blood. These treatment modalities have been reported to yield a high therapeutic efficacy in many patients of UC, including those who are refractory to steroids.

The pathogenetic mechanisms underlying the development of the two IBDs, UC, and CD, have not yet been clearly elucidated. However, it has been suggested that inflammation of the intestinal mucosa results from infiltration of the mucosa by inflammatory leukocytes, including neutrophils, monocytes, and lymphocytes. According to several reports, clustering of various leukocytes at the sites of inflammation along the intestinal mucosa and the resultant release of pro-inflammatory cytokines and reactive oxygen species lead to the tissue injury seen in these cases\cite{10,16,17}. Thus, GMA and LCAP were developed as treatment modalities for UC under the contention that removal of the inflammation-inducing activated leukocytes from the circulation might result in attenuation of the inflammation\cite{6,16,17}.

In the present study, the clinical effects of LCAP in cases of active UC were investigated, and the changes in the levels of cytokines and percentage of reactive-oxygen-producing leukocytes (granulocytes, monocytes, and lymphocytes) in the peripheral blood were determined to elucidate the possible mechanisms underlying the clinical efficacy of LCAP in patients of UC.

**MATERIALS AND METHODS**

**Patient background**

Nineteen patients with active UC were recruited for this study conducted to determine the efficacy of LCAP. The demographic characteristics of these patients are summarized in Table 1. The patients consisted of 9 males and 10 females, with a mean age of 37.3 years (range, 19-52 years); the mean age of the patients at the time of the first episode was 28.3 years (range, 14-50 years), and the mean duration of the disease was 5.1 years (range, 0.5-19 years).

| Demographic factors       | Measurement |
|---------------------------|-------------|
| Male/female               | 9/10        |
| Age (yr)                  | 37.3±16.9   |
| Age at first episode (yr) | 28.3±12.7   |
| Duration of UC (yr)       | 5.1±8.9     |
| Number of relapse         | 3.8±4.2     |
| Classification of severity|             |
| Severe                    | 2           |
| Moderate                  | 11          |
| Mild                      | 6           |
| CAI                       | 9.2 (6-16)  |
| Extent of UC              |             |
| Total colitis             | 12          |
| Left-sided colitis        | 7           |

The clinical activity index (CAI) and the endoscopic index (EI) were determined in conformance with the criteria proposed by Rachmilewitz\cite{16}. The mean CAI of the patients at the start of the LCAP treatment was 9.2 (6-16), and the CAI distribution in the patients was as follows: CAI: ≥12 (severe UC) in 2 patients, CAI: ≥8, <12 (moderate UC) in 11 patients, and CAI: ≥5, <8 (mild UC) in 6 patients. Twelve of the nineteen patients had involvement of the entire colon (total colitis), and 7 patients had involvement of the left-sided colitis.

**Leukocytapheresis (LCAP)**

LCAP was carried out using a column (Cellsorba E) filled with a non-woven fabric made up of polyester fibers. The fabric had a dual structure; an inner layer composed of superfine fibers 0.8-2.8 μm in diameter, and an outer layer composed of fibers 10-40 μm in diameter. The blood is filtrated from the outside into the inside of the non-woven fabric wound into a cylindrical shape in the column, and leukocyte components are removed. The blood, with leukocyte removed, is guided out from the column and heated, and then returned to the corresponding vein of the patient’s other arm or leg of the patient. The blood flow rate was set at 30-50 mL/min, and 2-3 L of blood was treated in each session of LCAP. The treatment was carried out in 1-h sessions, once a week, for 10 wk.

**Measurement of soluble IL-6 and IL-10**

Peripheral blood samples for the measurement of IL-6 and IL-10 were collected before the start of each LCAP session and then 60 min after the LCAP session. Both IL-6 and IL-10 were measured by an ELISA method. The human IL-6 ELISA kit was obtained from R&D Systems (Minneapolis, USA), while the ELISA kit for IL-10 (Human Interleukin-10 Ultrasensitive; hIL-10 US) was obtained from Biosource (CA, USA).

All the assays on the serum samples were performed in an SRL (Special Research Laboratory) in a blinded manner, and the data were processed by an individual who was blinded to both the clinical characteristics of the subjects and the purpose of the study.

**Determination of the percentage of reactive-oxygen-producing cells**

The percentage of reactive-oxygen-producing cells was determined in conformance with the method established by Bass et al\cite{19}. In brief, phosphate-buffered saline (PBS) mixed with 5 μmol/L of 2',7'-dichlorofluorescein diacetate (DCFH-DA) was added to the heparinized vein blood sample, and the sample was heated at 37 °C for 15 min under shaking. Then, PBS plus 20 mmol/L of ethylenediamine tetraacetic acid was added to the mixture to prevent agglutination of the neutrophils. The mixture was then centrifuged, washed, and subjected to hemolytic treatment. The reactive-oxygen-producing cells were counted with a flow cytometer using 2',7'-dichlorofluorescein, a product of oxidation with reactive oxygen species, as the indicator.

**Statistical analysis**

Student’s t-test or Mann-Whitney’s U-test was used for statistical analysis.

**RESULTS**

The therapeutic efficacy of LCAP treatment against active UC was investigated in 19 patients. The results revealed...
that the CAI, an indicator of the clinical severity of UC, decreased significantly ($P<0.01$) from the mean value of 9.2 before the treatment, to 2.8 following the treatment (Figure 1A). The serum level of C-reactive protein (CRP), a marker of inflammation, decreased significantly ($P<0.01$) from a mean level of 3.9 before treatment to 0.5 after the treatment (Figure 1B). Endoscopic examination revealed a significant decrease ($P<0.01$) of the EI score from a mean of 8.7 before treatment to 3.2 after the treatment (Figure 1C). Evaluation 12 wk after the LCAP treatment revealed that remission (CAI≤4) had occurred in 15 (79%) of the 19 patients; in the remaining 4 patients, even though remission had not occurred, the values of each of the above-described markers had nevertheless decreased, showing a beneficial therapeutic effect of the treatment against the disease. Dull headache as a side effect of LCAP treatment was observed in only one patient. Based on the above results, LCAP was confirmed to be a highly safe treatment modality.

The blood levels of IL-6, a pro-inflammatory cytokine, and IL-10, an inhibitory cytokine, were determined during the treatment in randomly selected patients. A decrease of IL-6 level in the peripheral blood during the treatment was observed in six patients (Figure 2A). The IL-10 level, on the other hand, was found to be increased in the blood in the outflow side of the LCAP column as compared to that in the inflow side of the LCAP column (Figure 2B). This elevation of IL-10 production was found to be particularly marked in the early stages of the treatment.

IL-10 has been reported to markedly inhibit the protein and mRNA expression of IL-1, a pro-inflammatory cytokine produced in response to lipopolysaccharide (LPS) stimulation of neutrophils and monocytes[20]. It was revealed that the production of IL-10 was stimulated by LCAP treatment. Based on this observation, the effects of LCAP on the functions of neutrophils and monocytes were investigated, using the percentage of reactive-oxygen-producing cells as an indicator. The percentages of reactive-oxygen-producing cells in the peripheral blood of healthy persons and UC patients prior to LCAP were determined first. The percentage of these cells was found to be significantly increased in the peripheral blood of UC patients as compared in that of healthy persons (7.1±4.9 vs 20.1±12.0) (Figure 3A). On the other hand, there were scarcely any reactive-oxygen-producing cells among the lymphocytes and monocytes in the peripheral blood of either healthy persons or UC patients in the absence of stimulation (data not shown). The percentage of reactive-oxygen-producing cells before LCAP treatment (one occasion) was compared with that determined after the treatment (one occasion) in the UC patients. The percentage was found to be significantly decreased after the LCAP treatment (20.8±12.4 vs 12.0±9.5) (Figure 3B). Representative results of fluorescein-activated cell-sorter (FACS) analysis before and after LCAP treatment in the UC patients are shown in Figure 4.

![Figure 1](image1.png) **Figure 1** A: Average CAI in the 19 patients at wk 5 and 10 following the start of LCAP treatment; B: Average CRP level in the 19 patients at wk 5 and 10 following the start of LCAP treatment; C: Average EI in 19 patients at wk 5 and 10 following the start of LCAP treatment.

![Figure 2](image2.png) **Figure 2** A: The serum IL-6 concentrations in six patients at wk 1, 2, 3, 5, and 10 following the start of LCAP treatment; B: The serum IL-10 concentrations measured at the Cellorba inflow (at the start of LCAP) and outflow (at the end of LCAP).

![Figure 3](image3.png) **Figure 3** A: Percentage of reactive-oxygen-producing granulocytes in normal subjects ($n=6$) and UC patients ($n=11$); B: Percentage of reactive-oxygen-producing granulocytes in the peripheral blood of 10 patients before and after LCAP treatment.
it does not affect the ability of these cells to produce IL-4, i.e., an anti-inflammatory cytokine, whereas LCAP enhances the ability of the peripheral blood lymphocytes in peripheral blood to produce cytokines before mechanism of actions of LCAP treatment, the ability of effects, and high safety with only a low frequency of side effects and mild side effects against inflammatory diseases by influencing the cytokine balance through adsorption and removal of interferon γ, a pro-inflammatory cytokine[23]. As for the third mechanism, it has been clarified that not only leukocytes, but also platelets are removed by LCAP (ca. 35% of platelets in the peripheral blood are removed during each session of LCAP); it has been reported that platelets induce leukocytes to produce reactive oxygen species[22], and that removal of platelets by LCAP, therefore, indirectly inhibits the production of reactive oxygen species by leukocytes, thereby preventing tissue injury.

Some mechanisms underlying the therapeutic efficacy of GMA have also been proposed. It has been suggested that GMA exerts its therapeutic effects by removing granulocytes and monocytes from the peripheral blood, thereby attenuating inflammation, similar to the mechanism proposed for LCAP[6,24]. Another explanation proposed was based on the observation that leukocytes in the peripheral blood showed decreased expression of membrane LECAM-1 and decreased ability to produce TNF-α, IL-6, and IL-8 under stimulation of LPS following GMA treatment. It has also been suggested that GMA exerts its therapeutic effects by inhibiting leukocyte infiltration at sites of inflammation and inhibiting inflammatory cytokine production[6,23]. Other mechanisms proposed include induction of immature monocytes in the bone marrow following GMA treatment[6,23].

In the present study, the cytokine levels in the peripheral blood after LCAP were investigated. The level of IL-6, a pro-inflammatory cytokine, was significantly decreased following LCAP treatment. The blood level of IL-10, an inhibitory cytokine, was found to be elevated following LCAP, as compared to the levels measured before treatment. This elevation was particularly marked in the early stages of the treatment, suggesting that the therapeutic efficacy might be closely related to this finding. IL-10 has been reported to functionally inhibit neutrophils and monocytes[23]. In the present study also, the blood levels of IL-6, produced from neutrophils and monocytes, was decreased following LCAP treatment. In other words, the results of the present study suggested that LCAP exerted anti-inflammatory effects by enhancing the production of IL-10, thereby inhibiting the functions of activated leukocytes. LCAP treatment exerts the best therapeutic efficacy when it is carried out once a week for 5-10 wk. It would be of interest to determine the duration of functional inhibition of leukocytes and elevation of IL-10 level following one session of LCAP.

LCAP has also been shown to exert high therapeutic efficacy in cases of CD[24], rheumatoid arthritis (RA)[25], and rapidly-progressive glomerulonephritis[26], in addition to those of UC. Hidaka et al[25] who conducted a study of the efficacy of LCAP in RA patients reported that the serum levels of IL-10 increased, whereas those of the pro-inflammatory cytokine IL-15 decreased, following LCAP treatment in RA patients, and that LCAP may influence some chemotactic factors as well. Based on the present results and a review of the literature, LCAP treatment is considered to exert its effects against inflammatory diseases by influencing the cytokine balance through adsorption and removal of activated leukocytes, and causing functional changes of inflammatory cells.

Another report has shown that LCAP removes large numbers of granulocytes and monocytes from the peripheral blood by removing these activated leukocytes from the peripheral blood, thereby allowing surgery to be avoided.

DISCUSSION
A previous clinical trial conducted in Japan revealed that LCAP yielded high therapeutic efficacy, that is, it yielded beneficial effects in 74% of UC patients who were refractory to steroid treatment[6]. In the present study, 73% of active UC patients entered into remission following LCAP treatment, and in the remaining patients also, the treatment was found to have beneficial therapeutic effects. In other words, LCAP was confirmed again to have a high therapeutic efficacy in cases of UC. Another extracorporeal circulation treatment strategy, GMA, has also been reported to exert high therapeutic efficacy in cases of UC (efficacy rate: 80% or higher), similar to LCAP. GMA was reported in one study to yield a beneficial effect in 100% of UC patients, in particular, steroid-naive UC patients[7]. Furthermore, both LCAP and GMA have been confirmed to be associated with only a low frequency of side effects and mild side effects, and high safety[6-9]. The use of these treatment methods has become more widespread since they were approved for reimbursement under the National Health Insurance in Japan, and they have been found to be useful for improving the quality of life of many patients, by inducing remission in a high percentage of patients, minimizing the side effects of steroids associated with high doses, and allowing surgery to be avoided.

However, the mechanisms underlying the therapeutic efficacy of these extracorporeal circulation treatment methods have not yet been clearly elucidated. Some mechanisms have been proposed, as follows: Marked infiltration by neutrophils and lymphocytes is observed at sites of inflammation along the colonic mucosa in UC; many of these leukocytes are activated, and adhere to hemangioendothelial cells via the enhanced surface expression of adhesion molecules, leading to penetration of the mucosa. LCAP has been suggested to cause attenuation of the process of inflammation and exert a therapeutic effect by removing these activated leukocytes from the peripheral blood[7]. In connection with the second mechanism of actions of LCAP treatment, the ability of lymphocytes in peripheral blood to produce cytokines before and after the treatment was investigated. It is shown that LCAP enhances the ability of the peripheral blood lymphocytes to produce IL-4, i.e., an anti-inflammatory cytokine, whereas it does not affect the ability of these cells to produce oxygen-producing cells in A and B were 22.9% and 7.1%, respectively.

Figure 4 Representative results of FACS analysis in the peripheral blood of one patient before (A) and after (B) LCAP treatment. The percentages of reactive-oxygen-producing cells in A and B were 22.9% and 7.1%, respectively.
blood. It has been revealed by many studies that granulocytes and monocytes are closely associated with the morbid condition in cases of UC. Some reports have shown that clustering of these cells at sites of inflammation along the colonic mucosa in cases of UC and the possibility that these activated granulocytes and monocytes induce tissue injury in response to production of various pro-inflammatory cytokines and reactive oxygen species. In fact, some reports have shown that the amount of reactive oxygen species is increased at the site of histological inflammation in cases of IBD. A report has recently described marked oxidation of actin, which may be caused by the accumulation of reactive oxygen species, at sites of inflammation in IBD. It has also been shown that such oxidation disrupts the cytoskeleton, causing tissue injury.

D’Odorico et al., have also reported that activation of neutrophils in the peripheral blood is associated with chronic intestinal inflammation in IBD. In other words, they indicated that neutrophils in the peripheral blood of UC or CD patients show higher chemiluminescence in response to stimulation, as compared to those of healthy persons, and produce larger amounts of reactive oxygen species. This phenomenon was particularly marked in cases with active UC and ileal CD, strongly suggesting its correlation with the morbid condition in these diseases. Some reports have also shown high levels of nitric oxide metabolites, i.e., one of the reactive oxygen species, in the serum, stool and colonic lumen of IBD patients. Based on these observations, it is believed that activated neutrophils and monocytes in the peripheral blood of IBD patients infiltrate the intestinal mucosa to produce reactive oxygen species and cause mucosal injury. There has also been a report discussing the absence of correlation of the amount of reactive oxygen species in the blood with the morbid condition in cases of IBD, even though the present study revealed an increase in the percentage of reactive-oxygen-producing cells in the blood in cases of active UC as compared to that in healthy persons, and a decrease in the percentage of these cells with improvement of the morbid condition, showing close involvement of the neutrophils in the peripheral blood with the morbid condition in cases of UC.

In conclusion, LCAP treatment was confirmed to exert high therapeutic efficacy against active UC in the present study, and it was clarified for the first time that LCAP directly decreases the percentage of reactive-oxygen-producing cells in the peripheral blood. The results suggested that the mechanisms underlying the therapeutic effects of LCAP include enhanced production of IL-10 associated with indirect inhibition of the functions of leukocytes, and potent anti-inflammatory effects by the direct removal of reactive-oxygen-producing neutrophils from the peripheral blood. Further studies in the future are proposed to clearly elucidate the underlying mechanisms.

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