Increased plasma lactoferrin levels in leucocytapheresis therapy in patients with rheumatoid arthritis

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Abstract

Objective. The aim of this study was to clarify the mechanism of leucocytapheresis (LCAP) in patients with RA.

Methods. Protein profiles of blood samples from two patients with RA obtained via LCAP column inlet and outlet lines were analysed by two-dimensional fluorescence difference gel electrophoresis and mass spectrometry. The lactoferrin (LTF) levels of peripheral and circulating blood samples from seven patients obtained via the LCAP column blood circuit were then determined by ELISA. Peripheral blood samples from 14 patients with RA were exposed to unwoven polyester fibre filters and the LTF level was determined. In addition, morphological changes in neutrophils after exposure to the filter were examined by optical microscopy, electronic microscopy and LTF immunostaining.

Results. LTF levels were increased in both samples from the LCAP column outlet and peripheral blood at the end of LCAP treatment. Furthermore, peripheral blood samples exposed to the filter revealed a decreased number of neutrophils and an increased level of LTF. Morphological analysis of the exposed neutrophils showed vacuolization of the cytoplasm and degranulation of LTF-positive granules. These data suggest that LTF stored in the granules of neutrophils is released from the neutrophils caught in the LCAP column.

Conclusion. Because LTF has been reported to have multiple anti-inflammatory properties, increased levels of LTF may contribute to the clinical effect of LCAP in patients with RA.

Key words: rheumatoid arthritis, neutrophils, inflammation, cytokines and inflammatory mediators, proteomics.

Introduction

RA is characterized by systemic inflammation with proliferation of synovial cells and destruction of joint bone. Its pathogenesis is associated with pro-inflammatory cytokines that activate macrophages, fibroblastic synovial cells and vascular endothelial cells and promote inflammation [1]. Recently it has been generally recognized that biologic agents for the inhibition of pro-inflammatory cytokines, such as TNF and IL-6, are effective in the control of RA disease activity [2–3]. In contrast, biologic agents are not of benefit to certain RA patient populations because of inefficacy or adverse effects.

Leucocytapheresis (LCAP) is a therapy involving extracorporeal circulation with a filter for the removal of white blood cells (WBCs) from the peripheral blood [4–6]. It has been speculated that the effectiveness of LCAP in inflammatory disease results from the removal of activated WBCs and platelets [7]. A decrease in inflammatory cytokines (TNF-α and IL-15) and an increase in anti-inflammatory cytokines (IL-10) in the sera of patients with RA after LCAP has been reported [8]. In addition, LCAP has been
reported to enhance the production of IL-4, an anti-inflammatory cytokine, from peripheral blood lymphocytes [9]. These data suggest that the therapeutic effect of LCAP is achieved through the modulation of cytokines and circulating T cells; however, the reason removal of WBCs induces the anti-inflammatory effect is still under investigation.

Neutrophils are known to have three types of granules that contain different bioactive molecules. These molecules are released from the cells when neutrophils are exposed to different stimuli, such as cytokines, bacteria and chemical materials. Physical stimulation with glass or synthetic fibre could be one of these [10]. In fact, we reported that an increase in neutrophil-derived microparticles was induced by LCAP in patients with RA [11].

These data raise the question of whether LCAP induces the release of molecules from neutrophil granules and whether those molecules in turn work beneficially in the treatment of RA. In the present study we compared the protein profiles of the blood flow between the LCAP column inlet and outlet and found that plasma levels of lactoferrin (LTF) derived from neutrophils were increased.

Patients and methods

Patients

The diagnosis of RA was based on the 1987 diagnostic criteria of the ACR [12]. To evaluate RA activity, we used the 28-joint DAS calculated by ESR (DAS28-ESR) [13], the simplified disease activity index (SDAI) [14] and the clinical disease activity index (CDAI) [15]. Seven Japanese patients (all female, median age 57 years) who received LCAP treatment were recruited into the present study (Table 1). The median DAS28 in these patients was 5.79 prior to LCAP. Five patients had high disease activity and two had moderate activity according to the European League Against Rheumatism (EULAR) response criteria [16]. Peripheral blood of 14 other Japanese patients with RA (five males and nine females, median age 63 years) was used for in vitro whole blood unwoven filter contact experiments. The median value of the DAS28 in these patients was 5.31. Seven patients had high disease activity, six had moderate disease activity and one had low disease activity. Written informed consent was obtained from all patients and the study was approved by the institutional review board of the University of Miyazaki.

LCAP procedure and sample preparation

The LCAP procedure is described in depth elsewhere [6]. Briefly, 100 ml/kg of body weight of whole blood was filtered using an LCAP column with filter (Cellsorba CS-180S, Asahi Kasei Medical, Tokyo, Japan) once per week for 5 weeks (five LCAP sessions in total) and returned to the patient. In the first week of LCAP treatment, peripheral blood samples were obtained from patients at the beginning and end of treatment. Samples were also taken from the LCAP column inlet and outlet after ~1500 ml of blood had been filtered. Plasma samples were stored at −80°C until use. EULAR response criteria were used to evaluate the activity of RA after LCAP treatment.

Comparison of protein profiles between plasma samples obtained via LCAP column inlet and outlet lines by two-dimensional fluorescence difference gel electrophoresis and mass spectrometry

Two hundred microlitres of plasma obtained from two patients with RA who received LCAP treatment as described above via LCAP column inlet and outlet lines were processed with the ProteoMiner Protein Enrichment Kit (BioRad Laboratories, Hercules, CA, USA) following analysis by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) [17]. The differences in intensity of each protein spot between the inlet and outlet samples were compared using Progenesis SameSpots software (Non-linear Dynamics, Newcastle, UK). If the intensity of the protein spot from the LCAP column outlet was >1.5 times that of the protein spot from the inlet, that protein spot was digested by trypsin and processed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or liquid chromatography-mass spectrometry (LCMS) and ion trap TOF (IT-TOF) MS for protein identification [18].

Measurement of LTF by ELISA

The level of LTF in the experiments described below was determined using BIOXYTECH Lactof-EIA (Oxis International, Beverly Hills, CA, USA) according to the manufacturer’s instructions.

In vitro whole blood unwoven filter contact experiment

To examine the effect of in vitro contact between the filter and whole blood, 5 ml of heparinized peripheral blood from 14 patients with RA were incubated with or without 4 × 4 cm unwoven polyester fibre filters in a 15 ml tube at 37°C for 60 min. After incubation, the number of WBCs was counted. Whole blood exposed to the filters was centrifuged at 1600g and plasma samples were
prepared. The level of LTF in the prepared plasma was determined using an ELISA kit as described above.

Morphological analysis of neutrophils after contact with filters
To visualize the morphological change in neutrophils after contact with the filter, neutrophils were isolated from the peripheral blood of a patient with RA using dextran sedimentation and the specific gravity centrifugal method with Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) [19]. The number of neutrophils was then adjusted to 2 × 10⁶ cells/ml in cell assay medium (RPMI1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin) in each tube and the cells were incubated with filters at 37°C for 60 min. After incubation, neutrophils were excised for Wright-Giemsa staining and observed by optical microscope. In addition, the level of LTF in cell assay medium was determined using an ELISA kit as described above. For electron microscopic analysis, cell pellets were prepared by centrifugation at 300 g and then fixed with 2% paraformaldehyde/2.5% glutaraldehyde/0.1 M phosphate buffer (PB) at room temperature for 60 min. After washing, they were fixed with 1% osmium tetroxide in 0.1 M PB at 4°C for 60 min. The specimen was dehydrated in a graded ethanol series and embedded in epoxy resin. Ultrathin sections (60–70 nm) of the specimen were cut, contrasted with 2% uranyl acetate in 70% methanol and Reynolds lead citrate, and observed using an HT-7700 (Hitachi High-Technologies, Tokyo, Japan) transmission electron microscope operating at 80 kV. Direct immunofluorescence analysis of LTF-positive granules was then performed. Incubated neutrophils were plated on slide glass with Cytospin (Thermo Scientific, Kanagawa, Japan) and fixed in 4% paraformaldehyde for 10 min. They were washed with phosphate buffered saline (PBS) containing 0.01% Triton X-100 and stained with fluorescein isothiocyanate (FITC)–labelled anti-LTF mouse monoclonal antibody (SC-53498, Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C for 180 min. They were washed with PBS containing 0.01% Triton X-100. The nuclei were counterstained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) (DAPI fluoromount-G, SouthernBiotech, Birmingham, AL, USA). Images were obtained by fluorescence microscopy (BIOREVO BZ-9000 microscope, Keyence, Osaka, Japan).

Statistical analysis
Data were analysed by STATview (SAS Institute, Cary, NC, USA). A non-parametric test (Wilcoxon signed rank test) was used to compare disease activity markers in patients receiving LCAP treatment. It was also used for comparison of the LTF level and WBC count in each experiment. A non-parametric test (Spearman’s correlation coefficient by rank) was used to test the association of plasma LTF increment with the decrement of DAS28. 

Results
Efficacy of LCAP
Clinical outcomes of seven patients with RA who received LCAP treatment (five sessions in total) are shown in Fig. 1. DAS28, SDAI and CDAI were evaluated at three time points: 0 weeks (before the first session), 4 weeks (before the fifth session) and 8 weeks (1 month after the fifth and final session). The median DAS28 decreased over time (0 weeks: 5.79; 4 weeks: 4.65; 8 weeks: 4.04) and the decrement from 0 weeks to 8 weeks was statistically significant, as shown in Fig. 1A (P = 0.02). Three and four patients, respectively, showed good and moderate responses based on EULAR response criteria. The median SDAI also decreased over time (0 weeks: 24.89; 4 weeks: 10.91; 8 weeks: 6.58) and the differences between the three time points were statistically significant, as shown in Fig. 1B (P = 0.02). The CDAI changed over time (0 weeks: 23.90; 4 weeks: 9.20; 8 weeks: 5.50) and the decrement from 0 weeks to 8 weeks was statistically significant, as shown in Fig. 1C (P = 0.02).

Comparison of protein profile between the plasma samples from the LCAP column inlet and outlet lines by 2D-DIGE and MS
The intensities of protein spots between the plasma samples from the LCAP column inlet and outlet lines were compared in two of seven patients who received LCAP treatment. Seven spots showed outlet sample intensities >1.5 times those of inlet samples in both patients. Six spots were observed to have the same molecular weight but different isoelectric points. These protein spots were processed by MALDI-TOF MS or LCMS IT-TOF and identified as LTF. Representative 2D-DIGE data are shown in Fig. 2. Six LTF spots were observed in the outlet samples (in the ellipse of Fig. 2B) but not in the inlet samples (in the ellipse of Fig. 2A). The remaining spot with a different molecular weight and isoelectric point was identified as an alpha chain of fibrinogen.

Measurement of LTF in patients who received LCAP treatment
Among WBCs, only neutrophils have been reported to possess LTF in their granules [20]. Therefore, based on the data described above, it was hypothesized that neutrophils caught in the LCAP column released LTF. To confirm this hypothesis, LTF levels in the LCAP column inlet and outlet lines of all seven patients who received treatment were measured by ELISA after 1500 ml of blood was filtered during the first LCAP session (Fig. 3A). The outlet LTF levels (median 1712.9 ng/ml) were significantly higher than inlet levels (median 216.0 ng/ml) (P = 0.02). The LTF levels of the patients at the beginning and end of the first session of LCAP were measured (Fig. 3B). Plasma LTF levels at the end of LCAP (median 954.6 ng/ml) were significantly higher than the levels at the beginning (median 102.1 ng/ml) (P = 0.02). Therefore the increased levels of plasma LTF in patients treated with LCAP were considered to be the result of the circulation of peripheral
blood in the LCAP column and the release of LTF from neutrophils caught in the column. In addition, we analysed whether there was an association between the increased plasma LTF and the decrease in DAS28; however, the correlation was not statistically significant (r² = 0.34, P = 0.34) (data not shown).

In vitro whole blood filter contact experiment

Next we examined whether direct contact of peripheral blood cells with the filters used in the LCAP column also resulted in an increase in LTF in the supernatant of incubated cells. Peripheral blood cells from 14 patients with RA were incubated with and without filters at 37°C for 60 min. After incubation the number of WBCs in the group with filters (median 400/µl) was significantly lower than in the group without (median 5775/µl, P < 0.01; Fig. 4A). LTF levels in the plasma isolated from the blood, which was incubated with and without filters, were 1178.0 ng/ml and 128.4 ng/ml, respectively, and the former was significantly higher than the latter (P < 0.01; Fig. 4B). These results suggested that only direct contact of neutrophils with the filter resulted in the attachment of neutrophils and an increase in LTF in the supernatant.

Morphological change in neutrophils after contact with filters

Neutrophils incubated with and without filters were examined for morphological changes. Representative neutrophils stained with Wright-Giemsa examined by optical microscope are shown in Fig. 5A and B. Compared with cells not exposed to the filter (Fig. 5A), neutrophils exposed to the filter had a greater degree of vacuolization in the cytoplasm (Fig. 5B). In addition, the concentration of LTF in the culture medium of the isolated neutrophils from 6 of 14 patients with RA incubated with and without the filter was measured. The median value of LTF with the filter (124.9 ng/ml) was significantly higher than that without the
filter (61.9 ng/ml) \((P=0.03; \text{data not shown})\). At the electron microscope level, the neutrophils not exposed to the filter exhibited a large number of granules in the cytoplasm (Fig. 5C). In contrast, a lower number of granules were observed in the neutrophils exposed to the filter and a greater number of vacuoles were formed in their cytoplasm (Fig. 5D). Moreover, staining with anti-LTF antibody showed that the majority of neutrophils not exposed to the filters tested positive for LTF (Fig. 5E), while the exposed cells did not (Fig. 5F). These data suggest that in vitro exposure to the filter resulted in the release of granules containing LTF from neutrophils.

Discussion

In the present study, 2D-DIGE showed LTF to be increased in the LCAP column outlet line in RA patients who received LCAP treatment. Increased LTF levels in the blood from the LCAP column outlet line as well as in the peripheral blood was confirmed by ELISA. These data suggest that LTF is released from the LCAP column and increased in the peripheral blood of the patients treated with LCAP. Because we hypothesized that neutrophils caught in the LCAP column released LTF, we performed an in vitro study to determine whether LTF was released from the peripheral blood cells of patients with RA by exposure to the LCAP column filter. This experiment clearly demonstrates that LTF levels are increased by exposure to the filter.

Vacuolization was observed by optical microscope and by analysis using an electron microscope in neutrophils exposed to the filter. Moreover, immunostaining of these neutrophils showed the loss of LTF-positive granules after exposure. These results suggest that increasing LTF levels both in the LCAP column outlet lines and in the peripheral blood of patients treated with LCAP is the result of the degranulation of neutrophils caught in the LCAP column. Unfortunately we did not perform a morphological analysis of the neutrophils derived from the LCAP column outlet samples or compare them with inlet samples. Yamasaki et al. [21] reported that 96% of neutrophils were removed by the LCAP filter. Only the neutrophils that did not contact the LCAP filter were thought to be in the outlet sample. Therefore these cells were assumed to have undergone no morphological change. Further study is required to determine whether this assumption is correct.

LTF is known to be an essential element of antimicrobial activity. In bacterial infection, LTF, which is a member of the transferrin family of iron-binding proteins, is released from neutrophils, particularly at the site of inflammation, impairing bacterial growth by the sequestration of iron [22]. LTF plays a key role not only against microbes, but also against excessive and harmful host responses in mammals. The anti-inflammatory properties of LTF may
be explained by its neutralization of endotoxins [23]. The protective anti-inflammatory activity of LTF is also based on its ability to bind free ferric ions and inhibit oxidative bursts [24]. LTF has also been reported to have multiple anti-inflammatory properties beyond those of infection. LTF has been shown to inhibit the proliferation and cytokine production of the antigen-specific TH1 cell line [25]. LTF reduces the number of infiltrating leucocytes in inflammation of the lung and suppresses the hyper-reaction of the host [26]. LTF may inhibit angiogenesis, probably by inducing IL-18 production [27]. LTF also inhibits IL-8 from binding with proteoglycans and their further presentation to leucocytes [28]. In skin allergies, LTF is thought to inhibit the release of TNF-α from keratinocytes [29]. Lastly, LTF may down-regulate TNF-α production in mononuclear cells through a mechanism involving LTF internalization, nuclear localization and interference with nuclear factor κB [30].

Some of these elements of inflammation, which have been shown to be inhibited by LTF, are also involved in the pathogenesis of RA [1]. Therefore increased LTF in the peripheral blood of patients with RA who received LCAP treatment can contribute to the improvement of RA, at least in part. If this is the case, the anti-inflammatory effect of LTF on inflammation in RA does not act directly on the inflammatory cells in the joints, but indirectly through the modulation of multiple inflammatory processes. The relatively slow improvement of RA with LCAP treatment may be due to this indirect effect. In fact, Hidaka et al. [8] measured serum TNF-α in 22 patients with RA before and after five sessions of LCAP treatment and reported that the latter decreased significantly compared with that in the former [8]. Unfortunately we were unable to measure the alteration of plasma cytokines in the present study due to a limitation of sample amount. Further study is required to clarify the relationship between the plasma cytokines and LTF in patients with RA who receive LCAP treatment.

There are several limitations to this study. First, the number of patients involved in this study was small. A larger number of patients is required to obtain conclusive results. Second, neutrophils have several types of granules containing many molecules [31]. Therefore it is natural to suspect that not only LTF, but also other molecules in the other types of neutrophil granules may be released from the LCAP column. The latter were not identified by 2D-DIGE and were not investigated in depth in this study; however, there is a possibility that these other molecules derived from neutrophils account for the effect of LCAP in patients with RA.

In conclusion, plasma LTF levels increased in patients with RA after receiving LCAP treatment. The increase in LTF was considered to be due to its release from neutrophils caught in the LCAP column. Exposure of peripheral blood to the LCAP column filter resulted in increased LTF levels. Morphological analysis of these neutrophils showing vacuolization of the cytoplasm and degranulation of LTF-positive granules supports this. Because LTF has been reported to have multiple anti-inflammatory properties, increased LTF levels may contribute to the clinical effect of LCAP in patients with RA. Clarification of the mechanism of anti-inflammatory activity of LTF in LCAP treatment requires further in vitro experiments to determine whether the LTF-rich serum in the LCAP outlet is capable of inhibiting the production of inflammatory cytokines.

**Rheumatology key messages**

- Plasma lactoferrin levels increased in patients with RA after receiving leucocytapheresis treatment.
- Degranulation of neutrophils from RA patients caught in the leucocytapheresis column resulted in increased plasma lactoferrin levels.
- Multiple anti-inflammatory properties of lactoferrin may affect the clinical effect of leucocytapheresis in RA patients.

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