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

Shunichi Miyauchi[1], Kunihiko Umekita[1], Toshihiko Hidaka[2], Kazumi Umeki[1], Yatsuki Aratake[1], Nobuyasu Takahashi[3], Akira Sawaguchi[3], Ayako Nakatake[4], Itsuki Morinaga[4], Kazuhiro Morishita[4] and Akihiko Okayama[1]

[1]Department of Rheumatology, Infectious Diseases, and Laboratory Medicine, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

[2]Institute of Rheumatology, Zenjinkai Shimin-No-Mori Hospital, Miyazaki, Japan

[3]Department of Anatomy, Ultrastructural Cell Biology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan

[4]Miyazaki University HTLV-1/ATL Research Facility, Miyazaki, Japan

Correspondence to: Akihiko Okayama, Department of Rheumatology, Infectious Diseases and Laboratory Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki, Japan, 889-1692

E-mail: okayama@med.miyazaki-u.ac.jp

Short title: Lactoferrin in leukocytapheresis therapy in RA

Abstract

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

Methods. Protein profiles of blood samples from 2 patients with RA obtained via LCAP column inlet and outlet lines were analyzed by two-dimensional fluorescence difference gel electrophoresis and mass spectrometry. Then, the lactoferrin (LTF) levels of peripheral and circulating blood samples from 7 patients obtained via the LCAP column blood circuit were determined by enzyme-linked immune sorbent assay. Peripheral blood samples from 14 patients with RA were exposed to unwoven polyester fiber filter (filter) and LTF level was determined. In addition, morphological change of neutrophils after exposure to filter was examined by optical microscopy, electronic microscopy and LTF immunostaining.

Results. LTF levels were increased both in the samples from the LCAP column outlet and in peripheral blood at the end of LCAP treatment. Furthermore, peripheral blood samples exposed to

filter revealed a decreased number of neutrophils and increased level of LTF. Morphological analysis of the exposed neutrophils showed vacuolization of the cytoplasm and degranulation of LTF positive granules. These data suggested that LTF stored in the granules of neutrophils was 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

Rheumatoid arthritis (RA) is characterized by systemic inflammation with proliferation of synovial cells and destruction of joint bone. Its pathogenesis is associated with proinflammatory cytokines which activate macrophages, fibroblastic synovial cells, and vascular endothelial cells, and promote inflammation.[1] Recently, it has been generally recognized that biologic reagents for the inhibition of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-6 are effective in the control of RA disease activity.[2-3] On the other hand, biologic reagents

are not of benefit to certain RA-patient populations because of inefficacy or adverse effects.

Leukocytapheresis (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-alpha 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 which contain different bio-active 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 fiber 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 LCAP column inlet and the 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 diagnostic criteria of the 1987 American College of Rheumatology.[12] To evaluate RA activity, we used disease activity score in 28 joints calculated by erythrocyte sedimentation rate (DAS28),[13] simplified disease activity index (SDAI)[14] and 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). 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 (5 males and 9 females, median age 63 years) was used for *in vitro* whole blood- unwoven filter (filter) contact experiment. The median value of 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 University of Miyazaki.

LCAP procedure and sample preparation

The LCAP procedure is described in depth elsewhere.[6] Briefly, 100ml/kg/body weight of whole blood was filtered using a LCAP column with filter (Cellsorba®CS-180S, Asahi Kasei Medical Co., Ltd, Tokyo, Japan) once per week for five 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 approximately 1,500ml 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 (2D-DIGE) and mass spectrometry

Two hundred microliters of plasma obtained from 2 patients with RA, who received LCAP treatment as described above, via LCAP column inlet and outlet lines were processed with ProteoMiner Protein Enrichment Kit (Bio-Rad Laboratories, Inc. Hercules, USA) following analysis by 2D-DIGE.[17] Then, the difference in intensity of each protein spot between inlet and outlet samples was 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 or more that of the protein spot from the inlet, that protein spot was digested by trypsin and processed by Matrix Assisted Laser Desorption/Ionization (MALDI)-Time of Flight Mass Spectrometry (TOF MS) or Liquid Chromatography-Mass Spectrometry (LCMS)-Ion Trap (IT)-Time of Flight (TOF) for protein identification.[18]

Measurement of LTF by Enzyme-Linked ImmunoSorbent Assay (ELISA)

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

***In vitro* whole blood- unwoven filter contact experiment**

To examine the effect of *in vitro* contact between filter and whole blood, five ml of heparinized

peripheral blood from 14 patients with RA were incubated with and without 4cm×4cm unwoven polyester fiber filters (filters) in a 15ml tube at 37°C for 60 minutes. After incubation, the number of WBCs was counted. Furthermore, whole blood exposed to the filters was centrifuged at 1600G and plasma samples were prepared. The level of LTF in the plasma prepared was determined using ELISA kit as described above.

Morphological analysis of neutrophils after contact with filters

To visualize the morphological change of neutrophils after contact with filters, 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] Then, the number of neutrophils was adjusted to 2×10^6 cells/ml in cell assay medium (RPMI1640 containing 10%FBS and 1%penicillin/streptomycin) in each tube, and the cells were incubated with filters at 37°C for 60 minutes. 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 ELISA kit as described above. For electron microscopic analysis, cell pellets were prepared by centrifuge at 300G and then fixed with 2%paraformaldehyde/2.5%glutaraldehyde/0.1M phosphate buffer (PB) at room temperature for 60

minutes. After washing, they were postfixed with 1% osmium tetroxide in 0.1M PB at 4°C for 60 minutes. The specimen was dehydrated in a graded ethanol series and embedded in epoxy resin. Ultrathin sections (60-70nm) 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 Corporation, Tokyo, Japan) transmission electron microscope operating at 80kV. Direct immunofluorescence analysis of LTF positive granules was then performed. Incubated neutrophils were plated on slide glass with Cytospin® (Thermo Scientific Co., Kanagawa, Japan) and fixed in 4% paraformaldehyde for 10 minutes. They were washed with phosphate buffered saline (PBS) containing 0.01%Triton-X-100 and stained with fluorescein isothiocyanate (FITC)–labeled anti-LTF mouse monoclonal antibody (SC-53498, Santa-Cruz Biotechnology, Inc., TX, USA) at 4°C for 180 minutes. 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, Southern Biotech, AL, USA). Images were obtained by fluorescence microscope (BIOREVO BZ-9000 microscope, Keyence, Osaka, Japan).

Statistical analysis

Data were analyzed by STATview® (SAS Institute Inc., NC, USA). A nonparametric test

(Wilcoxon signed-rank test) was used to compare disease activity markers in patients receiving LCAP treatment. It was also used for comparison of LTF level and WBC count in each experiment. A nonparametric test (Spearman's Correlation Coefficient by rank) was used to test the association of plasma LTF increment with the decrement of DAS28. Values of $P<0.05$ were considered significant.

Results

Efficacy of LCAP

Clinical outcome of 7 patients with RA who received LCAP treatment (five sessions in total) is shown in Figure 1. DAS28, SDAI and CDAI were evaluated at 3 time points; namely, 0w (before the 1st session), 4w (before the 5th session) and 8w (one month after the 5th and final session). Median DAS28 decreased over time (0w: 5.79, 4w: 4.65 and 8w: 4.04) and the decrement from 0w to 8w was statistically significant as shown in Fig 1A ($p=0.02$). Three and 4 patients, respectively, showed good and moderate responses based on EULAR response criteria. Median SDAI also decreased over time (0w: 24.89, 4w: 10.91 and 8w: 6.58) and the differences among the 3 time points were statistically significant as shown in Fig 1B($p=0.02$). CDAI changed over time (0w: 23.90, 4w: 9.20 and 8w: 5.50) and the decrement from 0w to 8w was statistically

significant as shown in Fig 1C (*p=0.02*).

Comparison of protein profile between the plasma samples from LCAP column inlet and outlet lines by 2D-DIGE and mass spectrometry

The intensities of protein spots between the plasma samples from LCAP column inlet and outlet lines were compared in 2 of 7 patients who received LCAP treatment. Seven spots showed outlet sample intensities 1.5 times or greater than those of inlet samples in both of the 2 cases. 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 Figure 2. Six LTF spots were observed in the outlet samples (in the ellipse of Figure 2B), but not in the inlet samples (in the ellipse of Figure 2A). The remaining spot with different molecular weight and isoelectric point was identified as an alpha chain of fibrinogen.

Measurement of LTF in patients who received LCAP treatment

Among WBC, only neutrophils have been reported to possess LTF in their granules.[20] It was, therefore, hypothesized that neutrophils caught in the LCAP column released LTF based on the

data described above. To confirm this hypothesis, LTF levels in the LCAP column inlet and outlet lines of all 7 patients who received treatment were measured by ELISA when 1,500ml of blood was filtered at the first session of LCAP (Figure 3A). Outlet LTF levels (median: 1,712.9 ng/ml) were significantly higher than inlet levels (median: 216.0 ng/ml) ($p=0.02$). Then, LTF levels of the patients at the beginning and end of the first session of LCAP were measured (Figure 3B). Plasma LTF levels at the end of LCAP (median: 954.6 ng/ml) were significantly higher than 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 analyzed whether there was an association between plasma LTF increment and the decrement of DAS28; however, the correlation was not statistically significant ($r^2 = 0.34$, $p=0.34$) (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 of 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

minutes. After incubation, the number of WBC in the group with filters (median: 400 /μl) was significantly lower than that without filters (median: 5,775 /μl) ($p<0.01$) (Figure 4A). LTF levels in the plasma isolated from the blood, which was incubated with and without filters, were 1,178.0 ng/ml and 128.4 ng/ml, respectively, and the former was significantly higher than the latter ($p<0.01$) (Figure 4B). These results suggested that only direct contact of neutrophils with the filter resulted in the attachment of neutrophils and the increase of LTF in the supernatant.

Morphological change of neutrophils after contact with filters

Neutrophils incubated with and without filters were examined for morphological changes. Representative Wright-Giemsa-stained neutrophils examined by optical microscope are shown in Figures 5A&5B. Compared to cells not exposed to the filter (Figure 5A), neutrophils exposed to the filter had a greater degree of vacuolization in the cytoplasm (Figure 5B). In addition, the concentration of LTF in culture medium of the isolated neutrophils from 6 of 14 patients with RA incubated with and without filter was measured. The median value of LTF with filter (124.9 ng/ml) was significantly higher than that without filter (61.9 ng/ml) ($p=0.03$) (data not shown). At the electron microscope level, the neutrophils not exposed to the filters exhibited a large number of granules in the cytoplasm (Figure 5C). In contrast, a lower number of granules were observed in

the neutrophils exposed to the filter, and a greater degree of vacuoles were formed in their cytoplasm (Figure 5D). Moreover, staining with anti-LTF antibody showed that the majority of neutrophils not exposed to the filters tested positive for LTF (Figure 5E), while the exposed cells did not (Figure 5F). These data suggested that *in vitro* exposure to 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 suggested that LTF was 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 demonstrated that LTF levels were increased by exposure to the filter.

Vacuolization was observed in neutrophils exposed to the filters by optical microscope and by analysis using electron microscope. Moreover, immunostaining of these neutrophils showed the

loss of LTF-positive granules after exposure. These results suggested that increasing LTF levels both in the LCAP column outlet lines and in the peripheral blood of patients treated with LCAP was the result of the degranulation of the neutrophils caught in the LCAP column. Unfortunately, we did not perform morphological analysis of neutrophils derived from LCAP column outlet samples or compare it to inlet samples. Yamasaki et al. reported that 96% of neutrophils were removed by the LCAP column.[21] Only the neutrophils, which did not contact with LCAP filter, were thought to be in the outlet sample. Therefore, these cells were assumed not to have undergone morphological change. Further study is required to determine whether this assumption was 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, and impairs 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 ion and inhibit oxidative burst.[24] LTF has also been reported to have multiple anti-inflammatory properties beyond the case of infection. LTF was shown to inhibit

proliferation and cytokine production by antigen specific TH1 cell line.[25] LTF reduces the number of infiltrating leukocytes 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 leukocytes.[28] In skin allergy, LTF is thought to inhibit the release of TNF-alpha from keratinocytes.[29] Lastly, LTF may down-regulate TNF-alpha production in mononuclear cells through a mechanism involving LTF internalization, nuclear localization and interference with NF-kappa B.[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 is not directly on the inflammatory cells in the joints, but acts indirectly through the modulation of multiple inflammatory processes. The relatively slow improvement of RA by LCAP treatment may be due to this indirect effect. In fact, Hidaka et al. measured serum TNF-alpha in 22 patients with RA before and after 5 sessions of LCAP treatment and reported that the latter decreased significantly compared to 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 received LCAP treatment.

There are several limitations to this study. Firstly, the number of patients involved in this study was small. A larger number of patients is required to obtain conclusive results. Secondly, 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 also be released from the LCAP column. The latter were not identified by 2D-DIGE and they 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 supported 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 experiment to determine whether the LTF rich serum in

the outlet of LCAP is or is not capable of inhibiting the production of inflammatory cytokines.

Key messages

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

Acknowledgements

We thank Ms. Y. Kaseda (University of Miyazaki) for her technical assistance. The unwoven polyester fiber filters used for in vitro contact experiment were supplied by Asahi Kasei Medical Co., Ltd, Tokyo, Japan

Disclosure statement: The authors have declared no conflicts of interest.

REFERENCES

1. Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest 2008;118:3537-45.

2. Lipsky PE, van der Heijde DM, St Clair EW, *et al*. Anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study group. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study group. N Engl J Med 2000;343:1594–1602.

3. Nishimoto N, Hashimoto J, Miyasaka N, *et al*. Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x ray reader-blinded randomised controlled trial of tocilizumab. Ann Rheum Dis 2007;66:1162-7.

4. Hidaka T, Suzuki K, Matsuki Y, *et al*. Filtration leukocytapheresis therapy in rheumatoid arthritis: a randomized, double-blind, placebo-controlled trial. Arthritis Rheum 1999;42:431-7.

5. Ueki Y, Sagawa A, Tanimura K, *et al*. A multicenter study of leukocytapheresis in rheumatoid arthritis. Clin Exp Rheumatol 2007;25:810-6.

6. Eguchi K, Saito K, Kondo M, *et al*. Enhanced effect of high-dose leukocytapheresis

using a large filter in rheumatoid arthritis. Mod Rheumatol 2007;17:481-5.

7. Yamaji K, Onuma S, Yasuda M, *et al*. Fluctuations in peripheral blood leukocyte and platelet counts and leukocyte recruitment with large volume leukocytaphresis in healthy volunteers. Ther Apher Dial 2006;10:396-403.

8. Hidaka T, Suzuki K, Kawakami M, *et al*. Dynamic changes in cytokine levels in serum and synovial fluid following filtration leukocytapheresis therapy in patients with rheumatoid arthritis. J Clin Apher 2001;16:74-81.

9. Noguchi M, Hiwatashi N, Hayakawa T, Toyota T. Leukocyte removal filter-passed lymphocytes produce large amounts of interleukin-4 in immunotherapy for inflammatory bowel disease: role of bystander suppression. Ther Apher 1998;2:109-14.

10. Klock JC, Bainton DF. Degranulation and abnormal bactericidal function of granulocytes procured by reversible adhesion to nylon wool. Blood 1976;48:149-61.

11. Umekita K, Hidaka T, Ueno S, *et al*. Leukocytapheresis (LCAP) decreases the level of platelet-derived microparticles (MPs) and increases the level of granulocytes-derived MPs: a possible connection with the effect of LCAP on rheumatoid arthritis. Mod Rheumatol 2009;19:265-72.

12. Arnett FC, Edworthy SM, Bloch DA, *et al*. The American Rheumatism Association

1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.

13. Prevoo ML, van't Hof MA, Kuper HH, *et al*. Modified disease activity scores that include 28-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.

14. Smolen JS, Breedveld FC, Schiff MH, *et al*. A simplified disease activity index for rheumatoid arthritis for use in clinical practice. Rheumatology 2003;42:244-57.

15. Aletaha D, Nell VP, Stamm T, *et al*. Acute phase reactants add little to composite disease activity indices for rheumatoid arthritis: validation of a clinical activity score. Arthritis Res Ther 2005;7:796-806.

16. van Gestel AM, Haagsma CJ, van Riel PL. Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. Arthritis Rheum 1998;41:1845-50.

17. Unlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997;18:2071-7.

18. Henzel WJ, Billeci TM, Stults JT, *et al*. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc Natl Acad Sci U S A 1993;90:5011-5.

19. Yuo A, Kitagawa S, Ohsaka A, Saito M, Takaku F. Stimulation and priming of human neutrophils by granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor: qualitative and quantitative differences. Biochem Biophys Res Commun 1990;171:491-7.

20. Baynes RD, Bezwoda WR, Khan Q, Mansoor N. Relationship of plasma lactoferrin content to neutrophil regeneration and bone marrow infusion. Scand J Haematol 1986;36:79-84.

21. Yamasaki S, Ueki Y, Nakamura H, *et al.* Effect of filtration leukocytapheresis therapy: modulation of white blood cell enzyme activities in patients with rheumatoid arthritis. Artif Organs. 2002;26:378-84.

22. Oram JD, Reiter B. Inhibition of bacteria by lactoferrin and other iron-chelating agents. Biochim Biophys Acta 1968;170:351-65.

23. Elass-Rochard E, Legrand D, Salmon V, *et al*. Lactoferrin inhibits the endotoxin interaction with CD14 by competition with the lipopolysaccharide-binding protein. Infect Immun 1998;66:486-91.

24. Annane D, Bellissant E, Cavaillon JM. Septic shock. Lancet 2005;365:63-78.

25. Zimecki M, Mazurier J, Spik G, Kapp JA. Lactoferrin inhibits proliferative response

and cytokine production of TH1 but not TH2 cell lines. Arch Immunol Ther Exp 1996;44:51-6.

26. Yamauchi K, Wakabayashi H, Shin K, Takase M. Bovine lactoferrin: benefits and mechanism of action against infections. Biochem Cell Biol 2006;84:291-6.

27. Shimamura M, Yamamoto Y, Ashino H, *et al*. Bovine lactoferrin inhibits tumor-induced angiogenesis. Int J Cancer 2004;111:111-6.

28. Elass E, Masson M, Mazurier J, Legrand D. Lactoferrin inhibits the lipopolysaccharide-induced expression and proteoglycan-binding ability of interleukin-8 in human endothelial cells. Infect Immun 2002;70:1860-6.

29. Cumberbatch M, Bhushan M, Dearman RJ, Kimber I, Griffiths CE. IL-1beta-induced Langerhans' cell migration and TNF-alpha production in human skin: regulation by lactoferrin. Clin Exp Immunol 2003;132:352-9.

30. Håversen L, Ohlsson BG, Hahn-Zoric M, Hanson LA, Mattsby-Baltzer I. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. Cell Immunol 2002;220:83-95.

31. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes Infect 2003;5:1317-27.

Figure 1. Time sequential changes of disease parameters in 7 patients with RA who received leukocytapheresis treatment

0w, before the 1st session; 4w, before the 5th session; 8w, one month after the 5th and final session. (A) Disease activity score in 28 joints calculated by erythrocyte sedimentation rate (DAS28). (B) Simplified disease activity index (SDAI). (C) Clinical disease activity index (CDAI).

Figure 2. Representative data from two-dimensional fluorescence difference gel electrophoresis analysis of plasma samples from a patient who received leukocytapheresis treatment

(A) Sample from the LCAP column inlet line. (B) Sample from the LCAP column outlet line of the same patient. pI: Isoelectric point, LCAP: leukocytapheresis.

Figure 3. Plasma lactoferrin levels determined by enzyme-linked immune sorbent assay in 7 patients with RA treated with leukocytapheresis

(A) Blood samples obtained from LCAP column inlet and outlet lines when 1,500ml blood was filtered by LCAP column. (B) Blood samples obtained at the beginning and end of one session of LCAP. LTF: lactoferrin, LCAP: leukocytapheresis.

Figure 4. Change of markers in blood samples after exposure to unwoven polyester fiber filters

(A) white blood cell (WBC) counts. (B) Plasma LTF levels. LTF: lactoferrin.

Figure 5. The morphological analysis of the neutrophils exposed and not exposed to unwoven polyester fiber filters

(A,B) Observation by optical microscope after Wright-Geimsa staining without and with exposure, respectively. <Bars=2.5μm>. (C,D) Electron microscope analysis without and with exposure, respectively. Arrow in D indicates a fiber. <Bars=2.5μm>. (E,F) Immuno-fluorescence analysis using antibody to LTF without and with exposure, respectively. LTF: lactoferrin, DAPI: 4',6-Diamidino-2-phenylindole dihydrochloride. <Bars=5.0μm>.

Table 1. Baseline characteristics of the patients with rheumatoid arthritis

	LCAP (n=7)	In vitro experiment (n=14)
Age, years	57 [11]	63 [14]
Gender (male/female)	0/7	5/9
Disease duration, years	5 [7]	6 [8]
Stage （I/II/III/IV）	0/4/3/0	1/8/2/3
DAS28	5.79 [1.74]	5.31 [2.20]
SDAI	24.9 [18.8]	23.9 [16.9]
CDAI	23.9 [19.0]	20.5 [18.0]

Values are presented as the median [IQR]. All other date represent the number of patients. DAS28: disease activity score in 28 joints calculated by erythrocyte sedimentation rate. SDAI: simplified disease activity index. CDAI: clinical disease activity index. LCAP: leukocytapheresis.

Figure 1

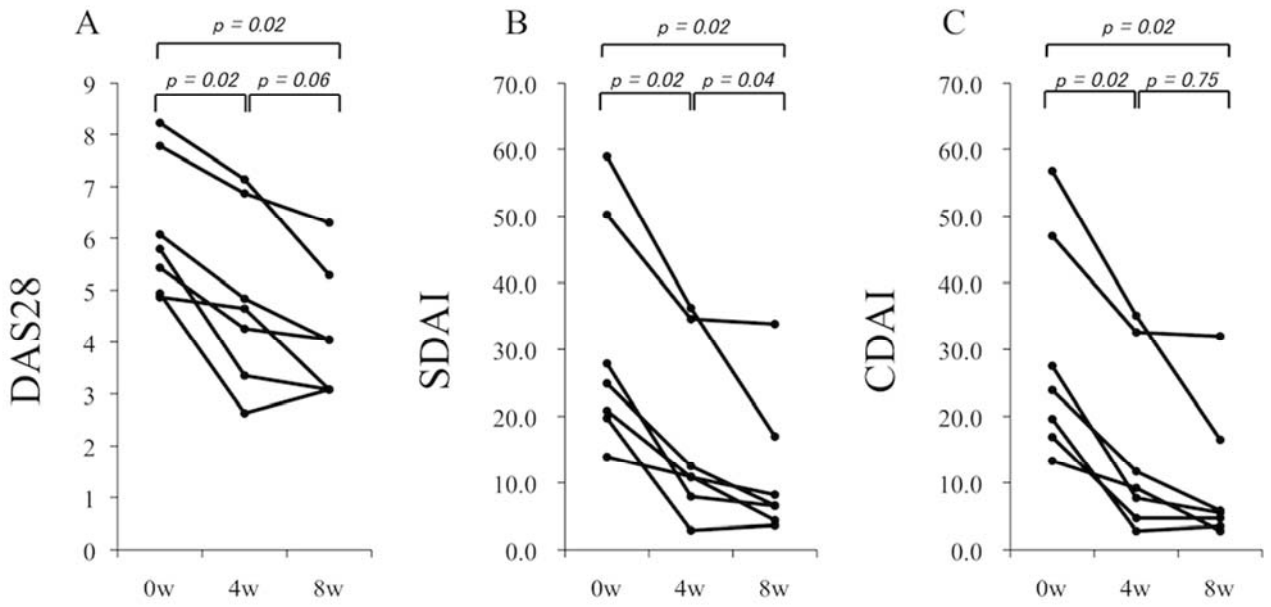

Figure 2

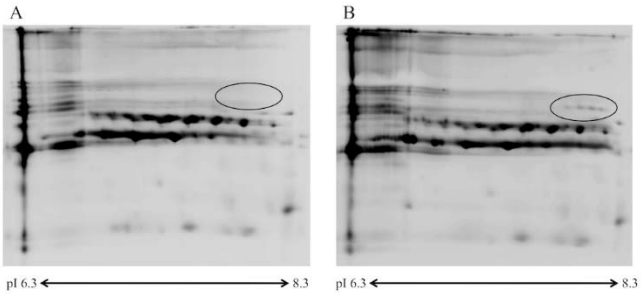

Figure 3

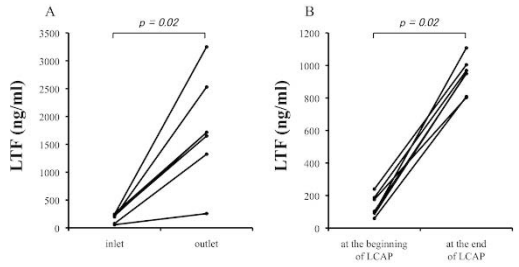

Figure 4

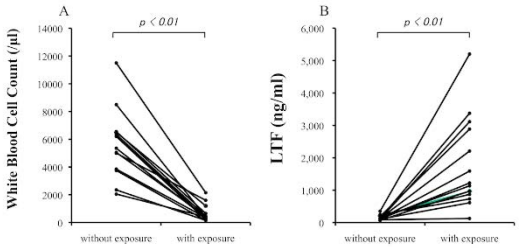

Figure 5