ORIGINAL ARTICLE

Serum proteome analysis in patients with rheumatoid arthritis receiving therapy with etanercept, a chimeric tumor necrosis factor-alpha receptor

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Abstract

Aim: Rheumatoid arthritis (RA) is a chronic inflammatory disorder of the synovium resulting in the destruction of affected joint cartilage and bone structures. Etanercept is a biological agent that blocks the tumor necrosis factor-α (TNF-α)-mediated inflammatory processes in RA patients, and has a regenerative effect on cartilage. In order to identify novel disease-related proteins and candidate biomarkers, we performed proteomic profiling of the serum in patients with RA who were treated with etanercept.

Method: Serum samples were obtained from eight RA patients before and after etanercept treatment. The low molecular weight proteins in the serum were concentrated and analyzed by liquid chromatography-tandem mass spectrometry. The results before and after etanercept treatment were compared by the spectrum count method.

Results: Among a total of 477 proteins identified, 12 were found to be decreased and five were increased by etanercept treatment. Some of the changed proteins were known to be related to RA, and most of the other changed proteins may play possible roles in the TNF-α signaling pathway or the state of cartilage and extracellular matrix.

Conclusion: The present proteomic study identified several proteins that could be involved in the pathogenesis of RA. These findings could thus lead to the identification of novel candidate disease-related protein biomarkers for RA, or indicate new targets for therapy.

Key words: etanercept, proteomics, rheumatoid arthritis, tumor necrosis factor.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of the synovium that induces the destruction of joint cartilage and bone structures. Although the precise molecular mechanisms underlying the pathogenesis of RA have not been fully elucidated, some inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are known to contribute to the onset and exacerbation of RA.

Recently, biological drugs targeting specific molecular pathways have been developed and are being applied for the treatment of RA. These drugs dramatically

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improve the clinical condition of RA patients compared with other traditional agents, such as anti-inflammatory drugs and disease-modifying antirheumatic drugs.

There are currently several biological agents that have been approved for the treatment of RA, including infliximab (an anti-TNF-α monoclonal antibody),[1] adalimumab (a fully human monoclonal antibody against TNF-α),[2] etanercept (a soluble TNF receptor),[3] tocilizumab (a humanized anti-IL-6 receptor monoclonal antibody)[4] and abatacept (a fusion protein composed of the immunoglobulin and the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4).[5] Although both infliximab and etanercept dramatically inhibit inflammation and control articular damage in RA patients by blocking TNF-α-mediated processes,[6] the molecular events induced by each drug are different.

Etanercept is a chimeric protein comprised of the human TNF receptor 2 and the Fc chain of human immunoglobulin G1 (ImG1).[3] It can bind both TNF-α and TNF-β (lymphotoxin alpha) and neutralize their activity. In contrast, infliximab, an anti-TNF-α monoclonal antibody, can bind only TNF-α. Importantly, infliximab can bind both the soluble and transmembrane forms of TNF-α.[7] Therefore, infliximab mediates the killing of cells expressing transmembrane TNF-α on their surface,[7] whereas etanercept cannot. Thus, the administration of these agents is expected to lead to different effects on the human body.

As drastic changes in the disease state occur after treatment with these agents, a comparative study before and after administration of these drugs would provide an effective way for elucidating the disease outcome and development of a novel diagnostic system. Previously, we performed a proteome analysis of the serum/plasma in RA patients treated with infliximab by use of mass spectrometry, and found that the detection profiles of several proteins, including proteins related to TNF-α and NF-kappa B responses, were changed by infliximab treatment.[8] Among the proteins changed, we focused on connective tissue growth factor (CTGF) and investigated its involvement in the pathogenesis of RA. As a result, we found that the serum concentrations of CTGF in RA patients were higher than those in normal healthy controls, and that CTGF promoted osteoclast differentiation and activation, which could induce bone and joint destruction.[9] The success of this proteomic analysis was partially due to the use of a sample pretreatment method based on ultrafiltration to reduce the highly abundant high molecular weight proteins.

Therefore, a proteomic analysis of the serum/plasma in RA patients with and without treatment with biological agents is a promising method for detecting novel candidate disease-related proteins and biomarkers.[10,11] In this study, we performed a comparative serum proteomic analysis of the samples obtained before and after etanercept treatment in order to identify novel proteins related to RA.

MATERIALS AND METHODS

Materials

Sequencing-grade modified trypsin and ammonium bicarbonate were obtained from Promega (Madison, WI, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. High perdormance liquid chromatography (HPLC)-grade water, methanol, acetonitrile, dithiothreitol, iodoacetamide and formic acid were purchased from Wako Pure Chemical Industries, (Osaka, Japan).

Patients and samples

Prior to this study, our local ethics committee, which conformed to the provisions of the World Medical Association's Declaration of Helsinki, approved the protocol for the research. Written informed consent was obtained from all patients who participated anonymously in this study. A profile of the RA patients is shown in Table 1. Eight female patients with RA (mean age: 46.5 years; range: 36–63 years) were included in this study. All patients were diagnosed to have RA according to the criteria of the American College of Rheumatology (1987).[12] The average duration of disease was 3.75 years (range: 1–12 years). The Steinbrocker's disease activity stage and the functional class of each patient[13] are also described in Table 1. At the time when they started etanercept treatment, all patients were being treated with prednisolone and/or methotrexate. Etanercept was administrated twice a week. All patients showed a moderate or good response to etanercept judging from the improvement rate based on the disease activity score 28 (DAS28).[14]

Serum specimens were obtained from each RA patient three times. The first, second and third blood samples were collected just before the first, fourth and eighth administration of etanercept, respectively. All blood samples were incubated at room temperature for 30 min, and were centrifuged for 7 min at 1600 g. Sera were stored at −80°C until analysis.

Serum pretreatment

The serum pretreatment and sample preparation for the proteomic analysis were performed as described previously, with slight modifications.[8] Briefly, the protein

Table 1 Profiles of patients with rheumatoid arthritis

No.	Age (years)	Sex	Duration of RA (years)	Class/Stage†	DAS28 (treatment)			Response to etanercept	PSL (mg/day)	MTX (mg/week)
					0	4	8			
1	38	F	2	3/4	5.90	4.11	4.32	Moderate	5	0
2	51	F	12	2/4	4.80	2.81	2.39	Good	5	4
3	63	F	3	3/4	5.45	2.98	2.89	Good	0	4
4	36	F	6	2/4	5.21	4.37	2.30	Good	5	6
5	54	F	1	3/4	6.39	6.26	3.55	Good	15	0
6	46	F	1	2/2	4.47	4.23	2.72	Good	5	6
7	43	F	1	2/2	4.75	3.39	2.51	Good	5	6
8	41	F	4	2/2	4.67	3.56	2.44	Good	5	0

†Functional (class) and radiological (stage) classifications by Steinbrocker.[13] DAS28, disease activity score 28.[12] The DAS28 was evaluated at the time of serum collection. 0: before first treatment, 4: before fourth treatment, 8: before eighth treatment. PSL, dose of prednisolone; MTX, dose of methotrexate.

concentration of each serum sample was measured by the Bradford method (Protein Assay Reagent; BioRad, Hercules, CA, USA) and then was adjusted to 12.5 mg/mL by adding 25 mmol/L ammonium bicarbonate buffer (pH 8.0). Four milliliters of each sample containing 50 mg protein was injected into a hollow fiber membrane-based ultrafiltration device (HFMD, Toray Industry, Tokyo, Japan).[15] With this device, the low molecular weight serum proteins were concentrated, and abundant high molecular weight serum proteins were depleted. The low molecular weight serum proteins obtained from the HFMD were then lyophilized and resuspended in 300 μL of 100 mmol/L Tris–HCl (pH 8.0).

Trypsin digestion

The samples were reduced with 10 mmol/L dithiothreitol at 80°C for 20 min and then alkylated with 30 mmol/L iodoacetamide at 37°C for 30 min in the dark. Trypsin was added to the samples at a protein: enzyme ratio of 50 : 1 (w/w), followed by incubation at 37°C for 2–3 h. The same amount of trypsin was added again, and the mixture was incubated at 37°C overnight. The digestion was terminated by adding formic acid to a final concentration of 0.1% (v/v). The digested samples were desalted using a reversed phase silica mini column cartridge (Oasis HLB cartridge 1 cc; Waters, Milford, MA, USA). After evaporation, the digested peptides were reconstituted in 0.1% v/v formic acid. The UV absorbance at 280 nm of each peptide sample was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA, USA), and the peptide concentration was calculated based on the assumption that UV absorbance at 280 nm was 1 for a 1 mg/mL solution.

Two-dimensional liquid chromatography coupled to tandem mass spectrometry (2D-LC-MS/MS) analysis

Fifty micrograms of each digested peptide sample were analyzed by a 2D nano LC-MS/MS using a direct nano LC system (DiNa; KYA Technologies, Tokyo, Japan) and a mass spectrometer (Q-Tof Ultima; Waters-Micromass, Manchester, UK). The tryptic peptides were diluted by adding 100 μL of 0.1% v/v formic acid, and were injected into a strong cation exchange chromatography (SCX) column (35 × 0.32 mm internal diameter [i.d.]; KYA Technologies, Tokyo, Japan) and washed with 0.1% v/v formic acid. The peptides were separated into eight fractions; one unbound fraction and seven fractions obtained from stepwise elution with 10, 15, 25, 35, 50, 100 and 500 mmol/L ammonium acetate. Each of the peptide fractions obtained from the SCX column was injected on-line into a C18 reversed-phase chromatography system (1 × 0.5 mm i.d. C18 trap column and 50 × 0.15 mm i.d. C18 separation column; KYA Technologies, Tokyo, Japan) and eluted with a linear gradient of acetonitrile with 0.1% v/v formic acid.

The eluted peptides were introduced on-line into the mass spectrometer with a nanospray emitter (New Objective, Woburn, MA, USA) and analyzed by MS scanning, followed by three data-dependent MS/MS scans of the three most intense peaks under a heated capillary temperature of 150°C and a spray voltage of 2 kV.

Data analysis

The acquired MS data were processed by the MassLynx software program ver. 4.0 (Waters-Micromass, UK) to

obtain the MS/MS peak list. The acquired MS/MS peak list was compared with the RefSeq human protein database (2009. 05. 18; National Center for Biotechnology Information, Bethesda, MD, USA) containing 39,071 entries for human proteins using the Mascot software program ver. 2.2 (Matrix Science, London, UK).[16] The search tolerance was set to 200 ppm for precursor ions and 0.3 Da for product ions. One trypsin miscleavage per peptide was allowed, and certain modifications (acetylation of the N-terminus of proteins, oxidation of methionine, pyroglutamation of the N-terminal glutamine of peptides, and carbamidomethylation of cysteine) were also considered. The criterion employed for protein identification was that the peptide match score should significantly ($P < 0.05$) exceed the peptide identification threshold in the Mascot MS/MS ion search.

The comparison of the protein content was performed by the spectrum count method.[17–19] The number of peptides thus identified for each protein was counted in each sample. Two sets of spectrum count data were compared. The difference in the early phase was given as a comparison between the samples collected before the first administration and before the fourth administration of etanercept, and the one in the late phase was given as a comparison between the samples collected before the first administration and before the eighth administration of etanercept.

The N_{inc} score was defined as the number of patients in whom the number of peptides identified by the eight series of analyses increased after etanercept treatment, while N_{dec} was the number of patients in whom the number of peptides decreased. The values of N_{inc} and N_{dec} were calculated separately for the early and late phases. The detection score (D) was calculated as $(N_{inc} - N_{dec})/n$ ($n = 8$).

The spectrum count of the identified proteins was also compared by a paired t-test for the early and late phases. In the comparison for either the early or late phases, proteins for which $D \geq 0.5$ and $P < 0.05$ in the t-test were defined as being increased by etanercept treatment, whereas proteins for which $D \leq -0.5$ and $P < 0.05$ in the t-test were defined as being decreased by etanercept treatment.

ELISA

The concentration of granulin in the serum samples was measured by Granulin Enzyme-Linked Immunosorbent Assay (ELISA) Kit E92513HU (Uscn Life Science Inc. Wuhan, China) following the manufacturer's instructions.

RESULTS

Table 1 shows the profiles of the RA patients who participated in this study. Compared with the untreated state, the DAS28 score was decreased after etanercept treatment in each of the patients, indicating that all patients showed a moderate or good response to etanercept treatment.

Samples were treated with HFMD ultrafiltration and trypsin digestion prior to the LC-MS/MS analysis, as described in the Materials and Methods section. A 50 mg sample of serum proteins yielded an average of 115 μg of digested peptides, and the coefficient of variation of pretreatment efficiency was 12.2%. Equal amounts (50 μg) of each digested peptide sample were applied to an LC-MS/MS analysis to adopt the spectrum count method for the semi-quantitation of proteins.

We performed a shotgun LC-MS/MS analysis of 24 samples from the eight patients, and the number of significantly ($P < 0.05$) identified peptides in each protein was counted. In total, we identified 477 proteins. The information about these proteins and the spectrum count of the identified peptides are listed in Supplementary Table 1. The average false positive rate for 24 analyses was 0.53%.

Figure 1 shows the distribution of the molecular weights of all of the proteins identified in this study. The indicated values of the molecular weights were theoretically calculated based on the amino acid sequence on the RefSeq database used for protein identification, and therefore it did not indicate the precise molecular weight of a mature or intact protein existing in the serum. Because we used a hollow fiber-based membrane ultrafiltration device for depletion of highly abundant proteins from serum, the low molecular weight proteins below 50 kDa were enriched. As expected, low molecular weight proteins were preferentially identified, thus indicating that the HFMD device worked efficiently in this study.

According to our scoring criteria described in the Materials and Methods section, the D of each protein, which indicates a change in the protein expression induced by etanercept treatment, was calculated. The score for the early phase was calculated for the differences between the samples collected before the first administration and before the fourth administration of etanercept, and the score for the late phase was calculated between the samples collected before the first administration and before the eighth administration. The distribution of the scores for all proteins is shown in Figure 2. The distribution of the scores for the early
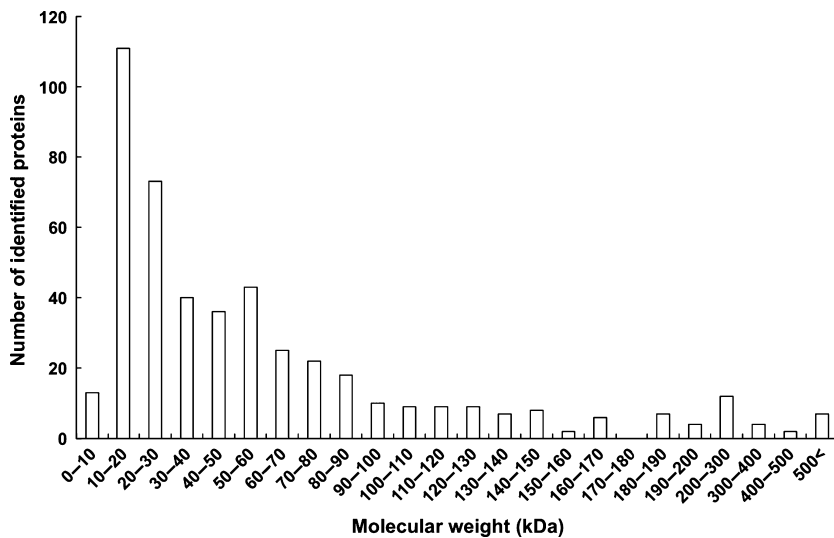
Figure 1 The distribution of the molecular weights of all the proteins identified in the serum samples from RA patients receiving etanercept treatment. The horizontal axis shows the range of molecular weights of the identified proteins. The molecular weight values were theoretically calculated based on the amino acid sequence entered in the database. The vertical axis shows the number of proteins identified in the indicated range of molecular weights.

phase is shown in Figure 2a, and that for the late phase is shown in Figure 2b. In both comparisons, more than 94% of the proteins were distributed in the range of $-0.375 \leq D \leq 0.375$. Based on our criteria, most of the proteins were judged to be unchanged, and the number of the proteins that showed $D \leq -0.5$ or $D \geq 0.5$ was 23 (4.8% of the total proteins) for the early phase and 26 (5.5% total proteins) for the late phase, respectively. We confirmed these results by paired *t*-tests ($P < 0.05$), and selected 17 proteins that were differentially expressed in patients before and after the etanercept treatment by our data analysis criteria. These differentially expressed proteins are shown in Table 2. Among them, the expression levels of 12 proteins were decreased by etanercept treatment, of which four were changed both in the early and late phases, and eight were changed only in the late phase. There were five proteins that were increased by etanercept treatment, of which two were changed both in the early and late phases, and three were changed only in the late phase. The proteins that showed significant changes from the early phase were concluded to respond more rapidly to the treatment.

The ELISA method was used to validate the concentration of granulin, one of the proteins with an altered detection profile following etanercept treatment in this study. Although statistical significance was not achieved, granulin was increased by etanercept treatment in RA patients, supporting the results of proteomic analysis (Fig. 3). The change in the detection profile of granulin protein by mass spectrometry was likely to show some influence by the disease-dependent change of the granulin validated by the ELISA method, although the detectable molecular form of granulin protein analyzed by these two methods might differ.

DISCUSSION

In this study, we investigated the influence of etanercept treatment on RA patients by a serum proteome analysis. Because abundant proteins, such as albumin and immunoglobulin exist in the serum in high concentrations,[20,21] the proteomic analyses of sera often show low comprehensiveness of identification. In order to overcome this obstacle, highly abundant proteins are usually depleted. One of the most frequently used approaches is multiple affinity chromatography by use of a column coupled with specific antibodies against these abundant proteins. However, the commercially available multiple affinity columns have some weak points in that the reuse of the column decreases the binding capacity of antibodies to the antigen proteins, and the concentrations of the highly abundant proteins remaining in the sample are somewhat changed. We therefore adopted a disposable HFMD ultrafiltration device for serum pretreatment, and efficiently depleted the abundant proteins with a high molecular weight.[8,15]

Using the HFMD, the polypeptide chains with a defined range of molecular weights, typically 3–50 kDa, were concentrated. Whereas some of these were mature proteins, there were also proteolytically processed or degraded polypeptide fragments derived from proteins with a larger molecular weight present in these samples.
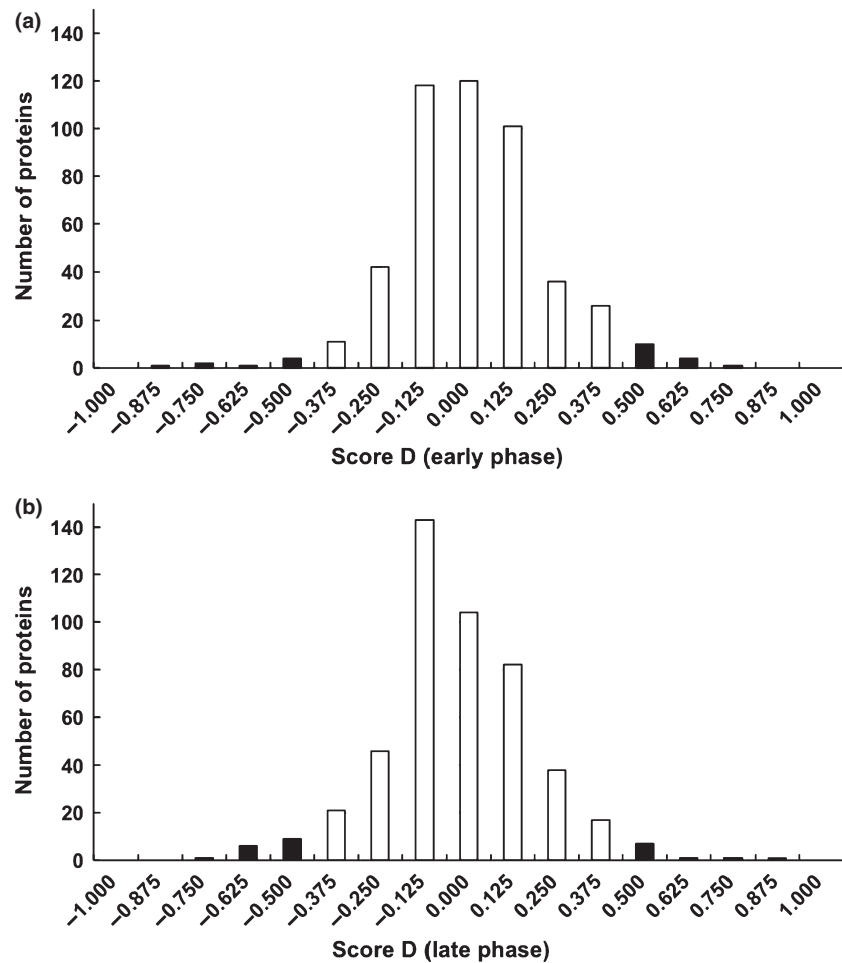
Figure 2 Distribution of the detection score D for all proteins. (a) A comparison between the first and second samples (early phase) and (b) a comparison between the first and third samples (late phase). Open column: $-0.5 < D < 0.5$, closed column: $D \leq -0.5$ or $D \geq 0.5$. The detection score D represented by the closed columns indicates that the proteins were differentially detected in the majority of the patients.

In fact, some of the identified proteins have large theoretical molecular weights based on the full-length amino acid sequence described in the database (Table 2). Therefore, the results of this analysis do not necessarily reflect the amount of total mature protein, but do provide a profile of polypeptides with molecular weights within the limited range. Proteins with a larger molecular weight may also have been detected due to leakage through the HFMD. Although the proteins with a higher concentration tend to leak much more, the ratio of leakage cannot be controlled. Therefore, the molecular species affected by the state of RA patients should be carefully assessed.

Among the differentially expressed proteins listed in Table 2, some of the proteins are known to be related to RA. For example, the serum levels of prostaglandin H2 D-isomerase[22] and kininogen 1[23] are known to increase in RA patients. Our results indicating that these proteins were decreased after etanercept treatment are

consistent with these reports. We have already found that the concentration of CTGF in the plasma was decreased by treatment with an anti-TNF-α drug.[9] In the present study, CTGF was also identified as a protein decreased by etanercept treatment. These results showed that the analysis method used in this study was appropriate for detecting disease-related proteins.

The other identified proteins may possibly play a role in the TNF-α signaling pathway or the state of cartilage and extracellular matrix, although their involvement in RA was not experimentally elucidated. Secreted and transmembrane 1 precursor and myotrophin, both identified as proteins that were decreased in this study, were functionally associated with NF-kappa B. The secreted and transmembrane 1 precursor was found to be related to NF-kappaB activation by a large-scale gene analysis of TNF-α activation.[24] The myotrophin protein is involved in cardiac hypertrophy and cerebellar morphogenesis via conversion of NF-kappaB p50-p65 hete-

Table 2 Proteins identified to be differentially expressed between patient samples collected before and after etanercept treatment

Protein name	Mass	Accession no.	Early phase		Late phase	
			D	t-test P	D	t-test P
Decreased						
Prostaglandin H2 D-isomerase	21,015	NP_000945	−0.88	**0.008**	−0.63	**0.033**
Kininogen 1 isoform 2	47,853	NP_000884	−0.75	**0.007**	−0.63	**0.038**
Proenkephalin	30,767	NP_006202	−0.50	**0.033**	−0.50	**0.033**
Alpha 1 type XI collagen isoform A preproprotein	180,954	NP_001845	−0.50	**0.049**	−0.50	**0.049**
Alpha 1 type V collagen preproprotein	183,447	NP_000084	−0.63	0.054	−0.63	**0.022**
Alpha 2 type I collagen	129,235	NP_000080	−0.50	0.072	−0.75	**0.026**
Beta actin	41,710	NP_001092	−0.13	0.685	−0.63	**0.011**
Rho GDP dissociation inhibitor (GDI) beta	22,974	NP_001166	0.13	0.451	−0.63	**0.041**
Connective tissue growth factor	38,043	NP_001892	0.38	0.170	−0.50	**0.033**
Secreted and transmembrane 1 precursor	27,022	NP_002995	−0.13	0.516	−0.50	**0.033**
Collagen, type II, alpha 1 isoform 1 precursor	141,698	NP_001835	−0.25	0.170	−0.50	**0.033**
Myotrophin	12,887	NP_665807	0.13	0.685	−0.50	**0.049**
Increased						
Proteoglycan 4 isoform A	150,984	NP_005798	**0.63**	**0.026**	**0.50**	**0.050**
Fibulin 5 precursor	50,147	NP_006320	**0.50**	**0.033**	**0.50**	**0.033**
Complement factor D preproprotein	27,016	NP_001919	0.50	0.138	**0.88**	**0.041**
Granulin precursor	63,500	NP_002078	0.50	0.250	**0.75**	**0.008**
Complement component 5 preproprotein	188,186	NP_001726	0.13	0.785	**0.63**	**0.021**

Mass: calculated molecular weight of the protein based on the full-length amino acid sequence described in the database; Accession no.: accession number in the human RefSeq protein database of the National Center for Biotechnology Information, USA; Early phase: a comparison between the first and second samples; Late phase: a comparison between the first and third samples; D: the detection score described in the Materials and Methods section, t-test P: P-value of the paired t-test. Bold: changed based on our criteria.
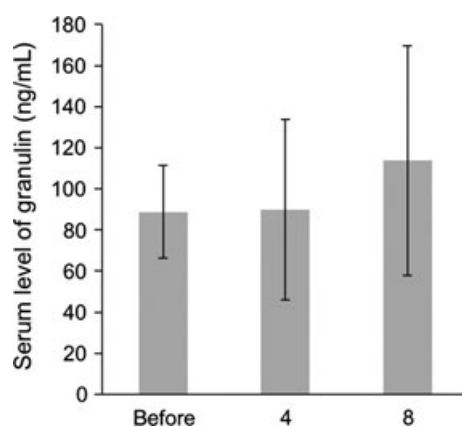

Figure 3 Concentration of granulin in the serum. The concentration of granulin in the serum of rheumatoid arthritis patients collected for the proteomic analysis in this study was measured by emzyme-linked immunosorbent assay. Before: before the etanercept treatment, 4: just before the fourth administration of etanercept, 8: just before the eighth administration of etanercept.

rodimers to p50-p50 and p65-p65 homodimers.[25] Therefore, the changes of the levels of these proteins would be related to the inhibition of the TNF-α pathway by etanercept.

The expression levels of several proteins related to cartilage and synovial membranes were changed by etanercept treatment. Proteoglycan 4 (PRG4), also known as lubricin or superficial zone protein, is a highly glycosylated protein secreted from synovial fibroblasts[26] that functions as a boundary lubricant in the synovial fluid between the surfaces of cartilage. It was reported that the concentration of PRG4 in synovial fluid is decreased in patients with acute injury,[27] and that early elevation in the coefficient of friction in the joints of rats with antigen-induced arthritis might result from the reduced synthesis of PRG4 by synovial fibroblasts.[28] However, the behavior of PRG4 in the serum of RA patients is unknown. Our data demonstrated that the level of PRG4 in the serum of patients with RA was increased after etanercept treatment. Since all of the patients participating in this study showed a decreased DAS28 score after etanercept treatment, the concentration of PRG4 in the serum may be influenced by the condition of the cartilage.

It should be noted that the PRG4 molecular species detected in this analysis were different from the one present in the synovial fluid, because the molecular weight of the mature PRG4 glycoprotein is about 300 kDa. Although the molecular form(s) of PRG4

present in our serum samples is unknown, we demonstrated that some PRG4-derived polypeptides with a molecular weight smaller than 50 kDa existed in the serum, and that the concentration of these peptides might depend on the pathological condition of the RA patient.

The granulin precursor was also increased after etanercept treatment. This protein is a 593-amino acid glycoprotein with a molecular weight of 80 kDa, and functions as a growth factor. It undergoes proteolytic processing, resulting in the production of an approximately 6 kDa granulin polypeptide.[29] It was reported that the granulin precursor binds to metalloproteinases ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-7 and ADAMTS-12 and inhibits their enzymatic activity, thereby preventing the degradation of cartilage oligomeric matrix proteins.[30] However, the relationship between granulin and the pathogenesis of RA is unknown. Similar to PRG4, there were processed granulin polypeptides with a smaller molecular size present in the patient serum, and the detection frequency was increased following improvement in the pathological condition of RA.

All four collagen proteins were decreased by etanercept treatment. Judging from the molecular weight of these proteins, fragmented molecular species of collagens existed in the serum of RA patients, and etanercept treatment decreased these fragments. Rho guanosine diphosphate -dissociation inhibitor beta was also identified as a decreased protein in this study. The small GTP protein Rho-mediated signaling promotes the proliferation of rheumatoid synovial fibroblasts.[31] Thus, the expression levels of these proteins related to cartilage and the extracellular matrix were changed by etanercept treatment.

We have already reported the results of a serum/plasma proteomic analysis in RA patients treated with infliximab by use of the same HFMD device applied in this study.[8] The results obtained after infliximab treatment were somewhat different from those observed after etanercept treatment. For example, the CTGF peptides were more easily detectable after infliximab treatment, whereas the etanercept treatment decreased CTGF detection in the sera of RA patients. This disparity may be due to the difference in the schedule for serum sample collection after treatment. This study collected the serum samples just before the fourth and eighth etanercept treatment. The previous study collected the serum samples 1 day after the first administration of infliximab. Therefore, the state of the RA patients could

be different. Another reason for this disparity may be the different mechanism of action between these agents. Both infliximab and etanercept bind to TNF-α in the plasma. However, infliximab can bind to the membrane-bound TNF-α on the cell surface and destroy the cells expressing it, whereas etanercept cannot.[7] Therefore, the differences might be due to the influence of these drugs on cellular destruction. Although there were some apparent disparities between the results of these studies, the change in the protein profile obtained by the mass spectrometric analysis of HFMD-processed samples were likely to have the information about the changes in the profile of the proteins, such as their molecular concentration, molecular size and molecular form.

Although there have been several comparative proteomic studies of RA patients treated with a biological agent,[8,10,32] this study is the first report of a mass spectrometry-based proteomic analysis of the serum in RA patients treated with etanercept. As described above, we identified several proteins that were differentially expressed in serum samples before and after etanercept treatment. However, these data obtained by large-scale proteomic analyses for patients may be insufficient to establish the individual involvement of each protein in the pathogenesis of RA. Based on these data obtained by the proteomic screening method, we will conduct a validation study, including a large number of patients, to elucidate whether these proteins are indeed related to RA. Further studies of these proteins could help to discover novel disease-related proteins and candidate biomarkers.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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