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Journal of Chromatography B, 855 (2007) 66–70
JOURNAL OF CHROMATOGRAPHY B
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Serum protein profile of rheumatoid arthritis treated with anti-TNF therapy (infliximab)☆

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Received 25 October 2006; accepted 12 February 2007
Available online 24 February 2007

Abstract

We analyzed the changes in the serum protein profile by infliximab using two-dimensional gel electrophoresis and mass spectrometry. More than 50 gel spots were seen to increase or decrease in correlation with clinical improvements of RA. The spots corresponding to CRP, C3, and Apo J showed reduced staining intensity, while the spots corresponding to Apo A-I, RBP, and transthyretin were enhanced. The protein profile of RA patients treated with infliximab was mostly similar to that of normal healthy controls except for several protein spots. This suggested that infliximab normalized the serum protein profile of RA patients, leading to modification in the serum lipid profile and antioxidant status in RA.
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Keywords: Tumor necrosis factor; Infliximab; Rheumatoid arthritis; Matrix assisted laser desorption ionization/time-of-flight mass spectrometry; Electrophoresis

1. Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by inflammation of the synovium and destruction of bone and cartilage. The pathogenesis of RA remains unclear, but inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α have been found to play an important role [1]. These inflammatory cytokines are produced abundantly by activated macrophages in synovial tissues from RA patients and stimulate mesenchymal cells such as osteoclasts, fibroblasts, and chondrocytes, which release joint-destructive matrix proteinase.

TNF-α is a pivotal mediator in inflammatory arthritis including RA [1]. TNF-α is an autocrine stimulator as well as a potent paracrine inducer of other inflammatory cytokines such as IL-1 and IL-6 [2]. The blockade and inhibition of TNF-α reduces the production of other inflammatory cytokines in cultured synovial cells from RA patients [3]. In animal polyarthritis, similar to rheumatoid synovitis, TNF-α inhibitors ameliorated disease activity and prevented the destruction of cartilage and bone [4,5].

Many reports have demonstrated the clinical efficacy of TNF-α inhibitors in the treatment of RA patients with active disease. Blocking TNF-α with monoclonal antibodies (infliximab) or a soluble TNF-receptor fusion protein (etanercept) reduced inflammatory biomarkers such as C-reactive protein (CRP) and serum matrix proteinase in parallel with clinical responses. However, little attention has been paid to the other biochemical changes brought about by TNF-α inhibitors.

The purpose of this study was to measure changes in serum proteins from RA patients treated with infliximab following a proteomics-based approach. We could analyze serum proteins including low-abundant proteins by depleting high-abundant proteins using a multiple-affinity column.

2. Materials and Methods

2.1. Patients and controls

Five patients (three women, two men) with clinically active RA were recruited from outpatient clinics at Osaka Medical College. Patient age at the time of study and the disease duration ranged from 35 to 68 years and from 1 to 16 years, respectively.

☆ This paper was presented at the 31st Annual Meeting of the Japanese Society for Biomedical Mass Spectrometry, Nagoya, Japan, 28–29 September 2006.

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doi:10.1016/j.jchromb.2007.02.028

Table 1
Clinical data of patients

Age/Sex	Disease duration	DAS28/MMP-3		Treatment during infliximab therapy
		Baseline	6th month	
66/F	4	6.02/802	3.04/82.6	MTX 8 mg/w, SASP 1000 mg/d PSL 10 mg/d, diclofenac sodium 50 mg/d
68/F	16	5.07/242	3.10/109	MTX 8 mg/w, SASP 1000 mg/d
35/F	1	6.31/527	2.68/197	MTX 8 mg/w, SASP 1000 mg/d, etodolac 200 mg/d
60/M	4	5.69/81.1	2.38/88.7	MTX 8 mg/w, PSL 5 mg/d, loxoprofen sodium 180 mg/d
67/M	2	6.76/207	3.38/213	MTX 8 mg/w, SASP 1000 mg/d PSL 10 mg/d, diclofenac sodium 75 mg/d

DAS28: Disease Activity Score 28 [6], MMP-3: matrix metalloproteinase-3 [normal range: 17.3–59.7 ng/mL (female) and 36.9–121 ng/mL (male)], MTX: methotrexate, SASP: salazosulfapyridine, PSL: prednisolone.

All patients received infliximab (3 mg/kg) on weeks 0, 2, and 6, and every 8 weeks thereafter in combination with methotrexate (8 mg per week), and showed good responses with infliximab (Table 1). Sera were obtained before and after 6 months of treatment with infliximab, and stored at −80 °C until assay. Five age and sex-matched controls (three women, two men) with no clinical or laboratory evidence of disease were recruited. Sera from five healthy controls were also obtained and stored at −80 °C until assay.

2.2. Clinical and laboratory assessment

Disease activity was assessed using the Disease Activity for 28 Joint Indices Score [6]. The serum CRP level was measured using the standard procedure at Osaka Medical College hospital, and serum levels of apolipoproteins A-I (Apo A-I) and retinol-binding protein (RBP) were measured at SRL Inc. (Tokyo, Japan).

2.3. Chemicals and materials

Urea, glycine, sodium dodecyl sulphate (SDS), iodoacetamide, CHAPS, acrylamide, *N*,*N*-methylene bisacrylamide, tris (hydroxymethyl)-aminomethane (tris), ammonium bicarbonate, glycerol, 2-mercaptoethanol, dithiothreitol (DTT), ammonium persulfate, silver nitrate, and ethylenediamine tetraacetic acid were purchased from Nakalai tesque (Kyoto, Japan). Methanol, ethanol, acetonitrile, acetone, acetic acid, formic acid, and distilled water were from Merck (Darmastadt, Germany). Immobilized pH3-10 non-linear gradient strips (17 and 7 cm long) were purchased from Amersham Pharmacia Biosciences (Arlington Hights, IL). Ten-twenty% gradient slub-gel (20 and 9 cm long) was obtained from Daiichi Chemical Co., Ltd. (Tokyo, Japan).

2.4. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

To deplete high-abundant proteins such as albumin, IgG, and haptoglobin from serum, sample serum was loaded onto an Agilent multiple-affinity column removal system (Agilent Technologies, DE) following the manufacturer's recommendations. The flow-through proteins as low-abundant proteins were collected, pooled, and concentrated. Samples of 50 μg protein were resolved by two-dimensional (2D)-PAGE and detected by silver staining, as previously described [7]. Isoelectric focusing (IEF) gel electrophoresis was conducted with pH3-10 non-linear gradient strips using an Ettan IPGphor II (Amersham Biosciences, CA) following the manufacturer's recommendations. After IEF gel electrophoresis, the IEF strip was equilibrated with 50 mM tris (pH 6.8) containing 10% glycerol, 2% SDS, 1% DTT, and bromphenol blue and the treated gel strip was loaded on a gradient slub-gel. Separated proteins were visualized using silver staining and the gel spots of interest were excised. Each piece of the gel spots was reduced, alkylated, and digested with TPCK-trypsin in Eppendorf tubes at 37 °C for 16 h. The gel pieces were alternately washed with 50 mM ammonium bicarbonate (pH 8.5) and acetonitrile and finally dehydrated with acetonitrile. These pieces were completely dehydrated in a Speedvac device at 30 °C and then covered with 25 mL of TPSK-modified trypsin (0.02 mg/mL, Promega Co., Ltd., Madison, WI) in NH_4HCO_3 buffer (40 mM, pH 8.5) and left at 37 °C overnight. After enzymatic digestion, the resultant peptides were extracted in 100 μL of 0.5% (v/v) formic acid (50/50). The extraction was conducted in an ultrasonic bath for 15 min each time. The extracts were concentrated and desalted in ZipTip C18 micro-columns (Millipore Co., Ltd., Bedford, MT).

2.5. Protein identification by mass spectrometry

Protein identification was performed as previously described [7]. The extracted peptides were loaded on the MALDI target plate by mixing 1 μL of each solution with the same volume of a matrix solution that was prepared fresh every day by dissolving 0.3 g/mL of a-cyano-4-hydroxycinnamic acid (Wako Purified Reagent Co. Ltd., Kyoto) in acetone-ethanol (1:1, v/v) solvent. Measurements were performed using an UltraFlex (Brucker-Daltonics, Germany). Calibration was accomplished using external peptide standards (Brucker-Daltonics) or the trypsin autodigestion peptide signal as an internal standard. The final mass spectra were produced by averaging 50–200 laser shots. The peptide mass fingerprint was used for protein identification from the tryptic fragment size using the Mascot Search engine based on the entire NCBIn and Swissprot protein

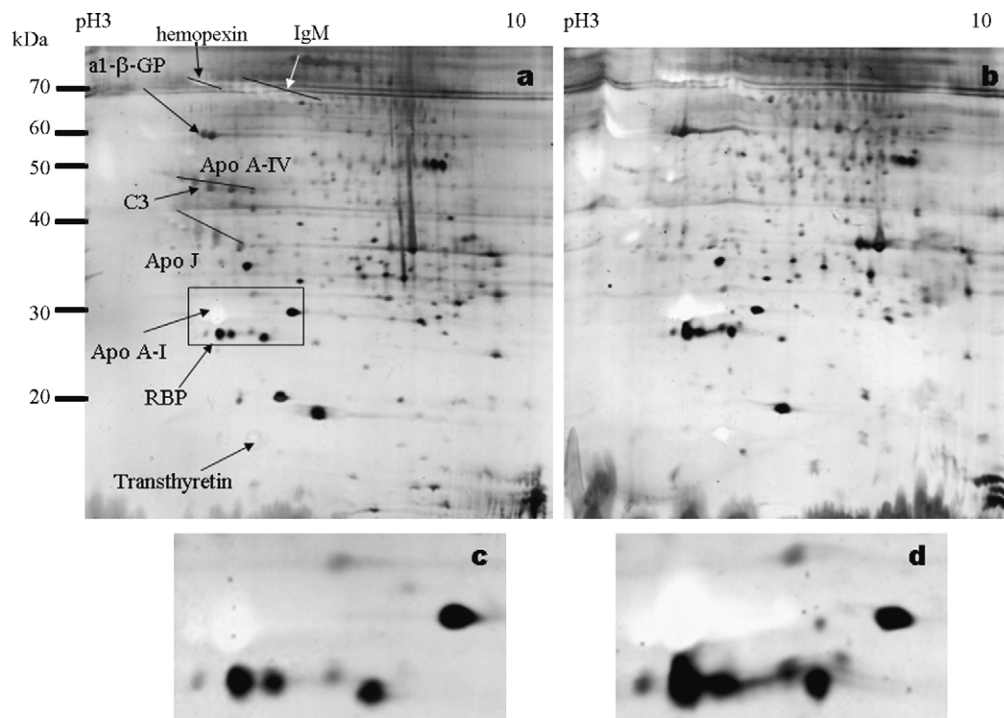

Fig. 1. Silver-stained 2D-PAGE gel showing 50 μg of serum sample from an RA patient treated with infliximab. Sera were obtained prior to infliximab (a, c) and at different times thereafter (b, d). The high-abundant proteins such as albumin and immunoglobulins were depleted from serum using the multiple-affinity column, as described in Materials and Methods. Serum samples were resolved on 2D-PAGE. Zoomed areas highlight the changes in Apo A-I and RBP (c, d).

databases, on the assumption that peptides are monoisotopic, oxidized at methionine residues, and carbamidemethylated at cystine residues. All proteins identified were in the expected size range by comparison with the PLASMA SWISS-2D PAGE map (http://www.expasy.ch/).

3. Results

Fig. 1 shows the silver staining profiles of the 2D-PAGE of serum proteins from an RA patient treated with infliximab. The most abundant proteins such as albumin and immunoglobulins were depleted using a multiple-affinity column removal system, and more than 1000 spots were visible. IgM, α1-β-glycoprotein, hemopexin, Apo A-I, Apo A-IV, Apo J, RBP, and transthyretin were identified by comparison with the PLASMA SWISS-2D PAGE map and the proteomics-based technique using MALDI-TOF/MS (Fig. 1). The administration of infliximab markedly changed the protein profile of serum from an RA patient (Fig. 1a and b). More than 50 gel spots were seen to increase or decrease in correlation with clinical improvements of RA. The spots corresponding to CRP, C3, and Apo J were reduced in staining intensity, while the spots corresponding to Apo A-I, RBP,

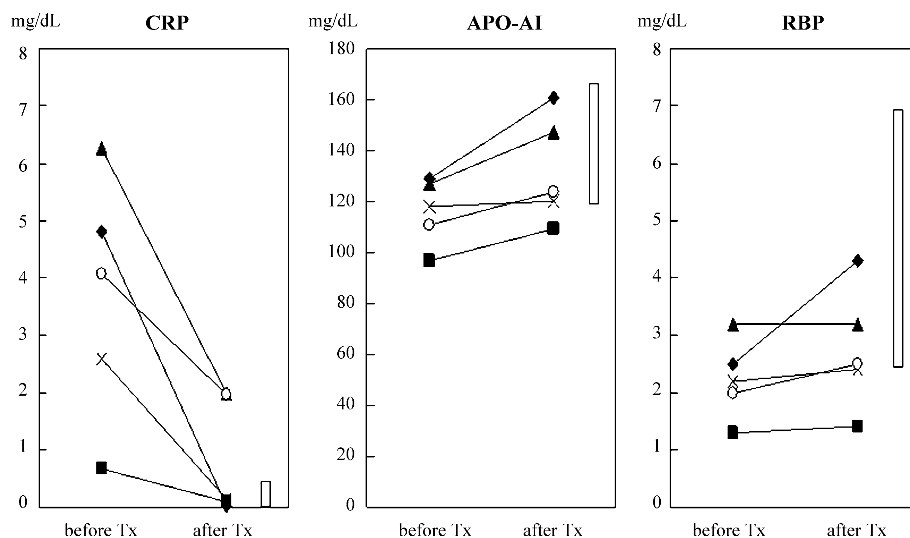

Fig. 2. Changes in the serum levels of CRP, Apo A-I, and RBP by infliximab. Open boxes show the normal ranges of CRP, Apo A-I, and RBP.

and transthyretin were enhanced. The protein maps of other patients were similar to that of this patient (data not shown). To confirm the serum levels of CRP, Apo AI, and RBP in RA patients, they were measured by laser nephelometry. Fig. 2 shows that the serum level of CRP was decreased (3.7 ± 2.2 to 0.84 ± 1.0 mg/dL; normal range <0.5 mg/dL) in all RA patients and that of Apo A-I was increased (116 ± 13 to 132 ± 21 mg/dL; normal range 119–165 mg/dL). Four of the five patients with RA showed increased RBP (2.4 ± 0.7 to 2.8 ± 1.0 mg/dL; normal range 2.4–7.0 mg/dL). These results were identical to protein profile changes of 2D-PAGE. Other spots of interest could not be identified because of the low concentrations.

To show the changes of the serum protein profile brought about by infliximab towards normal levels, we evaluate the differences in serum protein profiles between RA patients treated with infliximab and healthy controls. To exclude individual variations of serum proteins, pooled sera from five patients after 6 months of infliximab treatment and pooled sera from healthy controls were used. Fig. 3 shows that the protein profile of RA patients treated with infliximab was similar to that of healthy controls. No significant difference in staining intensity was observed in most spots of 2D-PAGE between RA patients and the healthy controls. However, there were several spots of 2D-PAGE in RA patients or healthy controls which could not be identified.

4. Discussion

We here present the changes in the serum protein profile of RA patients brought about by anti-TNF-α therapy using a proteomics-based technique. There have been many reports analyzing serum and synovial proteins from RA patients using 2D-PAGE and mass spectrometry [8,9]. However, the electrophoretic patterns of 2D-PAGE were inadequate because of the interference of high-abundant proteins such as albumin and immunoglobulins, and data used to assess the effect of anti-TNF-α on the protein profile of patients with RA was limited. In this study, the depletion of high-abundant serum proteins using a multiple-affinity column improved the electrophoretic patterns of serum proteins and could show the changes of serum low-abundant proteins brought about by anti-TNF-α therapy using 2D-PAGE.

TNF-α is a pivotal cytokine in the pathogenesis of RA [1]. Its blockade and inhibition improve the clinical manifestations as well as inflammatory biomarkers such as CRP. The levels of acute phase reactants, inflammatory cytokines, and matrix proteases were clearly decreased, as determined using conventional assays, but other biological information has not been reported. In this study, we could show the marked change of serum protein profiles by infliximab using 2D-PAGE. This suggests the presence of more alternative biomarkers than that determined using conventional assays. Although some spots could not be identified because of the low concentrations, we could show the increase or decrease of acute phase reactants and proteins related to the metabolism of lipids and vitamins by infliximab.

RA patients with active disease have an adverse lipid profile characterized by decreased serum levels of total cholesterol, high density lipoproteins (HDL), and Apo A-I [10–12]. This adverse lipid profile may be associated with an accelerated

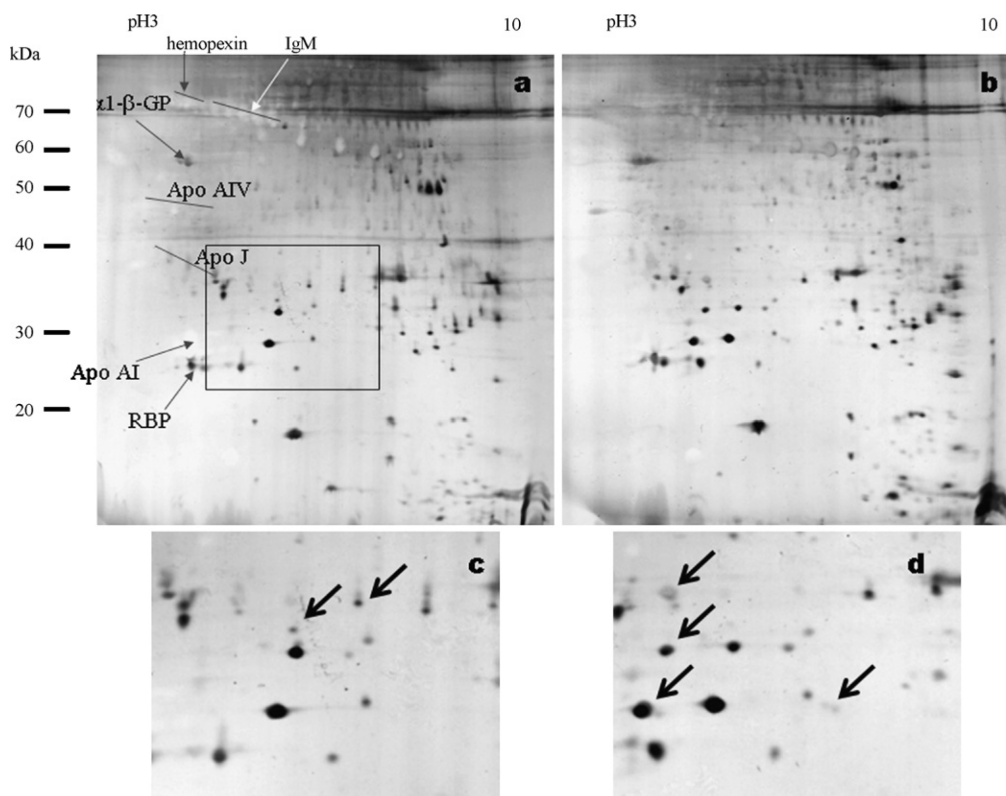

Fig. 3. Silver-stained 2D-PAGE gel showing 50 μg of serum samples from five RA patients treated with infliximab (a, c) and five healthy controls (b, d). Zoomed areas highlight the different spots (arrows) between RA patients (c) and healthy controls (d).

prognosis of atherosclerosis. Anti-TNF-α therapy reversed this profile [13–15], and serum HDL levels were modestly increased [13]. We showed that infliximab increased serum levels of Apo A-I, which is the main protein component of HDL. Recent reports showed that Apo A-I modifies the activation of monocytes/macrophages [16,17]. Apo A-I blocks the contact-mediated activation of monocytes by T-lymphocytes, resulting in the inhibition of IL-1β and TNF-α production. Abundant Apo A-I was identified in the perivascular cellular infiltrates of inflamed RA synovial tissue [18]. Infliximab changes lipid profiles, which may play a role in modulating inflammation indirectly.

Some studies have described lower serum concentrations of serum α-tocopherol and vitamin A in RA patients [19]. The decreased levels of these vitamins may lead to a markedly decreased antioxidant capacity and enhanced eicosanoid production. Nozaki et al. reported that all-trans-retinoic acid, a derivative of vitamin A, improved the clinical course and decreased the production of inflammatory cytokines, immunoglobulins, and chemokines in murine collagen-induced arthritis, similar to RA [20]. Serum RBP is also decreased in RA patients. Honkanen et al. suggested that hypovitaminosis of vitamin A involving its reduced transport from the liver to the blood is caused by the lower level of RBP synthesis [19]. In this study, serum RBP levels in RA patients were increased after infliximab. This increase of serum RBP levels may modify the antioxidant capacity and eicosanoid production in RA.

We firstly show the comparison of the serum protein profile between RA patients treated with infliximab and healthy controls. This suggested that changes in serum proteins including acute phase reactants towards normal levels were observed after infliximab. These changes were correlated with the clinical improvements of RA. However, there were several different spots between RA patients treated with infliximab and healthy controls. Although these spots could not be identified because of the low concentrations, they may be associated with RA and/or infliximab or methotrexate.

Proteomics-based approaches are useful tools for providing global disease information and identifying biomarkers in autoimmune diseases such as RA. We present the changes in the serum protein profile brought about by infliximab towards normal levels, which were correlated with clinical improvements. Changes in the protein profile may lead to modification of the serum lipid profile and antioxidant status. Further research is needed to confirm these possibilities.

Acknowledgements

We acknowledge Kyoko Fukamoto (Osaka Medical College, Osaka) for technical help.

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