Protein biomarker analysis by mass spectrometry in patients with rheumatoid arthritis receiving anti-tumor necrosis factor-α antibody therapy

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Abstract

Objective

To investigate the mechanism of action of anti-tumor necrosis factor-α (TNF-α) antibody in patients with rheumatoid arthritis (RA), we analyzed serum or plasma proteins by mass spectrometry system.

Methods

Ten RA patients who received treatment with anti-TNF-α antibody were studied. Samples obtained before and after therapy were analyzed by a two-dimensional liquid chromatography tandem mass spectrometry (2D LC-MS/MS) system after pretreatment by a recently developed method to remove high molecular weight proteins.

Results

Using this system, certain proteins were identified after treatment with anti-TNF-α antibody, including proteins related to the TNF-α-mediated pathway for nuclear factor kappa B (NF-κB) activation and/or to the metabolism (including regeneration) of articular cartilage.

Conclusion

Our mass spectrometry system appears to be useful for proteomic analysis. The efficacy of anti-TNF-α antibody therapy for RA may be related to various consequence of the inhibition of TNF-α activity.

Key words

Rheumatoid arthritis, anti-TNF-α antibody, proteomics, mass spectrometry, connective tissue growth factor (CTGF).

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Introduction

Infliximab (Remicade) is a chimeric monoclonal antibody directed against tumor necrosis factor-α (TNF-α). It is used to treat patients with rheumatoid arthritis (RA), and dramatically inhibits inflammation and controls articular damage by blocking TNF-α-mediated processes in these patients (1).

TNF-α has been suggested to act as a key proinflammatory cytokine in the patient with RA (2, 3). Blocking the activity of TNF-α is known to inhibit the cytokine/chemokine cascade and prevent articular destruction in RA patients (4-7). However, TNF-α is involved in many inflammatory pathways and it also regulates various physiological phenomena in RA patients (6, 7), and the precise mechanisms by which anti-TNF-α antibody inhibits the development of RA, including the prevention of damage to the articular cartilage, have not been clarified. Recent progression in mass spectrometry techniques has greatly contributed to biomedical studies, including the search for useful protein/peptide biomarkers in several diseases (8). In the present study, we investigated the serum protein/peptide profiles changed of infliximab-treated RA patients using a novel approach to proteomic research that employed a specially developed serum/plasma protein separation device and a linked two-dimensional liquid chromatography tandem mass spectrometry (2D LC-MS/MS) system (9). Here we report the results obtained by this method and discuss the possible mechanisms underlying the therapeutic effect of anti-TNF-α antibody in RA patients.

Materials and methods

Patients

A profile of the RA patients is shown in Table I. All patients were diagnosed as having RA according to the American College of Rheumatology (ACR) criteria (10). All patients gave written informed consent to the study, and this study was approved by our local ethics committee. Six women and four men patients with RA (mean age of 51 years and age range of 24-79 years) were included in this study. The average duration of disease was 7 years (range: 1-16 years). At the time of starting anti-TNF-α antibody treatment, nine patients were being treated with prednisolone at an average dose of 5.15 mg/day and methotrexate an average dosage of 5.6 mg/week. Four patients were also being treated with other disease-modifying antirheumatic drugs (DMARDs) or immunosuppressants (such as bucillamine, actarit, salazosulfapyridine, and mizoripine). Regarding laboratory findings, the average C-reactive protein (CRP) level, erythrocyte sedimentation rate (ESR), and rheumatoid factor (RF) before and after (two or six weeks later) treatment with anti-TNF-α antibody were as follows: CRP was 5.9±6.7 mg/dl and 1.5±1.9 mg/dl (normal range; n<0.3), ESR was 54.3±47 mm/h and 30.9±30.36 mm/h (n<20), and RF was 235±536 IU/ml and 218±531 IU/ml (n<20), respectively. Steinbrocker's disease activity stage and functional class of each patient (11) are also described in the Table, as well as the improvement rate based on the DAS28 and ACR score (12, 13).

Serum was collected from RA patients no. 1-5 and plasma was obtained from patients no.6-10 just before and 24 hours after administration of anti-TNF-α antibody.

Depletion of high molecular weight serum/plasma proteins

One milliliter of serum or plasma was diluted by adding 3 ml of 25 mM ammonium bicarbonate buffer (pH 8.0). Four milliliters of the diluted sample was injected into a hollow-fiber-membrane-based device (HFMD, Toray Industry, Japan) (9). After one hour, high molecular weight proteins were eliminated, while the solution containing low molecular weight proteins was recovered. The sample was collected from the HFMD, lyophilized, and resuspended in 100 μL of 20 mM ammonium bicarbonate buffer.

2D LC-MS/MS analysis

The sample was reduced by adding a 1/10 volume of 100 mM dithioerythritol at 80ºC for 20 min. After cooling to room temperature, the sample was alkylated by adding a 1/10 volume of 200 mM iodoacetamide at 37ºC. After

Table I. Profile of the patients with rheumatoid arthritis.

Patient No..sex(age)	Disease duration (years)	Stage/Class	Treatment [1] PSL (mg/day)	MTX (mg/week	DMARDs	Laboratory findings (after therapy) [2] CRP (mg/dl)	ESR (mm/h)	RF (U/ml)	DAS28 [3] (after therapy)	ACR [4] score
1. F (60)	9	IV/IV	5	6	-	14.4 (2.6)	107 (66)	228 (190)	6.25 (3.69)	70
2. M (79)	5	IV/III	5	6	-	19 (6.1)	111 (76)	1740 (1720)	5.30 (3.15)	70
3. F (75)	10	IV/III	4	6	ACT	0.9 (0.3)	30 (18)	76 (58)	3.47 (2.58)	50
4. F (64)	3	IV/III	3	6	MIZ	7.6 (2.8)	135 (77)	10 (10)	5.97 (3.41)	70
5. F (38)	3	III/II	0	8	-	0.5 (0.3)	25 (18)	95 (80)	4.32 (3.02)	50
6. F (48)	16	IV/III	7.5	6	-	10.9 (1.8)	68 (31)	227 (85)	5.87 (2.45)	70
7. F (24)	2	III/II	6	4	-	1.2 (0.3)	21 (11)	10 (10)	4.18 (2.67)	70
8. M (44)	1	II/II	5	6	SSP	1.85 (0.3)	22 (5)	10 (10)	4.55 (3.68)	20
9. M (54)	9	IV/II	8	4	BUC	0.5 (0.3)	8 (2)	12 (5)	4.96 (3.69)	50
10. M (32)	1	II/II	7.5	6	-	2.4 (0.3)	16 (5)	28 (17)	4.89 (1.53)	70

[1]PSL: prednisolone; MTX: methotrexate; DMARDs: disease-modifying antirheumatic drugs; ACT: Actarit; MIZ: mizoribine; SSP: salazosulfapyridine; BUC: bucillamine.
[2]CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor.
[3]DAS28: Disease activity score 28.
[4]ACR: American College of Rheumatology; 70, 50, and 20: 70%, 50%,and 20% improvement of the ACR score, respectively.

removing excess reagents by chloroform and methanol precipitation, trypsin was added to the sample at a protein: enzyme ratio of 50:1 (wt/wt), and it was incubated overnight at 37ºC. Digestion was terminated by adding formic acid at a final concentration of 0.5% (v/v).

The digested peptides were analyzed by 2D nano LC-MS/MS using a direct nano LC system (DiNa, KYA technologies, Tokyo, Japan) and a mass spectrometer (LCQ DECA XP plus, Thermo Electron, San Jose, CA). The peptide samples were diluted 3-fold with 0.1% v/v formic acid and injected into an SCX strong cation exchange chromatography column (35 mm x 0.32 mm i.d.). Each of five fractions obtained from SCX column, which were one unbound fraction and four other fractions eluted stepwise with 30 mM, 50 mM, 100 mM, and 500 mM ammonium formate, was injected on-line into a C18 reversed-phase chromatography system (1 mm x 0.5 mm i.d. C18 trap column and 50 mm x 0.15 mm i.d. C18 separation column). The C18 column was washed with solvent A (2% acetonitrile and 0.1% v/v formic acid) and eluted with a linear gradient of solvent B (70% acetonitrile and 0.1% v/v formic acid) from 0% to 50% over 120 min at a flow rate of 200 nl/min.

The eluted peptides were introduced on-line into the mass spectrometer and analyzed by an MS scanning, followed by 4 data-dependent MS/MS scans of the 4 most intense peaks under the following conditions: spray voltage of 1.6 keV, capillary temperature of 250ºC, and collision energy of 35%.

Data analysis

The acquired MS/MS spectra were compared with the RefSeq human protein database (NCBI, USA) containing 29,255 entries for human proteins using Mascot software ver. 2.1 (Matrix Science, MA). The search tolerance was set to 2.0 Da for precursor ions and 0.8 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, and carbamidomethylation of cysteine) were considered. The criterion employed for protein identification was that the peptide match score should significantly exceed ($p<0.05$) the peptide identification threshold in the Mascot MS/MS ion search. The number of peptides thus identified for each protein was counted in each patient and compared between before and after infliximab treatment. The score N_{inc} was defined as the number of patients in whom the peptides identified by 10 analyses were increased after infliximab treatment, while N_{dec} was the number of patients in whom the peptides decreased. The detection score (D), was calculated as $(N_{inc}-N_{dec})/n$. Proteins for which $D \geq 0.4$ and $N_{inc}/N_{dec} \geq 3$ were defined as being increased by infliximab treatment, whereas proteins for which $D \leq -0.4$ and $N_{dec}/N_{inc} \geq 3$ were defined as being decreased by infliximab treatment.

Results

Figure 1 showed the molecular weight distribution of all the proteins identified in the samples from RA patients receiving intravenous anti-TNF-α antibody therapy. The molecular weights of the full-length precursor proteins described in the human RefSeq protein database are indicated on the horizontal axis. Seventy percent of the proteins identified had a molecular weight below 50 kDa (Fig. 1), so high molecular weight proteins (approximately >30 kDa) were effectively removed by HFMD pretreatment. Figure 2 shows a list of proteins frequently identified by 2D LC-MS/MS analysis of the low molecular weight proteins in samples from the anti-TNF-α antibody treated RA patients. Proteins with a molecular weight of approximately 30 kDa or less were most frequently detected, although certain proteins with a higher molecular weight were also detected (Fig. 2). These may have been precursors that would be proteolytically processed into smaller functional proteins or randomly

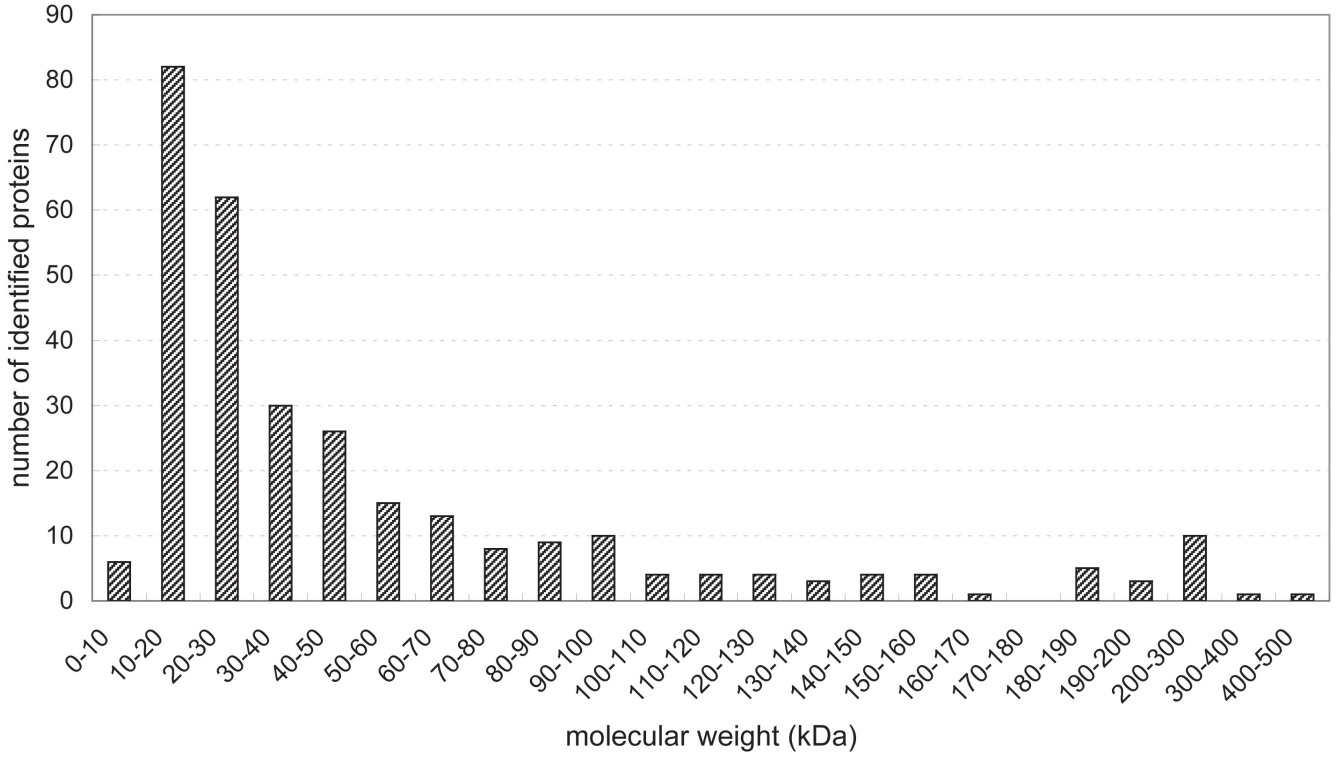

Fig. 1. Molecular weight distribution of the proteins identified by 2D LC-MS/MS analysis after HFMD pretreatment of samples.

protein	MW	pI		serum										plasma									
			patient #	1		2		3		4		5		6		7		8		9		10	
			treatment	before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after	before	after
			accession #																				
peptidylprolyl isomerase A isoform 1	18001	7.68	NP_066953																				
albumin precursor	69321	5.92	NP_000468																				
carbonic anhydrase I	28852	6.59	NP_001729																				
carbonic anhydrase II	29228	6.87	NP_000058																				
pro-platelet basic protein precursor	13885	9.04	NP_002695																				
fibrinogen, alpha chain isoform alpha-E preproprotein	94914	5.70	NP_000499																				
retinol-binding protein 4, plasma precursor	22995	5.76	NP_006735																				
PREDICTED: similar to Ig kappa chain	23723	6.06	XP_496488																				
SH3 domain binding glutamic acid-rich protein like 3	10431	4.82	NP_112576																				
cystatin C precursor	15789	9.00	NP_000090																				
prostatic binding protein	21044	7.01	NP_002558																				
complement factor D preproprotein	27016	7.64	NP_001919																				
prostaglandin H2 D-isomerase	21015	7.66	NP_000945																				
thioredoxin	11730	4.82	NP_003320																				
hemopexin	51643	6.55	NP_000604																				
heparan sulfate proteoglycan 2	468528	6.06	NP_005520																				
profilin 1	15045	8.44	NP_005013																				
hypothetical protein LOC29094	19000	4.99	NP_054900																				
alpha 1 type XV collagen precursor	141786	4.93	NP_001846																				
SH3 domain binding glutamic acid-rich protein like	12766	5.22	NP_003013																				
beta-2-microglobulin precursor	13706	6.06	NP_004039																				
chitinase 3-like 1	42586	8.69	NP_001267																				
lipocalin 2 (oncogene 24p3)	22574	9.02	NP_005555																				
acid phosphatase 1 isoform c	18031	6.30	NP_004291																				
S100 calcium-binding protein A8	10828	6.51	NP_002955																				
S100 calcium-binding protein A9	13234	5.71	NP_002956																				
SH3 domain binding glutamic acid-rich protein like 2	12318	6.29	NP_113657																				
biliverdin reductase B (flavin reductase (NADPH))	22105	7.13	NP_000704																				
complement factor B preproprotein	85479	6.67	NP_001701																				
complement component 4A preproprotein	192664	6.65	NP_009224																				
coagulation factor II precursor	69992	5.64	NP_000497																				
plasminogen	90510	7.04	NP_000292																				
serine (or cysteine) proteinase inhibitor, clade F	46283	5.97	NP_002606																				
myosin, heavy polypeptide 9, non-muscle	226392	5.50	NP_002464																				
insulin-like growth factor binding protein 4 precursor	27915	6.81	NP_001543																				
insulin-like growth factor binding protein 6	25306	8.15	NP_002169																				

Fig. 2. Frequently identified proteins. The number of significant peptides is as follows; : More than 6, : 4-6, : 1-3, and : 0.
MW: molecular weight, pI: isoelectric point, accession#: accession number in the RefSeq database.

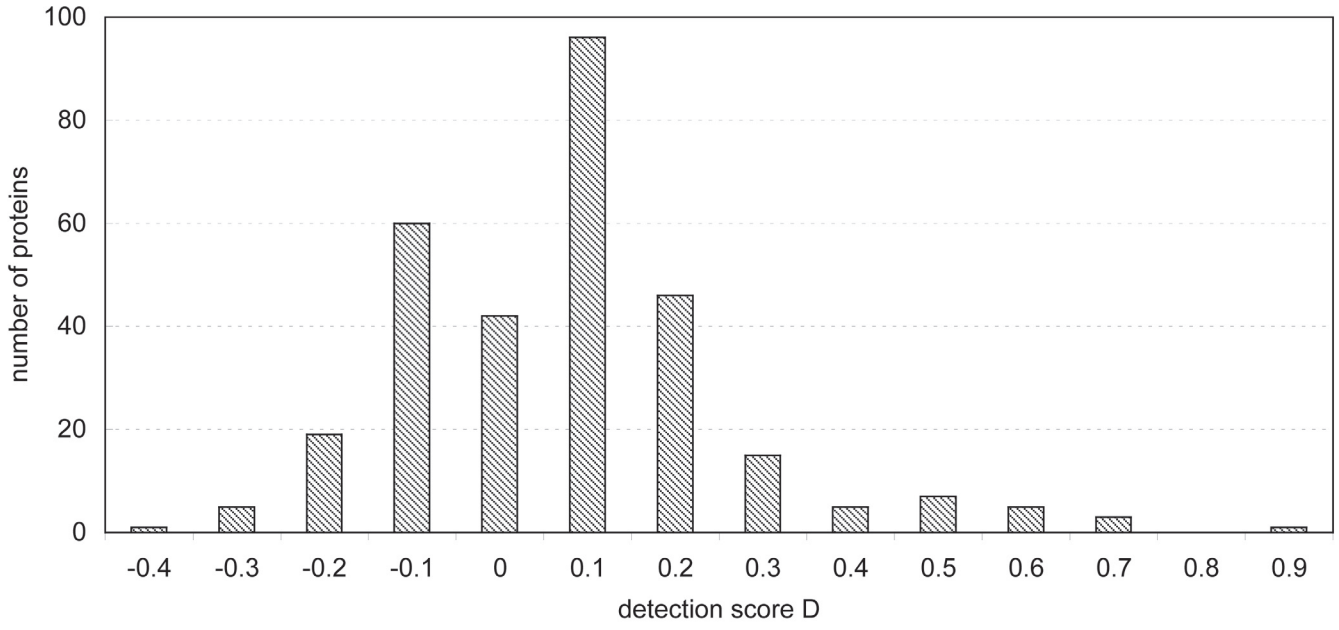

Fig. 3. Total detection score distribution of the identified proteins.

degraded by proteases. There were differences in the detection of some proteins before and/or after anti-TNF-α antibody therapy between serum and plasma samples (Fig. 2).

By using the scoring system as described in *Materials and methods*, the total scores of the proteins identified in serum or plasma samples were calculated and are shown in Figure 3. Ninety five percent of the proteins identified had a total score between –0.39 to +0.39. We focused on the proteins with a total score of +0.40 or more and –0.40 or less, which showed greater changes of expression after anti-TNF-α antibody treatment (Table II). As summarized in this Table, certain proteins like family with sequence similarity 62, member A/membrane-bound C2 domain-containing protein (FAM62A/MBC2) and connective tissue growth factor (CTGF) showed an increase of expression after treatment.

Table II. Proteins with great changes of expression by infliximab treatment.

Protein	MW	pI	Accession#	Detection Score
Increased				
Peptidylprolyl isomerase B precursor	23728	9.42	NP_000933	0.90
Caldesmon 1 isoform 1	93175	5.62	NP_149129	0.70
Family with sequence similarity 62 (C2 domain containing), member A	122780	5.57	NP_056107	0.70
Amylase, alpha 2A; pancreatic precursor	57670	6.60	NP_000690	0.70
Filamin 1 (actin-binding protein-280)	280586	5.73	NP_001447	0.60
Vasodilator-stimulated phosphoprotein isoform 1	39805	9.05	NP_003361	0.60
Cycteine and glycine-rich protein 1	20554	8.90	NP_004069	0.60
Myoglobin	17173	7.14	NP_005359	0.60
Transgelin 2	22377	8.41	NP_003555	0.60
Microtubele-associated protein, RP/EB family, member 1	29980	5.02	NP_036457	0.50
NCK adaptor protein 2 isoform A	42889	6.49	NP_003572	0.50
Tropomodulin 3 (ubiquitous)	39570	5.08	NP_055362	0.50
Connective tissue growth factor	38043	8.36	NP_001892	0.50
Latent transforming growth factor beta binding protein 1 isoform LTBP-1L	186716	5.63	NP_996826	0.50
Regenerating islet-derived 1 alpha precursor	18719	5.65	NP_002900	0.50
Peptidylprolyl isomerase A isoform 1	18001	7.68	NP_066953	0.50
Coronin, actin binding protein, 1C	53215	6.65	NP_055140	0.40
Triggering receptor expressed on myeloid cells-like 1	32658	5.70	NP_835468	0.40
Heparan sulfate proteoglycan 2	468528	6.06	NP_005520	0.40
Peptidoglycan recognition protein 1	21717	8.92	NP_005082	0.40
Superoxide dismutase 1, soluble	15926	5.70	NP_000445	0.40
Decreased				
Desmocollin 3 isoform Dsc3b preproprotein	93439	5.86	NP_077741	-0.40

Discussion

In this study, we examined the influence of treatment with an anti-TNF-α antibody (infliximab) on the protein profile in serum or plasma obtained from RA patients, in order to detect useful markers relating to the treatment of RA. As reported previously**,** 22 major protein species, such as albumin and immunoglobulins, accounted for approximately 99% wt/wt of the total serum proteins. While it has been estimated that up to 10,000 proteins are commonly present, most of these exist at very low levels (8, 14). Therefore, reduction of the complexity of the sample (*e.g.*, by depleting abundant proteins and accumulating rare proteins) is essential prior to any analysis of serum or plasma for proteins present at low levels, which potentially include protein biomarkers (9). For this purpose, we used a recently developed proteomics research system that depletes abundant serum proteins by using a high-performance hollow-fiber membrane device (9). The molecular weight distribution of the proteins identified in patients with RA suggested that this method of sample pretreatment worked well to select low molecular weight proteins (Fig. 1). Using this system, we identified proteins in the serum or plasma of RA patients by shotgun proteomic analysis and counted the number of significant peptides for each protein. Previous reports have suggested that counting of significant peptides can be used as a semi-quantitative index for estimation of relative protein levels (15-17). We adopted this method to estimate the changes of protein levels in the serum and plasma of RA patients receiving infliximab treatment.

As shown in Figure 2, there were differences in the frequency of detecting certain proteins between serum and plasma, although the reasons for this remain unclear. Certain proteins were only detected only in serum and not in plasma. In contrast, S100 calcium-binding protein A9 (which is reported to be widely detected in the plasma of RA patients and is possibly a useful biomarker) (18-20) was detected in plasma samples, but not in serum samples, by our assay system. Further investigations are required to understand the differences between serum and plasma with regard to these proteins that only exist at trace levels.

We paid attention to the serum or plasma proteins with relatively low (–0.40 or less) or high (+0.40 or more) total scores, which meant a greater change after anti-TNF-α antibody treatment, and found that the most frequently identified protein after antibody therapy was FAM62A/MBC2 (21) (Table II). Binding of TNF-α to its receptor can induce the activation of NF-κB, which is an intracellular DNA-binding protein that promotes gene transcription and is the basis of numerous physiological and pathological processes in RA patients. In its deactivated state, NF-κB is localized to the cytosol and is bound to IKB. FAM62A/MBC2 is one of the proteins that interact with IKB kinase gamma (IKBKG), which phosphorylates IKB (22). Phosphorylated IKB is thought to be degraded by proteasomes, releasing the free active form of NF-κB into the nucleus. Thus, FAM62A/MBC2 is related to translocation of NF-κB into the nucleus and the resultant activation of this transcription factor. NF-κB is reported to be abundant in RA synovium and is strongly activated in synovial lining cells because of the phosphorylation and degradation of IKB (23, 24). The reasons why FAM62A/MBC2 can be identified in the serum or plasma at 24 hours after administration of anti-TNF-α antibody are unclear. It is possible that inhibition of TNF-α signaling by this antibody induces an increase of FAM62A/MBC2 that is related to the effectiveness of antibody therapy. Because infliximab induces the death of TNF-α-producing cells by binding to the cell surface receptor for TNF-α (1), we cannot exclude the possibility that FAM62A/MBC2 was released from dying cells. Etanercept (Enbrel) is a soluble TNF-α receptor that improves RA-related symptoms by absorption of free TNF-α, and it does not destroy TNF-α producing cells, unlike inflixmab (25). Therefore, mass spectrometry analysis of samples from patients treated with etanercept may help to determine whether or not FAM62A/MBC2 was released after infliximab caused the death of TNF-α-producing cells. We are now performing a 2D LC-MS/MS analysis of HFMD-pretreated samples from RA patients, who received etanercept therapy.

Among the proteins listed in Table II, CTGF is interesting with respect to the mechanism of inhibition of bone destruction by infliximab. In certain patients, CTGF was identified after anti-TNF-α treatment. It is a member of the CCN family (CTGF/CCN2) that is reported to promote endochondral ossification and cartilage regeneration (26, 27). Several studies have investigated the relationship between TNF-α and CTGF production, but the results have varied, probably because of differences in the experimental systems used (28-31). Beddy *et al*. reported that anti-TNF-α antibody treatment for Crohn's disease may promote the formation of intestinal strictures because TNF-α suppresses fibrosis by downregulating CTGF expression in the fibroblasts of Crohn's patients (31). However, CTGF production mediated by infliximab may play an important role in blocking the cartilage destruction that can occur in RA. Analysis of serum CTGF levels in RA patients by enzyme-linked immunosorbent assay (ELISA) and western blot is now underway at our laboratory.

Several studies using mass spectrometry have already been performed to identify protein markers of disease activity in the synovial fluid and serum of RA patients (21, 32, 33). However, this is the first report regarding the effects of anti-TNF-α antibody therapy for RA based on proteomic analysis. Although the actions and importance of many of the proteins identified by this study remain unclear, once a useful biomarker has been discovered, it can be employed to predict disease activity and the efficacy of anti-TNF-α treatment. However, number of the patients with RA in this study is not enough to obtain reliable conclusions, and we cannot also deny influences of treatments and/or disease duration of the RA patients on the result of our study. Using larger number of the patients, further precise studies based on proteomic analysis are required to provide some useful clues to assist in the development of new treatments for RA as well as a deeper understanding of its etiology.

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