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Disease-specific Proteins from Rheumatoid Arthritis Patients

Rheumatoid arthritis (RA) is a chronic inflammatiory disease that mainly destroys cartilages or bones at the joints. This inflammatory disorder is initiated by self-attack using own immune system, but the detail of pathological mechanism is unclear. Features of autoantigens leading to autoimmune disease are also under veil although several candidates including type II collagen have been suggested to play a role in pathogenesis. In this report, we tried to identify proteins responding to antibodies purified from RA patients and screen proteins up-regulated or down-regulated in RA using proteomic approach. Fibronectin, semaphorin 7A precursor, growth factor binding protein 7 (GRB7), and immunoglobulin μ chain were specifically associated with antibodies isolated from RA synovial fluids. In addition, some metabolic proteins such as adipocyte fatty acid binding protein, galectin-1 and apolipoprotein A1 precursor were overexpressed in RA synovium. Also, expression of peroxiredoxin 2 was up-regulated in RA. On the contrary, expression of vimentin was severely suppressed in RA synoviocytes. Such findings might give some insights into understanding of pathological mechanism in RA.

Key Words : *Arthritis, Rheumatoid; Autoantigens; Fibronectins; Proteomics*

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INTRODUCTION

Rheumatoid arthritis (RA) is a common human autoimmune disease with a prevalence of about 1% throughout the world. It is discrete from osteoarthritis (OA) in terms of its pathological cause although both arthritic diseases mainly affect the joint. While there has been a progress in defining its etiology and pathogenesis, its molecular mechanism in pathology is still incompletely understood (1).

Rheumatoid arthritis is characterized by chronic inflammation at the synovial joint and infiltration by blood-derived cells, memory T cells, macrophages, and plasma cells, all of which show signs of hyper-activation of immune responses (2, 3). Generally, autoimmune diseases including RA are triggered by the immune response against own proteins leading to severe inflammation. In RA, such inflammation causes the cartilage destruction through the direct invasion of an inflammatory mass, called pannus. It is composed predominantly of macrophage and fibroblasts that secrete proteases and enzymes that can degrade the surrounding matrix and cartilage (4).

Several candidate antigens in RA such as type II collagen, glucose-6-phosphate isomerase and human chondrocyte glycoprotein 39 have been evaluated from studies to figure out how these molecules trigger to initiate the hyper-activation of immune response leading to RA in vivo or in vitro (5-7). However, only small subsets of RA patients exhibit immune response to these antigens, and no correlation in disease duration, activity or severity among patients has been observed. Therefore, identification of new candidate autoantigen proteins binding to antibodies obtained from RA patients' synovial fluid might give some insights into understanding the molecular mechanism during RA pathogenesis.

To identify new RA-specific proteins from patients' synovium or synovial fluid, we applied several different methods; two-dimensional gel electrophoresis, antibody affinity purification, and mass spectrometry. Here we report some proteins responding to RA antibodies or being specifically expressed in RA tissues.

MATERIALS AND METHODS

Materials

Human synovial fluids were obtained from the swollen knee of Korean RA patients (9 males and 16 females, average age: 50 yr) using syringe at the Gyeongsang National University Hospital during 2002-2003. RA (two females and a male, average age: 60 yr) or OA (15 females and 5 males, average

age: 67 yr) synovial tissues were obtained during the knee operation after obtaining informed consents of patients at the same institute for proteomic analysis. These patients were diagnosed as RA by a clinical specialist in the basis of RA clinical classification criteria (1987 ACR criteria). In addition, the joint area of these patients was erosive by radiography detection.

Purification of autoantibodies from synovial fluids

Synovial fluids were diluted 3 folds with binding buffer (10 mM Tris, pH 7.5) and applied to the 1 mL ImmunoPure Plus Protein A Column (PIERCE, Rockford, IL, U.S.A.) pre-equilibrated with 5 column volumes of the IgG binding buffer. The column was washed with 10-15 column volumes of the binding buffer. The bound IgG was eluted with 3-5 column volumes of the elution buffer (0.1 M glycine buffer, pH 2-3). The pooled protein fractions were immediately adjusted to a physiological pH by the addition of a suitable, more concentrated buffer (1.0 M Tris, pH 7.5, 100 μL of the buffer to 1 mL of sample). The eluted immunoglobulins were dialyzed with 1 × PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The concentration of purified IgG was estimated using a commercial Bradford reagent (Bio-Rad, Hercules, CA, U.S.A.). Commercial IgG (1.4 μg/μL) was used as a standard.

Western blot

Total cell proteins derived from tissue or cells were separated on a SDS-gel electrophoresis or two-dimensional gel electrophoresis and subsequently transferred to the nitrocellulose membrane using a Trans-Blot SD Semi-dry transfer cell (Bio-Rad) for 15-30 min at 15 V. SDS, acrylamide, methylene-bisacrylamide, TEMED, ammonium persulfate, DTT, urea, Tris, glycine, and glycerol, were purchased from Bio-Rad or USB (Cleveland, OH, U.S.A.). Silver nitrate, Coomassie Brilliant Blue G-250, iodoacetamide, and -cyano-4-hydroxycinnamic acid were from Sigma (St. Louis, MO, U.S.A.). The membrane was blocked for 1 hr at room temperature in 5% skim milk/TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20) and incubated with primary antibodies isolated from RA patients for 2 hr at room temperature (1:100 in 5% skim milk/TBST). After wash for 30 min with TBST three times, the membrane was incubated with anti-human rabbit IgG (Santa Craz Biotechnology, Santa Cruz, CA, U.S.A.) conjugated with HRP for 1 hr (1: 1,000 in 5% skim milk/TBST). Proteins were detected with an enhanced chemiluminescence system (ECL, Amersham, Buckinghamshire, U.K.) after wash with TBST three times.

Coupling of purified IgG to CNBr-activated Sepharose 4B

One CNBr-linked Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) was swollen in 1 mM HCl for 15 min, and washed with 40 mL of 1 mM HCl for 5 min five times, and then changed twice with 5 mL coupling buffer (0.1 M $NaHCO_3$, pH 8.3). The gel was mixed with 10 mL coupling buffer containing 10 mg purified IgG, and the mixture was rotated to end-over-end for 2 hr at room temperature. After centrifugation at 2,000 rpm for 2 min, excess ligands were washed out with at least 5 gel volumes of coupling buffer, and then any remaining active groups was blocked with 0.1 M Tris (pH 8.0).

Ab-affinity purification

50 μL of purified IgG coupled to CNBr-activated Sepharose 4B was used for this affinity isolation experiment. Protein mixtures obtained from OA or RA synvium was added to IgG-coupled resins equilibrated with binding buffer (Tris, pH 7.5, 150 mM NaCl) and incubated at 4°C for 2 hr. After spin, the resins were washed with the binding buffer twice. Proteins bound to IgG were eluted by the addition of SDS-sample buffer and separated by a 10% SDS-PAGE after boiling.

Two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry

Frozen synovium tissues (500-600 mg) were washed with PBS buffer to remove cell debris and blood and homogenized in 20 mL lysis buffer (8 M urea, 2% CHAPS, ampholytes, and 10% protease inhibitor cocktail). The protein solution was clarified by Beckman TL-100 table-top ultracentrifuge at 100,000 rpm at 4°C for 10 min. The collected supernatant was stored at -70°C until use. IPG strips of pH 3-10 and 4-7 were purchased from Bio-Rad (ReadyStrip, 0.53170 mm; Hercules, CA, U.S.A.). Bio-Lyte (pH 3-10) was from Bio-Rad. The general methods for two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry were described in our previous reports (8-10). Proteins were identified by peptide mass fingerprinting with a search program MS-FIT (http://prospector.ucsf.edu/ucsfhtml 3.4/msfit.htm).

RESULTS

High molecular weight proteins respond to antibodies isolated from RA patients

We here assumed most antibodies in the synovial fluid of patients with severe RA are auto-reactive because autoantibodis reacting with local synovial components are very crucial for the pathological process of RA. Local synovial fluids were collected from Korean RA's patients, and total antibodies in synovial fluids were purified using Protein-A affinity chromatography. Total twenty different antibodies have been independently isolated from RA patients (average age: 50 yr),

and all antibodies were purified to the near homogeneity (data not shown). Two of them were shown in Fig. 1A.

These purified antibodies were used for a primary antibody in blotting with total cellular proteins derived from RA synovium tissue and separated on a SDS-gel electrophoresis or two-dimensional gel electrophoresis. Some proteins with high molecular weight (over 100 kDa, indicated by arrows) were reacted to most RA's antibodies (Fig. 1B, lane 1-8). In addition, we detected not only heavy chains and light chains of immunoglobulins but also some proteins placed between heavy chain and light chain of immunoglobulin (Fig. 1B). Furthermore, we applied the 2-DE gel blot method to detect and isolate antigens using a RA antibody (marked as asterisk in Fig. 1B). Again we found some RA synovium proteins reacting with antibodies at the high molecular weight (Fig. 1C) but failed to identify them.

Protein analysis using antibody cross-linked chromatography

Due to the technical limitation for identification of autoantigens using 2-DE gel western blot as above-mentioned, we tried another approach to isolate proteins binding to RA-antibodies. Antibody-affinity purification is generally used for isolation of proteins that can interact with antibody. First, antibodies purified from RA patients were cross-linked to Sepharose resins using CNBr-activation. Then, whole cellular proteins solubilized from tissues (OA or RA) were mixed with antibody-bound beads. Antibody-bound proteins were analyzed by SDS-PAGE. As shown in Fig. 2A, several high molecular weight proteins were specifically bound to antibody beads (lane 1-4) but not to beads without antibody cross-link (lane C), suggesting that these are antibody-specific proteins. Some of these proteins have been identified using mass spectrometry. One of them was fibronectin, a connective tissue protein.

Identification of proteins bound to antibodies in the synovial fluid

Some antibodies in synovial fluid might be already bound to soluble antigen molecules. If these antigen-antibody complexes can be isolated, we can identify proteins being associated with antibody in rheumatoid synovial fluids. Synovial fluids containing antigen-antibody complexes were directly applied to Protein A resins, and antigen-antibody complexes were solubilized in SDS-sample buffer rather than eluted by low pH buffer after extensive wash in order to remove non-specific proteins or proteins with low affinity. The bound proteins were separated by a SDS-PAGE and each protein was identified by MALDI-TOF mass spectrometry. As shown in Fig. 2B, several proteins were bound to antibodies in synovial fluids (indicated by lane B). They were fibronectin, semaphorin 7A (CD108), nucleoside triphosphate phosphorylase 1, growth factor receptor binding protein 7 (GRB7), and μ heavy chain of immunoglobulin. They all were similarly found in most RA synovial fluids (data not shown). These proteins were dissociated from antibodies during the elution with low pH glycine buffer as shown in Fig. 2B (indicated by lane E).

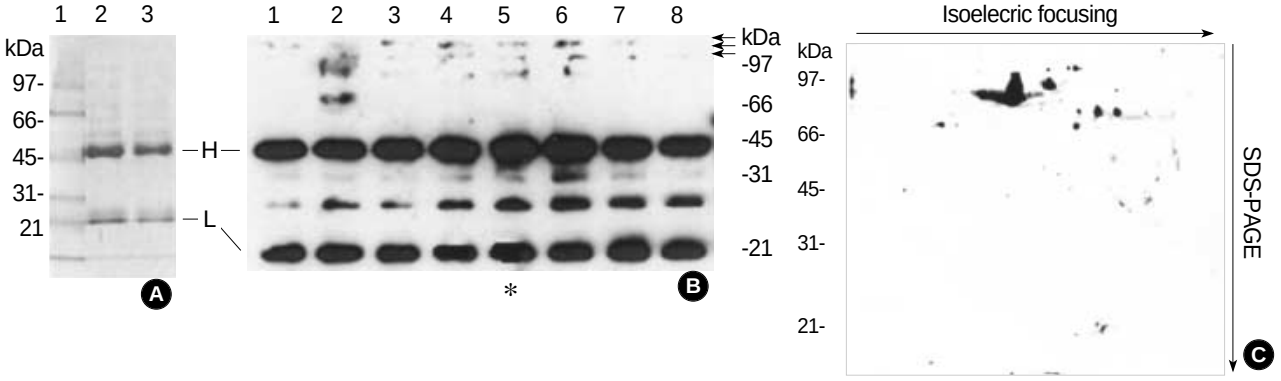

Fig. 1. Purification of autoantibodies and blotting. (A) Antibody purification. Antibodies were purified from RA patients' synovial fluid using protein A affinity chromatography as described in "Materials and Methods". Two samples of antibody (each 10 μg) was analyzed by 12.5% SDS-PAGE. Lane 1 is protein size marker. H and L indicate heavy chain and light chain of immunoglobulins, respectively. (B) Blotting on a one-dimensional gel. Total cellular proteins (20 μg each lane) derived from RA synovium tissue (63/M) were separated on a 12.5% SDS-gel electrophoresis. They were subsequently transferred to the nitrocellulose membrane. Eight different primary antibodies isolated from RA patients (1:500 in 5% skim milk/TBST) were incubated with the membrane for 2 hr at room temperature using multi-channel blotting system (Bio-RAD). Secondary antibody, rabbit anti-human IgG HRP (1:2,000 in 5% skim milk/TBST), was incubated with membrane at room temperature for 1 hr. Proteins were visualized by a ECL developing system. (C) Blotting on a two-dimensional gel. Total proteins 150 μg from the RA synovium protein (63/M) was separated on a 7 cm and pH3-10 IPG strip, in the first dimension and 12.5% SDS-PAGE at the second dimension and subsequently transferred to the nitrocellulose membrane. Primary antibody from a RA patient (40/F) (1:100 in 5% skim milk/TBST) and subsequent HRP-conjugated rabbit anti-human IgGs (1:1,000 in 5% skim milk/TBST) were incubated with the membrane. Proteins were visualized by a ECL detecting system.

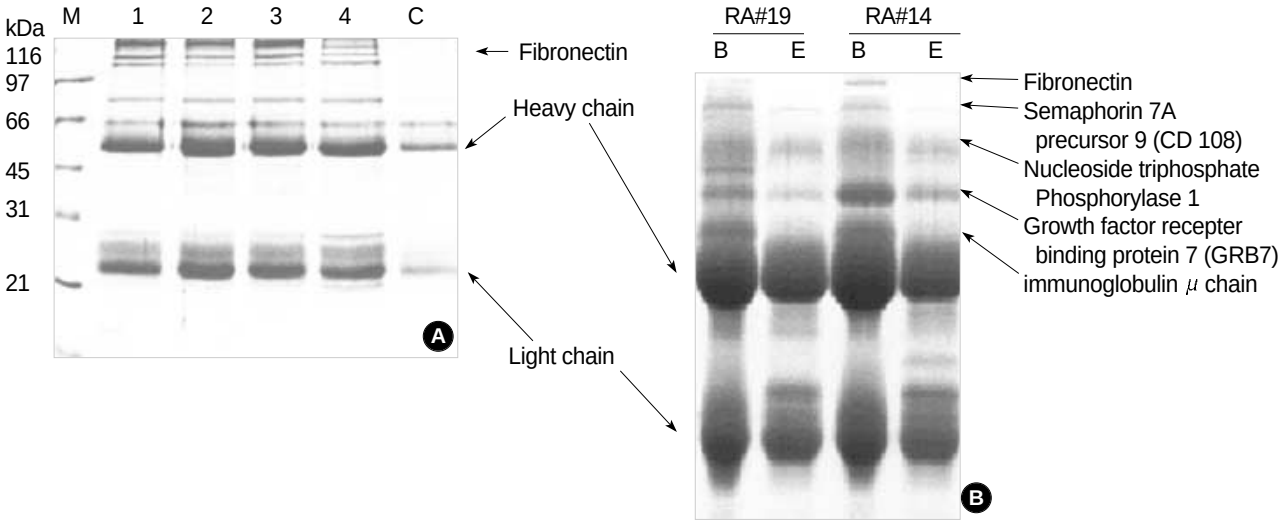

Fig. 2. Isolation of autoantogens. (A) Affinity purification of autoantigens. Four different purified IgGs coupled to CNBr-activated Sepharose 4B were incubated with total synovial proteins for 1 hr at room temperature. After wash with binding buffer three times, one bead volume of 2X SDS sample buffer was added to protein-bound resins. After boiling for 5 min, the supernatant was separated on a 10% SDS-PAGE. C is a control, no cross-linked antibody. M indicates protein size marker. (B) Analysis of antigen-antibody complexes in synovial fluids. Synovial fluid (RA patients #14 and 19) was incubated with protein A resins. After washing protein A bead with 10 mM Tris-HCl (pH 7.5), beads were mixed with one bead volume of 2X SDS sample buffer and boiled for 7 min. The solubilized proteins were separated by a 10% SDS-PAGE after centrifugation at 13,000 rpm for 5 min and visualized by Coomassie blue staining. The specific proteins were identified by MALDI-TOF mass spectrometry as described (8-10). Lane B indicates antibody-antigen mix isolated and solubilized by SDS-sample buffer, and lane E is antibody sample eluted by low pH buffer as described in antibody purification.

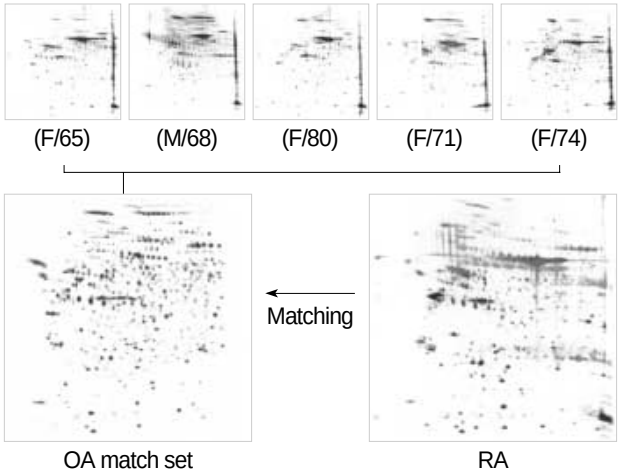

Fig. 3. An OA reference set for RA protein comparison. Total OA synovium proteins (40 μg) were separated on a two-dimensional gel: a linear IPG strip 17 cm and pH 4-7 at the first dimension and a 7.5-17.5% gradient SDS-PAGE at the second dimension. Protein spots were detected by silver staining. Five OA gels were matched together to create a reference set using the PDQuest program. This reference set was used for comparison with RA gels. Numbers in parentheses indicate age of patients.

Comparison of OA and RA synovium proteins

In order to isolate RA-specific synovium proteins, we first solubilized total proteins from RA patient's synovium and compared them with ones obtained from OA patients, as a control, in two-dimensional gel electrophoresis. Proteomic analysis has several drawbacks in experimental repeatability and statistical significance of data when in particular few samples are used for analysis. To minimize these experimental errors, we created a reference set, as a control, from five OA synovium samples composed of different sex and age and matched it with those from RA synovium of three different patients (Fig. 3). From these comparative analyses, we found that about 200 proteins were 3-fold more overexpressed in RA; and more than 100 proteins showed their expression to be decreased in RA. Some of these proteins were directly compared in the gel (Fig. 4). Proteins (#0204, 0212, 4008, 5001, 6003, 7001) dominantly expressed in RA have been identified by mass spectrometry and database search. They are tropomyosin β chain, fatty acid binding protein (adipocyte lipid binding protein), peroxiredoxin 2, galectin 1, and apolipoprotein A1 precursor (Table 1). Most of these proteins have been implicated to play some roles in RA pathogenesis. More details will be discussed. In addition, we examined total protein expression in synoviocytes sub-cultured from RA and OA synovium tissues using two-dimensional gel electrophoresis. Many proteins showed also their differential expression between OA and RA primary synoviocytes sub-cultured from each patient. In particular, a skeletal protein vimentin was much more highly overexpressed in OA than RA (data not shown).

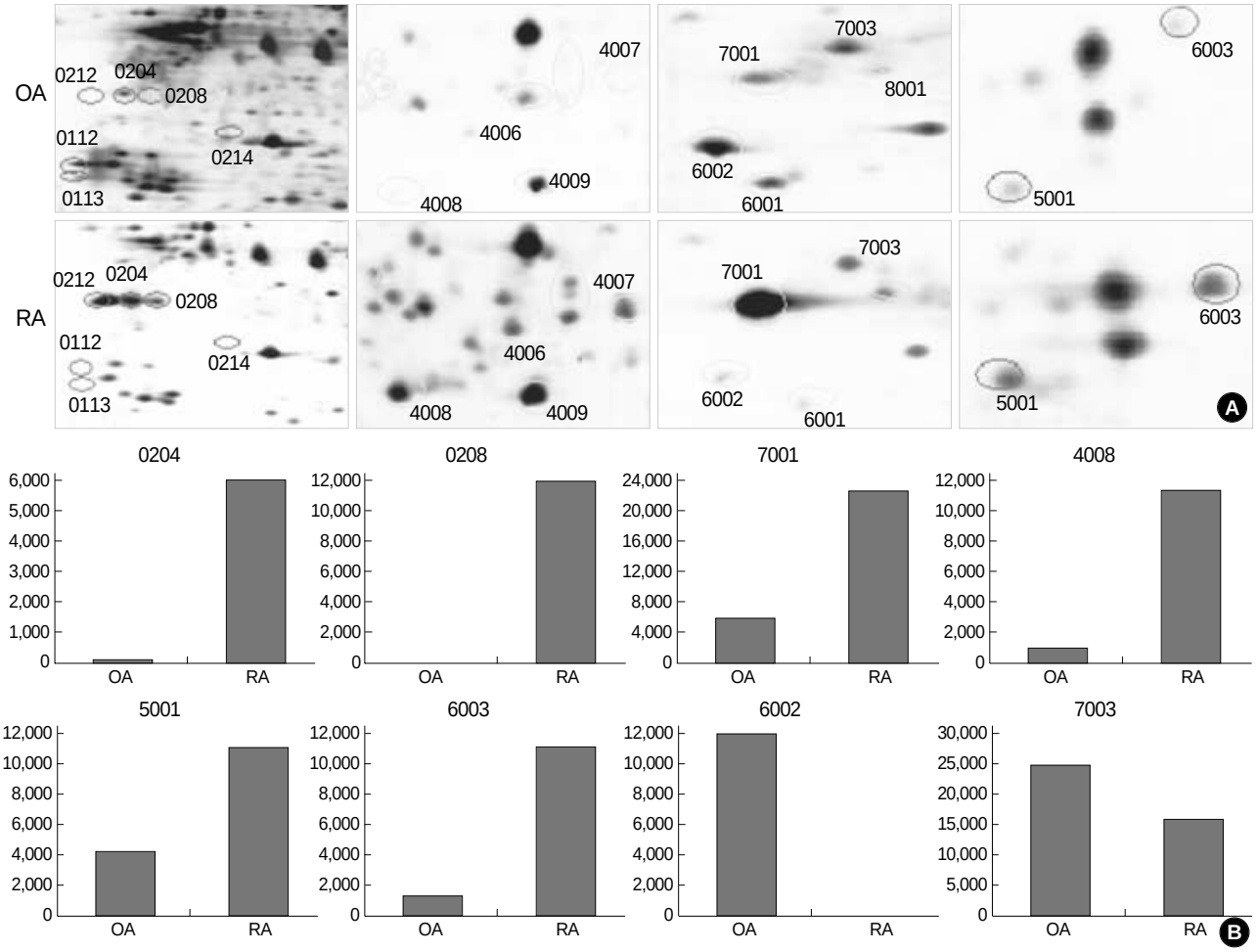

Fig. 4. Comparison of RA and OA proteins. Total RA or OA synovium proteins (40 μg) were separated on a two-dimensional gel: a linear IPG strip 17 cm and pH 4-7 at the first dimension and a 7.5-17.5% gradient SDS-PAGE at the second dimension. The RA gel was matched with the OA reference set (Fig. 3), and the relative values in (B) were obtained from the PDQuest analysis as described (8). The spot numbers were randomly given by the program.

Table 1. Mass spectrometry analysis of isolated protein spots

Spot number	Protein identified	Mass matched (%)	MOWSE score	MW (kDa)/pI	Accession number
5001	Galectin-1	20	6.0×10^3	14/5.3	P09382
6003	Peroxiredoxin 2	34	1.3×10^3	21.8/5.7	P32119
4009	Apo-A1 precursor	37	3.4×10^8	31/5.6	P02647
7001	Fatty acid binding protein	18	1.5×10^6	14.7/6.6	P15090
0212	Tropomyosin α chain	44	450	32/4.7	P09493
0204	Tropomyosin β chain	44	7.3×10^5	32/4.7	P07951

Protein spots were isolated from the gel and identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry as described previously (8, 9).

DISCUSSION

Rheumatoid arthritis, an autoimmune disease with chronic inflammation and destruction of cartilage and bone, has a prevalence of 1% over the world. Recently, the research in cytokines has provided some critical insights about their role in the RA development, but the immunological identities causing autoimmunity in RA pathogenesis are not clear (11, 12). To date, several candidates for autoantigen that might involve in occurrence of RA have been identified. For examples, type II collagen, glucose-6-phosphate isomerase, human cartilage protein gp39, and chaperone protein BiP have been isolated and studied for their roles in RA. In particular, the mouse injected by type II collagen showed the development of arthritis, relatively similar to RA (5). Also, antibodies reacting with collagen have been found in the synovial fluids of RA patients. However, only 25-45% of RA patients contain anti-collagen antibodies, and even these patients have different epitopes of collagen, suggesting that antibody production against collagen might be the secondary consequence of RA induction (7). Human cartilage protein gp39, a glycoprotein, is specifically expressed in synoviocytes of some

RA patients and sometimes found in synovial fluid (13). Furthermore, this protein binds to DR4 that have been considered as RA-specific haplotype. However, according to a recent paper, main CD4+ lymphocytes existing at the connective tissues of RA patients were not specific to gp39 protein (14). Antibody against glucose 6-phosphate isomerase has been found in RA patients and also in K/BxN mice developed RA-like arthritis by serum transfer (6, 15). But it is still not clear how this protein involves in RA pathogenesis. Besides, aggrecan, a component of cartilage, and epithelial filaggrin have been determined as autoantigen (16, 17). In addition, endoplasmic reticulum chaperone protein BiP is strongly expressed in the lining cells of RA synovium, and BiP stimulates T lymphocyte proliferation. Anti-BiP antibodies have been found in many RA patients (63%) (18, 19).

In this report we tried to isolate RA-specific proteins using antibody-antigen interaction and proteomic approach. About 5 mg/mL antibodies present in the synovial fluid of RA patients. Some molecules bound to these antibodies were high molecular weight proteins such as fibronectin, nucleoside triphosphate phosphorylase 1, semaphorin 7A precursor, and growth factor receptor-bound protein 7. In particular, fibronectin has been previously suggested as an autoantigen for RA (20, 21). It is a glycoprotein that is typically expressed on the surface of fibroblasts. A major function of the fibronectin is cell adhesion to the extracellular matrix or cell-cell contact. Also, fibronectin stimulates endocytosis and promotes the clearance of particular materials from the circulation (22). Therefore, production of antibody against fibronectin might cause cell destruction typically appearing during the RA pathogenesis.

Currently, many techniques to massively detect the differential expression of genes between normal and malignant state have been developed. Here we applied proteomic approach to isolate some RA-specific proteins by comparing RA patient's synovium with OA patient's one in a two-dimensional gel. Because acquisition of synovial tissues from normal person is limited, we used OA patient's synovium as a control. Many proteins were differentially expressed between RA and OA. Among them, vimentin was dominantly expressed in OA cultured-synoviocytes but its expression in RA cultured-synoviocytes was severely suppressed, suggesting that down-regulation of vimentin might play a role in RA pathology. Vimentin, an intermediate filament protein normally expressed in cells of mesenchymal origin, plays several roles in many biological reactions (23). For the most part, vimentin expression coincides with cellular growth and is cell-cycle-regulated (24). In addition, vimentin expression can contribute to the augmentation of motility and invasiveness in some cancers (25). Moreover, it has been suggested that vimentin can act as a signal transducer, relaying information from the extracellular matrix to the nucleus (26). Due to its diverse function, down-regulation in RA might cause some pathogenic phenotypes.

In this study, tropomyosin, adipocyte lipid binding protein, peroxiredoxin 2 (thioredoxin peroxidase 1), galectin-1 (beta - galactoside-binding lectin L-14-1), and apolipoprotein A-1 precursor (Apo-A1) were over-expressed in RA. These proteins have been previously suggested to play some roles in RA. Galectin-1 revealed a diverse range of activities in relation to cell survival and proliferation. Expression of galectin-1 was down-regulated in juvenile idiopathic arthritis characterized by hyperplasia of synovial cells, and galectin-3 was over-expressed (27). Also, galectin-3 expression was elevated in RA sera and synovial fluids (28). Antibody against peroxiredoxin 2, an anti-oxidative protein, has been detected in several systemic autoimmune diseases such RA or lupus erythematosus. In particular, peroxiredoxin 2 antibody has been observed in 19% of RA patients (29). Cytokines such as TNF-α and IL-1β have been implicated in pathogenesis of RA. Production of these cytokines was blocked by apolipoprotein-A1, and level of this protein was highly elevated in the synovial fluid of RA patients (30, 31). In addition, tropomyosin antibodies have been observed in RA patients, suggesting that there are some correlation between muscular proteins and RA activity (32). According to a recent proteome analysis using synovial fluid and serum of patients with RA, calgranulin A, B, C proteins were highly elevated in erosive RA patients (33). Taken together, most proteins isolated from this comparative analysis of three RA synovial tissues and an OA match set are likely associated with a number of metabolic alterations leading to inflammatory arthritis although further analyses are required to make a solid conclusion.

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