Identification of Specific Protein Markers of Rheumatoid Arthritis in Synovial Fluid and Serum

Keisuke Mihara¹, Sayuki Kohno¹, Shiho Hatanaka², Tsutomu Yamasaki³, Kei-ichiro Nishida⁴ and Tohru Nakanishi⁵

¹Shijitsu University School of Pharmacy, Okayama, Japan
²Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama University, Okayama, Japan
³Molecular Biology and Clinical Diagnosis, Shijitsu University Graduate School of Pharmacy, Okayama, Japan

Abstract: Rheumatoid arthritis (RA) is a common articular disease characterized by chronic inflammation, hyperproliferation of synovial cells, and bone destruction. Recently, we showed that small interfering RNA targeting CD81 (siCD81) is therapeutically effective for RA. CD81 belongs to a family of cell-surface proteins (tetraspanins) known to be up-regulated in RA synoviocytes, and siCD81 decreases the expression of synoviolin and TNF-α at the cellular level. We also previously established a highly sensitive sandwich ELISA system using monoclonal antibodies (MAbs) against CD81 by immunization with the outer membrane region of CD81 (LEL), which was overexpressed as a recombinant protein. This assay can be used to detect CD81 in synovial fluids and serum. Here, we identified several specific marker proteins and peptides for diagnosis of RA, including CD81 by using a protein chip system, 2D-gels, and western blotting. These markers can be used for early diagnosis of RA and might contribute to further effective treatment.

Key words: Rheumatoid arthritis, CD81, Synovial fluid, Protein chip, 2D-gel

Introduction

CD81 is a protein of the tetraspanin family that has four transmembrane domains. It regulates the immunological reactions of B and T cells via their adhesion, morphology, activation, proliferation, and differentiation. Our previous study using genome-wide DNA chips and immunostaining found that CD81 is involved with proteins up-regulated in rheumatoid arthritis (RA) synoviocytes in vitro. One RA specific protein, synoviolin, functions as a rheumatoid regulator and overgrowth factor of synovial tissues in RA. Mice in which synoviolin is overexpressed develop spontaneous arthropathy accompanied by synovial dysplasia. Thus, the expression of synoviolin appears to be highly associated with the progression of RA through apoptosis. We showed that CD81 siRNA decreases the expression of synoviolin in the joints of collagen-induced arthritis rats. We also showed that CD81 siRNA decreases the expression of TNF-α-induced expression of synoviolin in SW982 cells, suggesting that TNF-α stimulates the expression of synoviolin via CD81. These results showed CD81 might be an effective target for treatment of RA and an effective marker for diagnosis of RA by quantitation of CD81 or its derivatives. In the present study, to find specific markers including CD81 derivatives and other proteins and peptides for early and effective diagnosis of RA, we performed protein chip analysis, 2D-gel analysis, and western blotting using RA synovial fluid and serum.

Materials and Methods

Preparation of synovial fluid and serum

Synovial fluid and serum from RA and osteoarthritis (OA) patients (also serum from normal subjects) were obtained by informed consent and approved by the Bioethics Committee of Okayama University and Shijitsu University. Three samples from each patient were centrifuged and the supernatant was collected. Before using these samples for experiments, albumin and globulin were eliminated by using an AlbuVoid Albumin Depletion Kit (Biotech Support, Monmouth Junction, NJ). The concentration of proteins in samples was quantitated using BSA as a standard following Bradford method.

Protein chip analysis

Proteins in each serum sample were added to ProteinChip arrays (Ciphergen Biosystems, Fremont, CA), and proteins bound to chemical docking sites on the ProteinChip surface through affinity interactions. Proteins that bound non-specifically to the chips were washed away. Then the chips were dried, and an energy absorbing material (EAM) was applied to the chips to facilitate desorption of retained proteins. Proteins were eluted from arrays by laser desorption and ionized in the ProteinChip reader. Finally, proteins were detected by time-of-flight mass spectrometry (TOF-MS).

2D-gel analysis

2D-gel electrophoresis was performed and 2D electrophoresis gels were stained following separation for protein visualization and analysis. Briefly, samples of synovial fluid were applied to IPG strips (Bio-Rad, Hercules, CA), and isoelectric focusing (IEF) was performed by using a PROTEAN IEF system (Bio-Rad). In the next step, IPG strips were applied to a Criterion system (Bio-Rad) to run SDS-polyacrylamide gel electrophoresis (PAGE). Then, proteins on 2D separated gels were stained with ProteoSilver (Sigma-Aldrich, St. Louis, MO). Protein spots were counted by Molecular Imager FX (Bio-Rad).
Western blotting

Each synovial fluid sample was separated by SDS-PAGE for 30 min at 200 V. SDS-PAGE Molecular Weight Standards Broad Range (Bio-Rad) was used as a standard. Before heating, 2-mercaptethanol (2ME) was added to the sample in reduced condition. Proteins were transferred to PVDF membranes for 50 min at 100 V, then stained by Ponceau S and destained by TBS-tween 20 (TBS-T). After blocking with 3% bovine serum albumin (BSA) for 1 hr, anti-CD81 monoclonal antibodies (MAbs) were added to the membrane and incubated overnight at 4 °C, washed with PBS-T, then anti-mouse IgG-HRP was added to the membrane and incubated for 1 hr at room temperature. The membrane was washed with TBS-T, and Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA) was added to the membrane to detect CD81.
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**Results**

**Protein chip analysis**

Proteins in serum from three RA joints, three OA patients, three RA patients, and two normal subjects were applied to the ProteinChip array, and the mass of retained proteins was determined by TOF-MS. Fig. 1 shows the principle of the TOF-MS protein detection and analysis system. Fig. 2 shows the results of protein chip analysis of RA, OA, or normal-derived serum by using anion exchange SAX2 chips at pH 8. There are several protein peaks and two arrows that indicate RA-specific markers and normal/OA markers. The large arrow indicates a candidate RA-specific marker. Although we compared these data with the database of Ciphergen, we could not identify these markers as known peptides.

**2D-gel analysis**

Samples of synovial fluid were applied to IEF and SDS-PAGE. Fig. 3 shows the results of 2D-gel electrophoresis. The pH range of IEF was 5.0-8.0. There are several known proteins (apolipoprotein A1, a1 antitrypsin, IgG light chain, haptoglobin 2-a chain, and transthyretin) in the 2D gel. We compared the patterns of 2D-gel electrophoresis between RA-derived and OA-derived synovial fluids as shown in Fig. 4. We found several RA- or OA-specific proteins, for example at MW/ pl of 83k/7.3, 76k/6.17, 77k/6.23, 56k/5.9, and 43k/6.0. Among these, 56k/5.9 was the only candidate that was RA-specific in this experiment. We could not find known proteins corresponding to these MW and pl.

**Western blotting**

Synovial fluids from RA and OA joints were analyzed by western blotting using anti-CD81 MAbs. Fig. 5 showed the results of western blotting under two different conditions (reducing and non-reducing). We found a 26-kDa band corresponding to the whole CD81 molecule only in RA-derived synovial fluid under reducing conditions. There are no RA-specific smaller molecules in this result. In non-reducing conditions, there were several proteins detected in the gel, and one of the RA samples showed a CD81 molecule (26 kDa) and three smaller molecules. The molecular weights of two of the three smaller proteins are 23 kDa and 20 kDa. In the control condition (eliminated anti-CD81) no such proteins were observed.

**Discussion**

In this study, we searched for RA-specific protein/peptide markers to facilitate early and sensitive diagnosis of RA. We used three different methods, a protein chip, 2D-gel, and western blotting. As a result, we found several RA-specific protein and peptide marker candidates. In the protein chip analysis, we used serum samples from two normal subjects and three each from RA and OA patients. We tried different cation and anion exchange chips and different washing conditions. Finally, we found one RA serum-specific molecule that was not present in OA serum and existed in a very small amount in normal serum. Although we could not find this molecule in the database of Ciphergen, we are currently analyzing the amino acid sequence of this peptide to identify it.

Next, we performed 2D-gel electrophoresis and analyzed the protein patterns of synovial fluid from RA and OA joints. In conditions of pH 5.0-8.0 of IEF, we found one RA synovial-fluid-specific protein with MW 56 kDa and pl 5.9. Because the content of this protein is very low, we cannot yet identify its amino acid sequence. Further experiments are necessary to identify this protein, and we plan to raise antibodies against it.

Finally, we performed western blotting to find CD81 and its derivatives in RA synovial fluids. To establish anti-CD81 MAb, we previously overexpressed and purified the large outer membrane region of CD81 (LEL)11,12. Although this MAb recognizes the LEL region of CD81, we detected the whole molecule (26 kDa) and did not find LEL or smaller molecules in western blotting under reducing conditions, indicating that CD81 exists in its entirety on the membranes of microsomes in RA synovial fluid, and is not processed or degraded in RA joints. RA-specific microsomes containing CD81 might be useful for early diagnosis of RA. In non-reducing SDS-PAGE, we found the whole molecule of CD81 and two smaller molecules in one synovial fluid sample of RA. We did not analyze whether these two smaller molecules are processed forms or degradation products of CD81, though we suppose they have some relation to CD81 because we cannot detect them under reducing conditions in which the protein structure is denatured.

We previously showed that inhibition of CD81 production by CD81 siRNA ameliorated arthritis in rats10. We have also previously shown that CD81 mediates TNF-α production in SW982 cells11. Given this, diagnosis using CD81 or its derivatives might open new avenues for RA treatment. We have already established a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) system for quantitating CD81 by using two MAbs against CD81 that recognize different epitopes11. Further improvement of this system and the identification of possible molecules for RA diagnosis are in progress.

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**Conflict of interests**

The authors have no COI existed.

**References**

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