Serum samples of patients with rheumatoid arthritis contain a specific autoantibody to "denatured" aldolase A in the osteoblast-like cell line, MG-63

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Abstract

Objective—To identify rheumatoid arthritis (RA) specific autoantibody and its antigen in the human osteoblast-like cell line, MG-63.

Methods—MG-63 cell extract was subjected to western blotting by using RA and normal serum samples as probes. The autoantigen was purified and its N-terminal sequence was determined by automated Edman degradation. The reactivity of denatured aldolase A was evaluated by immunoblotting. Screening by enzyme linked immunosorbent assay (ELISA) using the autoantibody was performed.

Results—40 kDa protein was found only in the RA serum samples and it was identified as aldolase A. A polyclonal antibody for rabbit muscle aldolase A bound to the 40 kDa protein and reacted in preference with the denatured enzyme. Using ELISA for denatured rabbit aldolase A, the autoantibody was found in approximately 10% of RA patients, whereas it was not found in the other arthropathy and healthy adults.

Conclusion—This 40 kDa anti-aldolase A autoantibody, which was identified only in serum samples of RA patients with severe bone erosion, could be related to a certain event that induces RA specific joint destructions.

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Rheumatoid arthritis (RA) is a chronic disease characterised by inflammation of the synovial joints and infiltration of blood derived cells, such as memory T cells, macrophages, and plasma cells.¹ Many molecules including inflammatory cytokines, growth factors, and autoantibodies have been implicated as pathogenic factors for RA.² There have been many observations of circulating autoantibodies in serum samples of patients with RA. Rheumatoid factors that recognise the Fc region of IgG have been most extensively studied, and various other autoantibodies such as antikeratin,³ antiperinuclear,⁴ antinuclear,⁵⁻⁷ and anticalpastatin^{8,9} have been reported. The specific nature of the antibodies including antikeratin antibody, are indicative for a possible role of citrulline containing epitopes in the pathogenesis of RA.¹⁰ These studies used syno-

vial tissue or the tissues of other organs as autoantigens for the screening. Recent studies reported the presence of circulating antibodies against extracellular matrix components of chondrocytes, for example, type II collagen, in RA serum samples.^{11,12} In addition, Sakawa *et al*¹³ found antibodies to cell surface proteins (105 kDa, 68 kDa, and 47 kDa) of human chondrocytes, and a relatively close relation between these antibodies and the degree of erosion in affected joints. To date, however, no studies have examined autoantibodies to osteoblasts. We considered that erosion of the periosteum occurs before the start of cartilage destruction in RA and before the appearance of autoantibodies to chondrocyte components, and then we started a study for identification of autoantibodies to osteoblasts using the osteoblast-like cell line, MG-63.

In this study, we identified target antigens in MG-63 cells and demonstrated that one of the autoantibodies was aldolase A, a well known enzyme that takes a major role in glycolysis and fructose metabolism. This is a report of the biochemical and immunological characterisation of the aldolase A that was detected by serum samples of RA patients, and discusses a possible mechanism of autoantibody production.

Methods

SUBJECTS

Serum samples were obtained from 62 Japanese RA patients who fulfilled the revised criteria of the American Rheumatism Association¹⁴; and from 69 arthropathy patients including 32 osteoarthritis (OA) patients, 15 systemic lupus erythematosus (SLE) patients, and 22 gout patients. Control serum samples were obtained from 82 healthy Japanese adults (age: 24-68 years). The 62 RA patients consisted of 22 men and 40 women, their ages ranged between 25 and 78 years old (mean age: 53.6 years), the mean disease duration was 7.8 years, and bone distractions on the carpal or knee joints was radiologically confirmed in 41 of the 62 (66%). RA activity was assessed using a 28 point score system by one skilled clinician. Radiological destruction of the hands and feet was quantified with the Larsen grade classification¹⁵ and with the Steinbrocker stage classification¹⁶ by one technical expert.

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Figure 1 Detection of 40 kDa protein by using RA serum as a primary antibody. The MG-63 (20 µg/lane) was separated by SDS-PAGE on 10% polyacrylamide gel, and the obtained proteins were electroblotted. The membranes were incubated with serum of RA patients or healthy controls (1/100 dilution). The 40 kDa protein was chemiluminescently recognised only in the serum samples from six RA patients who were in a progressive bone destruction stage. RA: with serum of RA patients. Normal: with serum of healthy persons.

PROTEIN EXTRACTION AND WESTERN BLOTTING

To search for RA specific autoantibody to osteoblast-like cell line, MG-63 (ATCC CRL 1427), we analysed the cell extract by western blotting by using serum samples of RA patients as a primary antibody, and compared the findings with those when using serum samples from healthy people. MG-63 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin, at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. The cells were lysed in 50 mM TRIS-HCl buffer pH 8.0 containing 1% Nonidet P-40 (NP-40) and 1 mM phenyl-methylsulphonyl fluoride. The aliquots of cell extract were separated on 10% polyacrylamide gel with SDS (SDS-PAGE). After electrophoresis, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and the membranes were blocked by PBS with 5% skimmed milk. Each membrane was incubated for an hour at room temperature with serum samples from patients (1/100 dilution), or with goat antirabbit muscle aldolase A antibody (1/10 000 dilution) (Rockland Inc, Gilbertsville, PA), and then incubated with peroxidase conjugated antihuman IgG antibody or antigoat IgG (Olganion Teknika, Co, West Chest, PA) for an hour at room temperature. Immunoreactive proteins were visualised by using enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK).

PURIFICATION OF 40 KDa PROTEIN

We used two types of columns to purify 40 kDa protein—that is, the ion exchange column, TSKgel Super Q-5PW column (7.5 × 75 mm) (Tosoh Co, Tokyo, Japan) and the reverse phase column, TSKgel Phenyl-5PW RP column (4.6 × 75 mm) (Tosoh Co). TSKgel Super Q-5PW column was equilibrated with 50 mM

TRIS-HCl buffer pH 8.0 containing 1% NP-40. MG-63 extract was applied to this column and the column was washed with equilibrium buffer at a rate of 0.5 ml/min. The flow through fractions were collected, and an aliquot of each fraction was subjected to the western blot analysis using RA serum as the probe. Fractions that contained immunoreactive 40 kDa protein were pooled and concentrated by Centricon-10 (Amicon Inc, Beverly, MA), to which five volumes of cold acetone (−20°C) was added and it was cooled at −80°C for 30 minutes. Precipitated proteins were recovered by centrifugation and dissolved in 80% formic acid. TSKgel Phenyl-5PW RP column was equilibrated with 0.05% trifluoroacetic acid (TFA). The sample collected from the TSKgel Super Q-5PW column was applied to this column, and the column was eluted with a linear gradient of acetonitrile in 0.05% TFA at 1 ml/min, and protein fractions were collected. An aliquot of each fraction was applied to western blotting. The protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL)¹⁷ using bovine serum albumin (BSA) as a standard.

AMINO ACID SEQUENCE ANALYSIS

The fractions collected by TSKgel Phenyl-5PW RP column were resolved by SDS-PAGE and electroblotted onto a PVDF membrane (Bio-Rad Laboratories). Proteins on the membrane were detected by Coomassie brilliant blue (CBB) and 40 kDa proteins were excised from the membrane. N-terminal sequence was determined by automated Edman degradation.¹⁸

IMMUNOADSORPTION AND IMMUNOPRECIPITATION

RA serum samples were preincubated for an hour at room temperature in 5 or 30 µg/ml of rabbit muscle D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase A (aldolase A, EC 4.1.2.13) (Wako Pure Chemical Industries Ltd, Osaka, Japan) or in 30 µg/ml of BSA, before being used in western blot analysis. One hundred µl of MG-63 extract (protein concentration, 2 mg/ml) was incubated with antirabbit muscle aldolase A antibody or control goat IgG at 4°C for 16 hours. Then protein G agarose (Boehringer Mannheim GmbH, Germany) was added, and incubated for another hour. Immune complexes were collected by centrifugation and analysed by western blotting.

DENATURATION OF ALDOLASE A

Rabbit muscle aldolase A solution was dialysed against PBS containing 0.1% Tween 20 (PBS-Tween) to remove ammonium sulphate, and then 8 M urea and 5% 2-mercaptoethanol were added. After one hour incubation at room temperature, the solution was dialysed against PBS-Tween, 0.1% SDS, and 0.4% 2-mercaptoethanol.

ELISA

Denatured aldolase A was diluted to 20 µg/ml with washing buffer (PBS containing 0.05% Tween 20). This antigen solution (100 µl) was

Figure 2 Purification of 40 kDa protein. The MG-63 cell extract (lane C) was applied to the TSKgel Super Q-5PW column and a flow through fraction was collected (lane Q). After concentration, the sample was separated into three peaks by the TSKgel Phenyl-5PW RP column (lane 1–3). Aliquot of each fraction was resolved by electrophoresis on 10% SDS/polyacrylamide gels. Western blotting was performed by using RA serum (1/100 dilution) as the probe. Arrow: 40 kDa protein.

added to each well of Maxi-Sorp Nunc-immunoplate (Nalge Nunc International, Denmark) and incubated at 4°C for 16 hours. After blocking with PBS containing 1% skimmed milk (Morinaga Nyugyo, Tokyo, Japan) and 1% Tween 20, 110 µl of serum samples that were diluted to 1/11 with PBS containing 1% casein, 1% skimmed milk, and 2% emulsit 25 (Daichi Kogyo Seiyaku Co, Kyoto, Japan) were added, and they were incubated for an hour at 37°C. This high level of the primary antibody (serum, 1/11 dilution) is necessary when skimmed milk and emulsit are included in the dilution, but these additives were effective to reduce non-specific reactions. One hundred µl of peroxidase conjugated antihuman IgG antibody (diluted to 1/1000 with washing buffer containing 1% BSA) were added and incubated for an hour at 37°C. After washing, the amount of bound IgG was determined by the absorbance at 405 nm using 2,2-azino-di(3-ethyl-benzthiazoline sulphonate) as a peroxidase substrate. For competitive inhibition ELISA, 10 µg/ml of denatured or native

aldolase A was preincubated with RA serum samples for two hours at room temperature, and their reactivity to the coated aldolase A was examined. The percentage of binding inhibition was calculated from the absorbance value with or without each inhibitor (that is, denatured or normal aldolase A), and the rate shows the difference in the binding affinity.

Results

DETECTION OF RA AUTOANTIBODY TO MG-63 CELL EXTRACT

Both the serum samples of RA patients and healthy subjects reacted with approximately 20 proteins at various concentrations, however, 40 kDa protein (fig 1, arrow) was strongly recognised only in the serum samples of the six RA patients whose ELISA findings were higher than 0.5 OD (fig 5). There were no significant differences in the mean disease duration between the six antibody positive RA patients (6.6 years) and the 56 antibody negative RA patients (6.2 years).

IDENTIFICATION OF 40 KDa PROTEIN BY AMINO ACID SEQUENCING

The 40 kDa protein was recognised only in the serum samples of the six RA patients who had severe bone destruction. To identify this protein, we purified cell extract by column chromatography. The cell extract was applied to the TSKgel Super Q-5PW column and 40 kDa protein was recovered in the flow through fraction (fig 2). For further purification, the samples collected through the TSKgel Super Q-5PW column were concentrated and applied to the TSKgel Phenyl-5PW RP column. The proteins were then eluted from the column at 45–50% acetonitrile and separated into three peaks. Each peak was collected and an aliquot of each was analysed. As shown in fig 2, RA serum reactive 40 kDa protein was detected in the fractions 2 and 3. Sequence analysis of the 40 kDa protein in these two fractions revealed that both band sequences were exactly identical to N-terminal sequence of aldolase A (that is, Pro-Tyr-Gln-Tyr-Pro-Ala-Leu-Thr), which is a tetrameric enzyme composed of 40 kDa monomer, and has a pI value 8.04 (calculated from the amino acids composition). These results strongly suggested that 40 kDa protein is a subunit of aldolase A.

IDENTIFICATION OF 40 KDa PROTEIN BY IMMUNOLOGICAL METHOD

Because of high homology between rabbit aldolase A and human aldolase A (only five residues of 363 amino acids are different, 99% identity), we used commercially available rabbit muscle aldolase A and antirabbit muscle aldolase A antibody to immunologically identify 40 kDa protein. The reactivity of RA serum samples to rabbit muscle aldolase A was evaluated by western blotting. As shown in fig 3A, RA serum recognised rabbit aldolase A, while normal serum did not react or reacted very weakly with rabbit aldolase A. These data suggested the presence of an RA antibody that recognises rabbit muscle aldolase A. The reactivity of the RA serum to the 40 kDa protein in

Figure 3 Identification of 40 kDa protein. (A) RA serum recognised rabbit muscle aldolase A. Purified rabbit muscle aldolase A (0.1 µg/lane) was applied to SDS-PAGE on 10% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated with either RA or normal serum, and bound IgG were detected. Normal serum reacted weakly with rabbit aldolase A. (B) Immunoabsorption of RA serum with rabbit muscle aldolase A. RA serum samples were preincubated with BSA (30 mg/ml) or rabbit muscle aldolase A (5 or 30 mg/ml), and reactivity to the 40 kDa protein was examined by western blotting. Lane 1: no adsorption. Lane 2: adsorbed by BSA (30 mg/ml). Lane 3 (30 mg/ml) and Lane 4 (5 mg/ml): adsorbed by rabbit muscle aldolase A.

Figure 4 Recognition of the RA serum reactive 40 kDa protein by antirabbit aldolase A antibody. The MG-63 cell extract (100 μ l, 2 mg/ml) was immunoprecipitated with the antirabbit aldolase A antibody or with the control IgG. The immunoprecipitates were analysed by western blotting with RA or normal serum. Upper panel: immunoprecipitation (IP) with the anti-aldolase A antibody (Ab). Lower panel: IP with the control IgG. Lane 1: antirabbit muscle aldolase A antibody. Lane 2: control IgG. Lane 3–7: RA serum. Lane 8 and 9: normal serum. The arrow shows the position of 40 kDa protein.

MG-63 cell extract was specifically blocked by the incubation with the rabbit muscle aldolase A (fig 3B). This finding indicated that RA serum that reacts with rabbit aldolase A would also react with 40 kDa protein in the MG-63 cell extract.

We then investigated whether the RA serum reactive 40 kDa protein is recognised by anti-rabbit aldolase A antibody. Immunoprecipitation of MG-63 cell extract was performed by using the antirabbit aldolase A antibody or the control IgG, then immunoprecipitates were analysed by western blotting (fig 4). The 40

Figure 5 Detection of anti-aldolase autoantibody in various serum samples by ELISA. Multititre wells were coated with 2 μ g of denatured rabbit aldolase A. The diluted serum samples (1/11) were applied to the wells and the amounts of bound IgG were determined. The cut off value ($A_{405\text{ nm}} = 0.3$) was calculated from the data of seronegative normal serum samples (mean (2SD)). RA: rheumatoid arthritis. OA: osteoarthritis. SLE: systemic lupus erythematosus.

kDa protein was specifically recovered by the antirabbit aldolase A antibody, which indicated that the 40 kDa protein was recognised by the antirabbit aldolase A antibody as well as the serum of RA patients. Therefore the 40 kDa protein and autoantibody from RA serum were identified as aldolase A and anti-aldolase A antibody, respectively. The antigen to antikeratin antibody was previously reported to have the same molecular weight as an acidic/neutral isoform of filaggrin (40 kDa).³ We investigated whether the 40 kDa protein's bands on SDS-PAGE include the band of filaggrin by means of western blotting of the cell extract using anti-filaggrin monoclonal antibody. However, the band was not detected and it was confirmed that osteoblasts do not express filaggrin (data not shown).

DETECTION OF ANTI-ALDOLASE AUTOANTIBODY BY ELISA

We developed an ELISA using rabbit muscle aldolase A as an antigen, and examined the presence of anti-aldolase autoantibody in various serum samples (fig 5). The cut off value ($A_{405\text{ nm}} = 0.3$) was determined by the antibody negative data of healthy people (mean (2SD), confirmed by western blotting). The anti-aldolase A autoantibody positive cases were found only in RA patients (6 of 62 patients), and not in the other patients with arthropathy including OA ($n = 32$), SLE ($n = 15$), gout ($n = 22$) and healthy subjects ($n = 82$). Bone erosions were present in the six antibody positive RA patients, who were in a progressive bone destruction stage (four patients were in grade II and two patients in grade III in the Larsen grade classification. Three patients were in stage II and three patients in stage III in the Steinbrocker stage classification). The radiographs of the six antibody positive patients clearly depicted destructions and erosion of the carpal joints or the knee joints. The six patients consisted of two men and four women, mean age was 58.3 years old, and mean disease period was 6.6 years. None of the six patients had been treated with corticosteroids. Four of them were antinuclear antibody positive, three of them were positive to rheumatoid factor, and all six patients were positive to C reactive protein and their erythrocyte sedimentation rate (ESR) was high, 77.3 mm 1st h (table 1). Among the 56 antibody negative RA patients, bone destruction was also radiologically confirmed in 35 patients (eight patients were in grade I, 13 patients in grade II, seven patients in grade III, four patients in grade IV, and three patients in grade V in the Larsen grade classification). Between these antibody positive and negative patients, there were no significant differences in the age of disease onset, mean disease duration, male/female ratio, and complications; however, the antibody positive patients had higher concentration of C reactive protein and ESR, and inflammatory changes that were more severe (table 1).

Table 1 Clinical characteristics and laboratory data of the patients

	Duration of disease (y)	Functional stage	Larsen grade	Steinbrocker stage	Anti-aldolase A antibody (OD)	Autoantibodies (titre)	ESR (mm 1st h)	CRP (mg/dl)
<i>Anti-aldolase A antibody positive patients (n=6)</i>								
patients/sex/age								
1/F/55	6.4	II	III	II	2.49	80	88	5.5
2/F/67	10.3	III	III	III	0.38	80	52	3.8
3/F/52	5.3	II	II	III	1.74	40	65	4.8
4/F/45	4.8	II	II	II	1.21	80	95	9.8
5/M/64	5.6	II	II	II	0.68	80	102	12.5
6/M/67	7.2	II	II	III	0.55	40	62	4.0
(age)								
58.3 (9.0)	6.6 (2.0)	2.2 (0.40)	2.43 (0.51)	2.5 (0.54)	1.18 (0.81)	66.6 (20.6)	77.3 (20.3)	6.73 (3.57)
<i>Anti-aldolase A negative patients with bone destruction (n=35)</i>								
(age)								
55.7 (7.5)	5.9 (5.4)	2.8 (0.57)	2.46 (0.87)	2.8 (0.88)	0.021 (0.09)	58.4 (15.6)	52.2 (18.4)	4.22 (2.58)
p value	NS	NS	NS	NS	<0.02	NS	<0.05	<0.05

Figures represent mean (SD). ESR: erythrocyte sedimentation rate. CRP: C reactive protein.

ALDOLASE A ACTIVITY IN THE CIRCULATION

We investigated the relation between the amount of aldolase A and anti-aldolase A autoantibody. By using diagnostic reagents, activity of aldolase A in the serum samples from the six antibody positive RA patients and antibody negative people (56 RA patients and 86 normal healthy controls) was determined, and the aldolase activity in the serum samples of both group was found to be in the normal range (1.7–5.7 IU/l). Furthermore, in the antibody negative RA patients, aldolase A was not detected in the serum and synovial fluid by western blotting (data not shown). There was no correlation between the aldolase A in the circulation and anti-aldolase A autoantibody in the serum.

ENHANCED BINDING TO DENATURED ALDOLASE A

The reactivity of anti-aldolase A autoantibody in RA serum to the denatured and native aldolase A was examined by the competitive inhibition ELISA (fig 6). This autoantibody recognises both native and denatured aldolase A, but bound more tightly to the denatured type than

to the native type. This indicates the presence of an RA specific event that leads to denaturation of aldolase A.

Discussion

In this study, we demonstrated that one of the autoantibodies in RA serum reacted with a 40 kDa protein in the osteoblast-like cell (MG-63). Only six of the 62 serum samples from RA patients reacted with the 40 kDa protein. The six antibody positive patients were in a progressive bone destruction stage and whose joints showed severe inflammatory activity. As this 40 kDa protein was not recognised in the serum samples from the other patients with arthropathy, including OA, SLE, and gout, this autoantibody is specific to RA with progressive bone destructions. We identified this 40 kDa protein as a D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase A (aldolase A, EC 4.1.2.13).

Aldolase is a tetrameric enzyme that catalyses reversible aldose cleavage/condensation reaction in the glycolytic pathway. In mammalian tissue, aldolase exists in three forms. Aldolase A is found in a variety of tissues, but predominantly in muscle; aldolase B is expressed in the liver and kidney; and aldolase C is present in the brain, together with various hybrids of A and B subunits. O'Hara *et al*¹⁹ showed that aldolase reacts non-specifically with immune complexes. However, we confirmed that the immune complex that is present at a high level in SLE, and the immune complex found in non-RA arthropathy, did not react to aldolase (data not shown). Therefore, we consider that the possibility of non-specific reaction attributable to an immune complex and aggregated antibodies is low. We investigated the relation between the amount of aldolase A in the circulation and that of anti-aldolase A autoantibody in the serum, but no correlation between them was observed. Reactivity of anti-aldolase A autoantibody in RA serum was evaluated by using denatured and native aldolase A, and this autoantibody was found to bind predominantly to the denatured enzyme. These findings suggest that anti-aldolase A autoantibody would be produced in response to the structural change in aldolase A, but not to the increase of the enzyme itself; furthermore, the presence of this autoantibody

Figure 6 Reactivity of anti-aldolase autoantibody in RA serum samples with denatured and native aldolase A. The serum samples (1/11 dilution) were preincubated with the denatured or native aldolase A (10 µg/ml), and the reactivity were evaluated as the reactivity against coated aldolase A. The percentage of inhibition was calculated from absorbance value with and without each inhibitor. The autoantibody bound more tightly to the denatured aldolase A. RA1: patient 1 with RA. RA2: patient 2 with RA. RA3: patient 3 with RA. RA4: patient 4 with RA.

would be related to a specific event that induces bone destructions in RA.

The same reactivity of autoantibody has been reported in Sjögren's syndrome. Itoh *et al*²⁰ described that anti-52 kDa SSA/Ro autoantibodies in serum of patients bound predominantly to the denatured antigens. Tsuzaka *et al*²¹ reported that 61% of anti-SSA/Ro autoantibody positive serum samples were positive for the anti-denatured protein but none of their serum samples immunoprecipitated the native 52 kDa antigen.

In RA serum, we were able to identify an autoantibody directed to aldolase A. The autoantibody reacted predominantly with the denatured aldolase A, whereas commercially available anti-aldolase A antibody reacted equally with the denatured and native aldolase A (data not shown). The epitope that is recognised by anti-aldolase A autoantibody in RA serum has been carefully examined in our laboratory by using various recombinant deleted aldolase A. At present, the dominant epitope region is determined in the N-terminal 100 amino acids region. On the other hand, commercially available anti-aldolase A antibody recognises all recombinant proteins that we prepared. Further experiments are being carried out to determine precise epitope regions. These findings, together with the results shown in figure 6, show that a certain event may occur in the antibody positive RA patients, and it may lead the dominant epitope region of aldolase A, which may locate inside the molecule (normal), to the outside (denaturation), and may promote the production of the autoantibody that could also be involved in the reactions found in Sjögren's syndrome. The mechanism of this autoantibody production during joint destruction may relate to the production of various autoantibodies in RA.

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