

Ribosomal P protein P0 as a candidate for the target antigen of anti-endothelial cell antibodies in mixed connective tissue disease

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Abstract Objective

To evaluate the presence of anti-endothelial cell antibodies (AECA) in patients with mixed connective tissue disease (MCTD) compared to those with systemic sclerosis (SSc) and to determine the candidates for the endothelial autoantigen that reacts with AECA in patients with MCTD using a molecular cloning strategy.

Methods

AECA were measured by a cellular enzyme-linked immunosorbent assay (ELISA) using fixed human umbilical vein endothelial cells (HUVEC) in 47 MCTD patients, 68 SSc patients, and 52 normal controls. A HUVEC cDNA expression library was immunoscreened with pooled sera from 6 patients with high AECA levels determined by cellular ELISA to explore the endothelial autoantigens in MCTD. An ELISA assay for anti-ribosomal protein P0 antibodies was used to assess the correlation with AECA levels.

Results

The candidate target proteins recognized by AECA in MCTD included: (i) ribosomal protein P0; (ii) a putative oncogene derived from *dek* mRNA; (iii) SS-B/La protein; (iv) U1 RNA-associated 70K protein; and (v) DNA-binding protein B. A significant correlation between the levels of AECA and anti-ribosomal protein P0 antibodies was demonstrated in MCTD, but not in systemic sclerosis. The sera containing high levels of AECA from patients with MCTD frequently cross-reacted with ribosomal protein P0. On the other hand, sera without AECA activity from patients with MCTD never reacted with ribosomal protein P0.

Conclusion

AECA were more frequently seen in patients with MCTD than in patients with SSc. Ribosomal protein P0 may be one of the major target antigens of AECA in patients with MCTD.

Key words

Mixed connective tissue disease, endothelial cells, autoantibodies, ribosomal protein P0, cDNA library, immunoassay, enzyme-linked immunosorbent assay.

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Received on November 12, 2006; accepted in revised form on April 6, 2007.

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Introduction

Mixed connective tissue disease (MCTD) is commonly characterized by overlapping features with other connective tissue diseases, including systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), high levels of circulating autoantibodies, and diffuse proliferative intimal and/or medial vascular lesions resulting in a narrowing of the lumen of the small vessels and the medium-size arteries in many organs (1). Anti-endothelial cell antibodies (AECA) represent a heterogeneous group of antibodies directed against a variety of antigenic determinants on endothelial cells (2). Higher prevalences and concentrations of AECA have been described in patients with SLE, SSc, and systemic vasculitis (3-6). Furthermore, sera containing high concentrations of AECA from patients with SLE or SSc have been shown to increase the expression of adhesion molecules on human umbilical vein endothelial cells (HUVEC) (7). These observations indicate that AECA may be implicated in the pathogenesis of the vascular lesions in these disorders. Recently, high levels of AECA in the serum have also been demonstrated in MCTD patients (8, 9). However, very little published data is available regarding the target antigens of AECA in connective tissue diseases, including MCTD.

In this study we investigated the prevalence of AECA using a cell-based enzyme-linked immunosorbent assay (ELISA) in patients with MCTD compared to those with SSc and normal controls, and identified the endothelial cell autoantigens in MCTD by immunoscreening a HUVEC cDNA expression library with sera from patients with high AECA levels. We show that the anti-ribosomal protein P0 antibody (anti-P0) is a candidate for the endothelial autoantigen in patients with MCTD. We also found a correlation between the serum levels of anti-P0 and AECA, which suggests that ribosomal protein P0 may be one of the target antigens of AECA in MCTD.

Materials and methods

Patients

The MCTD group consisted of 47 pa-

tients (2 males and 45 females) who fulfilled the Kasukawa criteria (10) for MCTD. The SSc group consisted of 68 patients (13 males and 55 females) who fulfilled the American Collage of Rheumatology 1980 criteria for SSc (11), but not Kasukawa's MCTD criteria. Thirteen out of 47 patients from the MCTD group also fulfilled the criteria for SSc. As normal controls, 52 healthy age- and sex-matched individuals were also enrolled in this study. The mean ages of the subjects in the three groups were as follows: MCTD: 42.0 yrs, SSc: 58.0 yrs, and normal controls: 49.0 yrs. Patients with SSc were divided into two groups: 31 had diffuse cutaneous SSc (d-SSc) while 37 had limited cutaneous SSc (l-SSc), according to LeRoy's criteria (12). All of the subjects were Japanese and provided their written informed consent. The sera used in this study were stored at -80° until used.

Cell culture and cellular ELISA assay for AECA

Commercially available HUVEC and endothelial culture media were used (Cambrex, East Rutherford, NJ). All cultures were incubated at 37° in 5% CO₂. On reaching sub-confluence after the 3rd passage, the cells were harvested using 0.025% trypsin, 0.01% EDTA 0.01% in HEPES buffered saline (Cambrex), and seeded to wells of gelatine-coated 96-well microtiter plates (Sumitomo Bakelite, Tokyo, Japan) at a concentration of 5 x 10³ cells. These were allowed to grow until confluence, washed gently 3 times with phosphate buffered saline, pH 7.4 (PBS), and fixed with 100 µl/well of 0.1% glutaraldehyde (Sigma, St. Louis, MO) for 10 minutes at 4°C, followed by washing 2 times with PBS. Non-specific protein binding sites were blocked by adding 100 µl/well of PBS containing 3% bovine serum albumin fraction V (Roche Diagnostics GmbH, Mannheim, Germany, BSA/PBS) to each well and incubating overnight at 4°C.

The wells were then flooded in triplicate with 50 µl of sera diluted 1:500 in BSA/PBS and incubated overnight at 4°C. After extensive washes with PBS containing 0.05% vol/vol of Tween20 (Sigma, PBS-T), the bound antibodies

Competing interests: none declared.

were detected with 1/3,000 horseradish peroxidase-conjugated goat anti-human IgG-gamma chain antibodies (Zymed Laboratories, South San Francisco, CA) using 0.4 mg/ml o-phenylenediamine (Sigma). Optical density was measured at 492 nm in an ELISA plate reader (SPECTRAMax 340, Molecular devices, Sunnyvale, CA). All assays included at least one positive and one negative reference sample on each plate. The intra-assay and inter-assay coefficients of variation were 5% and 9%, respectively.

The results were expressed as an ELISA ratio = $100 \times (S-A)/(B-A)$, where S = the absorbance of the sample, A = the absorbance of a negative reference sample, and B = the absorbance of a positive reference sample. The mean +3 SD of the ELISA ratio obtained from the normal control subjects was taken as the cut-off limit for positivity. We performed preliminary comparable experiments using unfixed HUVEC, and found no significant differences between the use of fixed and unfixed cells (data not shown).

Immunoscreening a recombinant endothelial cell expression library with sera from MCTD patients

Poly (A)+mRNA was purified from 5×10^7 HUVEC and employed to construct the cDNA expression library in the ZAP Express vector arms of a lambda phage vector using a cDNA library construction kit (Stratagene, La Jolla, CA, USA). A library consisting of approximately 6.5×10^5 recombinants was constructed and 1×10^5 recombinants were screened with the pooled sera of 6 MCTD patients with high AECA levels determined by cellular ELISA. Prior to immunoscreening, the pooled sera were pre-absorbed with *Escherichia coli* phage lysate (Stratagene) according to the instruction manual provided by the manufacturer to reduce the background sera activity. *Escherichia coli* host strain XL1-Blue MRF⁷ transfected with lambda-phages were plated on agar plates. After the emergence of visible plaques, nitrocellulose membranes (Schleicher & Schuell, Keene, NH) soaked in isopropyl-D-thiogalactosidase (Sigma) for protein induction were placed on plates and cultured for 3.5 hours. β -galactosi-

dase fusion proteins induced by isopropyl-D-thiogalactoside were transferred to nitrocellulose membranes. Thereafter, the membranes were extensively washed in Tris buffered saline (TBS, 20 mM Tris [tris(hydroxymethyl) aminomethane] (pH 7.5), 150 mM NaCl) with 0.05% vol/vol of Tween 20 (TBST) and blocked for 1 hour with 1% wt/vol non-fat dry milk in TBST. Nitrocellulose membranes with the transferred proteins were incubated overnight at 4°C with a 1:500 dilution of the pre-absorbed pooled sera of the MCTD patients. After washing, the membranes were incubated with 1/3,000 peroxidase-conjugated goat anti-human IgG-gamma chain secondary antibodies (Zymed laboratories) for 1 hour, and the reactive phage plaques were visualized by incubating with diaminobenzidine tetrahydrochloride (Sigma). Clones identified as positive in this first round of screening were subjected to two further rounds of screening to ensure that they were positive for reactivity to the patients' sera and to purify them to homogeneity.

Sequencing and alignment of positive clones

Positive phage clones were subcloned, purified, and excised *in vitro* to pBK-CMV plasmid forms (Stratagene). Plasmid DNA was purified using the Wizard Miniprep DNA Purification System (Promega, Madison, WI, USA). Sequencing was performed according to the manufacturer's instructions using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Database searches for matches of the DNA sequences of positive clones were performed with the GenBank databases using the BLASTN and BLASTX algorithms.

ELISA assay for anti-P0

A ribosomal P ELISA kit (MBL, Nagoya, Japan) was used to detect anti-P0 with an ELISA plate reader (SPECTRAMax 340) according to the manufacturer's instructions. The antigenic substrate of this ELISA plate is recombinant ribosomal protein P0 containing

carboxyl-terminal peptide 22 amino acids, which has been shown to cross-react with antibodies reacting with carboxyl-terminal peptide 22 amino acids of the 60S acidic ribosomal protein P2 of *Artemia Salina*, which was originally described as an anti-ribosomal P antibody by Elkouf *et al.* (13, 14) The mean +3 SD of the antibody level obtained from normal control subjects was taken as the cut-off limit for positivity.

Statistical analysis

Statistical analyses were performed using Fisher's exact probability test for the analysis of frequencies, Student's two-tailed t-test for the comparison between antibody levels in each group, and Spearman's rank correlation test for the analysis of the correlation between the antibody levels of AECA and anti-P0. P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS11J for Windows (SPSS Japan Inc., Tokyo, Japan).

Results

Prevalence of AECA in SSc and MCTD patients

Assuming that the cut-off level is the mean of the normal controls +3 SD, the prevalences of AECA were as follows; 59.6% (28/47) in MCTD, 26.5% (18/68) in total SSc, 32.3% (10/31) in d-SSc, 16.2% (6/37) in l-SSc, and 0% (0/52) in normal controls. If the mean +2 SD was used as the cut-off limit for positivity, AECA were found to be present in 38 patients with MCTD (80.9%), 30 patients with SSc (44.1%), 19 patients with d-SSc (61.3%), 11 patients with l-SSc (29.7%), and 4 normal controls (7.7%). AECA levels were significantly higher in patients with MCTD or SSc than in controls ($p < 0.001$). AECA levels in patients with MCTD were significantly higher than in patients with SSc ($p < 0.001$, Fig. 1). No statistically significant differences in AECA levels were found between patients with d-SSc and l-SSc.

Immunoscreening a recombinant endothelial cell expression library with sera from MCTD patients

To identify the autoantigens recognized by AECA in MCTD patients, we

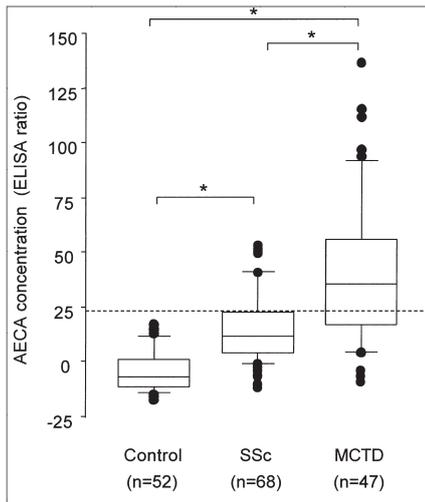


Fig. 1. Box plots of the levels of anti-endothelial antibodies (AECA) expressed as an ELISA ratio in patients with MCTD (n = 47), patients with SSc (n = 68), and normal controls (n = 52). The central horizontal line in the box, the box edges, and the ends of the vertical lines indicate the median of each group, the upper and lower quartiles, and the 10th and 90th percentiles of the data, respectively. The horizontal dashed line represents the mean +3 standard deviations of AECA ELISA ratio for normal controls (ELISA ratio = 22.8). AECA levels were significantly higher in patients with MCTD and SSc patients than in normal controls. In MCTD patients, AECA levels were significantly higher than in SSc patients. The asterisks indicate significant differences at the level of $p < 0.001$ between each group.

screened a HUVEC cDNA expression library with the pooled sera from the 6 MCTD patients with high AECA levels. Plaques (1×10^5 pfu) were immunoscreened, resulting in the isolation of 28 independent seroreactive clones. DNA sequencing demonstrated that 16 of the sero-reactive clones encoded the ribosomal P protein P0 mRNA, 5 encoded a putative oncogene derived

from dek mRNA, 3 encoded SS-B/La protein, 2 encoded a U1 RNA-associated 70K protein, and 2 encoded DNA-binding protein B (Table I).

Anti-P0 in MCTD and SSc and its correlation with AECA

Since more than half of the seroreactive clones encoded ribosomal protein P0, we investigated the correlation between the levels of IgG AECA and anti-P0 in MCTD, SSc, and normal controls. The prevalences of anti-P0 were 23.4% (11/47) in MCTD, 16.1% (5/31) in d-SSc, 10.8% (4/37) in l-SSc, 13.2% (9/68) in the total SSc group, and 0% (0/52) in normal controls. The levels of anti-P0 were significantly higher in patients with MCTD than in the total SSc group ($p = 0.015$), the d-SSc group ($p = 0.014$), or normal controls ($p = 0.024$) (Fig. 2). Patients with MCTD tended to have high levels of anti-P0 compared to those with l-SSc ($p = 0.073$). A significant correlation between the levels of AECA and anti-P0 was demonstrated in the MCTD patients (correlation coefficient $R = 0.731$, $p < 0.001$) (Fig. 3), but not in those with d-SSc ($R = 0.208$, $p = 0.233$), l-SSc ($R = 0.215$, $p = 0.203$), or total SSc ($R = 0.200$, $p = 0.089$).

Discussion

In this study we have demonstrated a higher prevalence and higher levels of AECA in MCTD compared to SSc and identified five candidates for the endothelial cell autoantigen in MCTD by means of a molecular cloning strategy: (i) human ribosomal protein P0,

which is reported to be associated with SLE (13-15); (ii) human dek protein, an autoantigen associated with SLE (16, 17); (iii) human La/SS-B protein, a disease-specific marker for Sjögren's syndrome (18); (iv) human U1 RNA-associated 70K protein, an autoantigen of U1-RNP autoantibodies (19); and (v) human dbpB-coded protein, an autoantigen associated with SSc (20). The identified endothelial autoantigens screened with MCTD sera in this study have been previously reported to be autoantigens associated with SLE or SSc. These endothelial autoantibody profiles may help to explain the overlapping features seen in MCTD.

More than half of the 28 independent positive clones derived from a non-amplified endothelial cell cDNA expression library screened with sera from the 6 MCTD patients with high AECA concentrations encoded ribosomal protein P0. This finding indicates that the mRNA transcripts coding ribosomal protein P0 may be the most abundant of those coding the target proteins recognized by AECA in MCTD, and ribosomal protein P0 may very likely be a major target protein in the cultured HUVEC recognized by AECA in MCTD. A significant correlation between the levels of AECA and anti-P0 was demonstrated in MCTD patients, but not in SSc patients. These findings suggest that ribosomal P0 protein is one of the major targets of AECA in MCTD patients, especially in those with high serum concentrations of AECA, and is less important in SSc patients.

Ribosomal P proteins have been re-

Table I. Genes identified by the immunoscreening of an HUVEC cDNA expression library with the sera of patients with MCTD who had high AECA levels.

Gene	Accession no.	No. of clones	Associated disease	Reference
1 <i>Homo sapiens</i> ribosomal protein, large, P0, transcript variant 1, mRNA	BC015690	16/28	SLE	(13-15)
2 <i>Homo sapiens</i> dek mRNA	X64229	5/28	SLE, JRA	(16, 17)
3 <i>Homo sapiens</i> Sjögren's syndrome antigen B (autoantigen La) (SSB), mRNA	NM_003142	3/28	Sjögren's syndrome	(18)
4 Human mRNA for U1 RNA-associated 70K protein	X04654	2/28	MCTD, SLE (paucity of nephritis)	(19)
5 Human DNA-binding protein B (dbpB) gene, 3' end.	M24070	2/28	SSc	(20)

JRA: juvenile rheumatoid arthritis; MCTD: mixed connective tissue disease; SLE: systemic lupus erythematosus; SSc: systemic sclerosis.

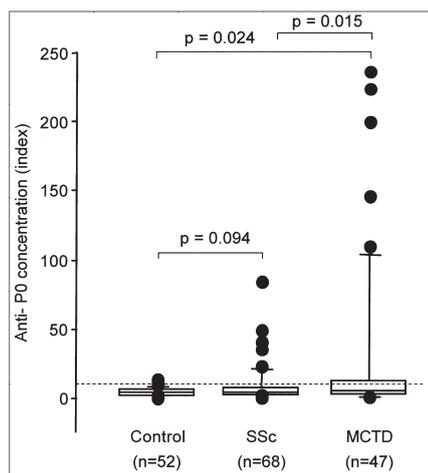


Fig. 2. Box plots of the levels of anti-ribosomal protein P0 antibodies (anti-P0) expressed as an ELISA index in patients with MCTD ($n = 47$), patients with SSc ($n = 68$), and normal controls ($n = 52$). The central horizontal line in the box, the box edges, and the ends of the vertical lines indicate the median of each group, the upper and lower quartiles, and the 10th and 90th percentiles of the data, respectively. The horizontal dashed line represents the mean +3 standard deviations of anti-P0 ELISA index for normal controls (ELISA index = 13.3). Anti-P0 levels were significantly higher in patients with MCTD than SSc ($p = 0.015$) or normal controls ($p = 0.024$).

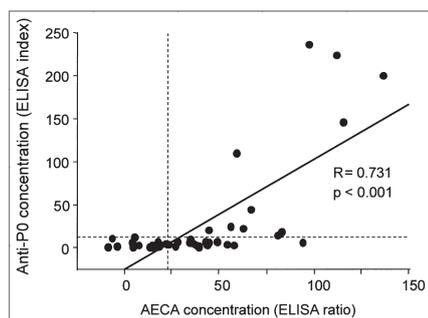


Fig. 3. Correlation between the levels of anti-endothelial cell antibodies (AECA) and anti-ribosomal protein P0 antibodies (anti-P0) in patients with MCTD. Figure 3 shows a significant correlation between the levels of AECA and anti-P0 in MCTD patients. In contrast, no significant correlation between the levels of AECA and anti-P0 in patients with SSc was observed (data not shown). According to the Spearman's rank correlation test for comparison of the two series of data (AECA levels and anti-P0 levels), the correlation coefficient (R) was 0.731 ($p < 0.001$) for data from samples of MCTD patients ($n = 47$), 0.200 ($p = 0.089$) for all SSc patients ($n = 68$), 0.208 ($p = 0.233$) for diffuse SSc patients ($n = 31$), and 0.215 ($p = 0.203$) for limited SSc patients ($n = 37$). The vertical dashed line represents the mean +3 SD of the AECA ELISA ratio for normal controls (ELISA ratio = 22.8), the horizontal dashed line represents the mean +3 SD of the anti-P0 ELISA index (ELISA index = 13.3), and the solid oblique line represents the regression line.

ported as autoantigens reacting with the sera of lupus patients (13-15). Several clinical studies indicate that autoantibodies to ribosomal P proteins have a highly specific association with SLE, particularly in renal and hepatic involvement, but their presence in other connective tissue diseases has only occasionally been reported (15) and there are no reports on the clinical association between anti-P0 and MCTD. Recently, Frampton *et al.* investigated endothelial autoantigens in SLE using a similar approach and identified a panel of candidate endothelial cell autoantigens in patients with SLE, which includes ribosomal protein P0 (21).

Although the methodology of AECA measurement remains to be standardized, mild cell fixation with glutaraldehyde has been widely used in order to prevent the detachment of cells from 96-well microtiter plates during the extensive washing step with detergents that is required to avoid non-specific binding. Ribosomal P proteins are known to be located in cytoplasm. As the fixation and washing steps may permeabilize the cell membrane and permit antibodies to react with cytoplasmic antigens, the high level of AECA measured by fixed cell ELISA in sera containing a high level of anti-P0 antibodies might be at least partially due to the interaction of cytoplasmic ribosomal protein P0 and its antibodies. However, there have been several reports suggesting that autoantibodies to ribosomal P proteins bind not only to intracellular antigen but also to a plasma membrane-associated target on human cells, including human neuroblastoma cells, human fibroblasts (22), mesangial cells (23), and T-cells (24). Yoshio *et al.* showed that anti-P0 that had been affinity-purified from the sera of lupus patients reacted with the surface of HUVEC (25). Frampton *et al.* observed the direct binding of rat IgG anti-ribosomal peptide antibodies to HUVEC, which was confirmed by immunofluorescence and fluorescence-activated cell sorter analysis (21).

To examine whether anti-P0 displays true AECA activity, we recently performed a series of absorption studies using recombinant ribosomal protein

P0 as substrate, and BSA as a control. The absorption of 4 MCTD sera containing high AECA and high anti-P0 titers with recombinant ribosomal protein P0 resulted in a significant decrease in AECA titers determined by cell ELISA using not only fixed, but also unfixed cells. On the other hands, the absorption of 4 MCTD sera containing high AECA titers but lacking anti-P0 activity with recombinant ribosomal protein P0 resulted in an insignificant decrease in AECA titers (T. Naniwa *et al.*, unpublished results). These observations suggest that antibodies to ribosomal P proteins bind to endothelial cells by recognizing not only cytoplasmic ribosomal protein P0, but also ribosomal protein P0 itself or other antigenically related molecules located on the plasma membrane.

It has been suggested that antibodies to ribosomal P proteins may have possible pathogenic effects, such as the inhibition of protein synthesis and augmenting apoptosis (26, 27). Although evidence regarding the effects of anti-P0 on endothelial cells in the pathogenesis of MCTD remain to be confirmed, these observations, together with the literature concerning the possible pathogenicity of anti-P0, suggest that anti-P0 may play a role in the vascular damage seen in MCTD.

In conclusion, we have demonstrated significantly increased concentrations of AECA in MCTD patients compared to SSc patients and controls, and have found a panel of candidates for the endothelial autoantigens in MCTD, including ribosomal protein P0, by means of a molecular cloning strategy. We also showed a significant correlation between the levels of anti-P0 and AECA. These results suggest that ribosomal protein P0 is an important endothelial autoantigen in MCTD and warrants further study regarding the biological effects of AECA and anti-P0 on endothelial cells.

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