Mod Rheumatol (2008) 18:570–577
DOI 10.1007/s10165-008-0094-4

ORIGINAL ARTICLE

Human voltage-dependent anion selective channel 1 is a target antigen for antiglomerular endothelial cell antibody in mixed connective tissue disease

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Received: 25 March 2008 / Accepted: 14 May 2008 / Published online: 21 June 2008

Abstract The purpose of this study was to identify the endothelial cell antigens that react with circulating antiendothelial antibody (AECA) in mixed connective tissue disease (MCTD). We screened serum AECA reactivity in 23 patients with MCTD using a human glomerular endothelial cell (HGEC) cellular ELISA. Proteomics, two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry were used to identify the endothelial cell antigens of HGECs that reacted with serum antibodies from MCTD patients. Sera from 12 patients (52.0%) were positive for anti-HGEC antibody based on cellular ELISA. MALDI-TOF mass spectrometry used in combination with immunoblotting using serum antibody revealed one protein spot that represented a 36-kDa cell component of HGECs, with an isoelectric point (IP) of about 9, which had a high homology with the voltage-dependent anion-selective channel 1 (VDAC-1). This protein spot was confirmed to react with the antibody specific to VDAC-1. This is the first report of the presence of antibody to VDAC-1 from HGECs in the sera from MCTD patients. Although future studies will be needed to clarify the disease specificity of the a-VDAC-1 antibody in MCTD, the results show that modern proteomics technology is useful for identifying antigens that react with AECA in autoimmune diseases such as MCTD.

Keywords Antiendothelial cell antibody · HGEC · MCTD · Proteomics · VDAC-1

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Introduction

The term "mixed connective tissue disease" (MCTD) was initially used to refer to a new distinct type of connective tissue disease by Sharp in 1972 [1]. The criteria for the disease consist of the following three conditions: (1) either Raynaud's phenomenon or swollen fingers or hands; (2) positive anti-nRNP antibody; (3) mixed findings of at least two of the connective tissue diseases (SLE, PSS, and PM). Proliferative vascular lesions in the intima of muscular arteries and arterioles followed by vast mononuclear cell infiltration in different tissues is often found [2]. Patients with MCTD show a high level of serum ET-1 and endothelial cell proliferation in the vascular wall, suggesting that endothelial cell damage may be critical to the pathogenesis of MCTD [3]. While antiendothelial cell antibodies (AECA) have been detected in MCTD [4], the endothelial antigen recognized by AECA in MCTD still requires characterization, and in particular the antigenic specificity for glomerular endothelial cells must be elucidated.

The present study was undertaken to identify the endothelial cell antigens that react with AECA in MCTD via two-dimensional gel electrophoresis and immunoblotting in combination with mass spectrometry. This line of approach using modern proteomics technology allows us to deepen our understanding of the pathogenesis of glomerular injury

in autoimmune vascular diseases including MCTD and to develop novel diagnostic techniques for AECA assays.

Materials and methods

Patients

This study included 23 MCTD patients who satisfied the diagnostic criteria of Kasukawa et al. [5]. Sera from seven healthy volunteer donors were used as controls. Among the 23 patients, the serum from a MCTD patient with thrombotic thrombocytopenic purpura (TTP) [6] was used as a representative serum.

Culture of human endothelial and mesangial cells

Human glomerular endothelial cells (HGECs) isolated from normal human kidneys were purchased from Cell System Corp. (Kirkland, WA, USA). HGECs were cultured as described previously [7].

Human mesangial cells (HMCs) were prepared as described previously [8, 9]. Glomeruli were isolated from the normal cortices of human kidneys obtained at the time of nephrectomy due to renal tumors using the serial sieving method. HMCs were cultured as described previously [10].

Immunofluorescence microscopy

For immunofluorescence microscopy, cells cultured on an eight-well Lab-Tech chamber slide (Nalge Nunc, Naperville, IL, USA) were fixed with 2% paraformaldehyde for 2 h. They were incubated with sera, diluted with phosphate-buffered saline (PBS) at 1:800, from patients with MCTD and healthy controls for 60 min at room temperature (RT). After three washes with PBS, FITC-conjugated anti-human immunoglobulin antibody (DAKO, Copenhagen, Denmark) diluted 1:600 with PBS was added and incubated for 60 min at RT. The immunostained samples were examined under an Olympus AH3-RFC microscope (Tokyo, Japan) equipped for epifluorescence.

Cellular enzyme-linked immunosorbent assay (ELISA)

As described previously [11], HGECs were plated on gelatin-coated 96-well ELISA plates (Iwaki, Tokyo, Japan) and allowed to grow to subconfluency. Cells were fixed with 1% paraformaldehyde for 2 h at RT, and blocked with 0.5% bovine serum albumin (BSA) in 0.1 M phosphate buffer (PB) for 1 h at RT. Then sera from the normal controls and MCTD patients, diluted 1:20 in 0.5% BSA-containing PB, were added to the wells and incubated for 1 h at RT. After three washes with PB, peroxidase-labeled anti-human immunogloblins (DAKO) were added to each well and incubated for 1 h at RT. The plates were washed, developed with *O*-phenylenediamine dihydrochloride (Sigma Chemical Co., Tokyo, Japan), and measured for absorbance at 492 nm with a microtiter plate reader (Bio-Rad, Hercules, CA, USA). Positivity was defined as an absorbance that is greater than the mean plus 2SD for the seven normal control sera.

SDS-PAGE and immunoblotting

HGECs and HMCs were lysed in RIPA buffer (1% Triton X-100, 1% DOC, 0.1% SDS, 0.15 M NaCl, and 0.05 M Tris–HCl, pH 7.4) on ice for 30 min. After centrifugation at 15,000×*g* for 20 min at 4°C, the supernatant was collected and pooled with another extract taken at a different passage. The supernatant was mixed with an equal amount of 2× SDS sample buffer (4% SDS, 0.125 M Tris–HCl, pH 6.8, 20% glycerol, 0.01% bromophenol blue, and 4% 2-mercaptoethanol), boiled for 2 min, and rapidly cooled.

The cell lysate was then subjected to separation on 12% SDS gel electrophoresis using a Laemmli buffer system. A mixture of molecular weight (MW) standards of 27, 36, 42, 55, 66, 97, 116 and 159 kDa was included in each run. The separated proteins were transferred to a PVDF membrane by a tank transfer apparatus at 80 mA for 3 h. The membrane was washed with Tris-buffered saline (TBS) and blocked for 1 h at RT in TBS supplemented with 2% nonfat milk. The transblot was incubated with patients' sera, which had been diluted 1:500 with TBS containing 2% nonfat milk, overnight at 4°C. The membrane was washed four times with TBS containing 0.1% Tween-20 and then incubated with HRP-conjugataed anti-human immunoglobrin, which was diluted 1:2,000 for 1 h at RT. After washing, immunoreactive protein bands were visualized on X-ray films using ECL Western blotting detection reagents (GE Healthcare, Chalfont, St Giles, UK).

Two-dimensional gel electrophoresis

Cell lysate proteins were first concentrated by methanol and chloroform and dissolved in rehydration buffer (9.8 M urea, 2% NP-40, 0.2% Pharmalyte (pH 3–10), 100 mM DTT, and 0.1% bromophenol blue) supplemented with protease inhibitor cocktail (0.5 μg/ml E-64, 0.5 mM PMSF, 40 μg/ml TLCK, 1 μg/ml aprotinin, 0.5 mM EDTA, and 10 μg/ml chymostatin). Isoelectric focusing in the first dimension was carried out in a horizontal apparatus, an IPGphor (GE Healthcare), using a 7-cm immobiline DryStrip (IPG strip) with a linear pH range of pH 3–10 (GE Healthcare), according to the

standard protocol described by the manufacturer. The IPG strips were rehydrated for 12 h at 20°C with 125 μl of rehydration buffer containing sample protein (20 μg). After isoelectric focusing, IPG strips were reduced by incubation for 30 min at RT with 10 ml of SDS equilibration buffer (6 M urea, 2% SDS, 50 mM Tris–HCl, pH 8.8, 30% glycerol, and 0.01% bromophenol blue) containing 100 mg DTT, and alkalized by incubation for 30 min with 10 ml of SDS equilibration buffer containing 250 mg iodoacetamide. Following equilibration the IPG strips were embedded on 12% SDS-PAGE gels with agarose using the Laemmli buffer system. Gels were run at a constant voltage of 200 V for 70–80 min in a Model 3000Xi apparatus (Bio-Rad). The separated proteins were transferred onto a PVDF membrane using a semidry Western blotting apparatus (Trans-Blot SD, Bio-Rad) at 2 mA/cm^2 for 55 min.

Detection of protein spots that are immunoreactive with sera from MCTD patients

After two-dimensional electrophoresis, the gels were transferred onto a PVDF membrane as described above. The PVDF membranes were stained with fluorescent dye, deep purple (GE Healthcare), and visualized by scanning with a Typhoon 9400 (GE Healthcare). After scanning, the PVDF membrane was processed for immunoblotting with sera from MCTD patients and visualized on an X-ray film. To identify the protein spots that are immunoreactive with the sera, the immunoblot image on X-ray film and the image of the deep purple-stained PVDF membrane were superimposed with the aid of Photoshop version 7.0 (Adobe Systems, San Jose, CA, USA). The immunoreactive protein spots on deep-purple-stained PVDF membrane were then matched to the corresponding spots of the silver-stained gel run in parallel with the same cell lysate. The matching spots in silver-stained gels were subjected to identification by mass spectrometry. When immunoblotting analysis of proteins separated on two-dimensional gels was conducted to validate the protein identified by mass spectrometry, the blotted membranes were similarly processed using deep purple staining and immunoblotted with anti-VDAC1 antibody (Abcam Ltd., Cambridge, UK).

Protein identification by MALDI-TOF MS

Protein spots that reacted with patient sera were manually excised with a scalpel from silver-stained two-dimensional gels equilibrated with ultrapure water. The excised spots were cut into small pieces and washed four times in 0.5 ml of 50 mM ammonium bicarbonate and 50% acetonitrile for 10 min. The gel pieces were dehydrated in 0.1 ml of acetonitrile for 10 min and air-dried. The dried gel pieces were swollen for 10 min in 0.1 ml of 10 mM DTT and 50 mM ammonium bicarbonate, and incubated for 1 h at 56°C. They were then dehydrated again with acetonitrile as above, swollen in 50 mM iodoacetamide and 50 mM ammonium bicarbonate, and incubated at RT for 45 min.

Excess DTT and iodoacetamide were removed by washing twice in 0.1 ml of 50 mM ammonium bicarbonate, and then they were dehydrated yet again with acetonitrile. To the dried gel pieces, 2–4 μl of trypsin (16 μg/ml, proteomics sequencing grade, Sigma-Aldrich, Tokyo, Japan) in 10 mM ammonium bicarbonate were added. After the trypsin solution was completely absorbed into the gel pieces, the minimum amount of 25 mM ammonium bicarbonate needed to just cover the gel pieces was added, and the gel pieces were incubated for 16 h at 37°C in an air incubator.

The tryptic peptides were extracted twice with 40 μl of acetonitrile/water/trifluoroacetic acid (66:33:0.1, by volume) in a sonicator for 10 min. The gel pieces were finally dehydrated with 30 μl of acetonitrile for 15 min, and then recovered and combined with the extract. The combined extracts were dried for 2 h at 50°C in a vacuum centrifuge.

Mass spectral data for tryptic peptides were obtained with a MALDI-TOF MS (AXIMA-CFR, Shimadzu Biotech, Kyoto, Japan) used in positive ion reflection mode. α-Cyano-4-hydroxycinnamic acid (99% grade, Sigma-Aldrich) was used as a matrix throughout the experiment. The mass axis was calibrated using the following authentic peptides (Sigma-Aldrich) as external standards: bradykinin 1–7 with a monoisotopic mass of 757.3997; angiotensin II of mass 1046.5423; $P_{14}R$ (synthetic peptide) of mass 1533.8582; human ACTH (18–39) of mass 2465.1989. The mass spectra were processed with Kompact version 2.3.2 in order to obtain accurate monoisotopic peaks. Blank gel experiments were performed to remove those masses derived from the trypsin, the matrix, as well as other known (typically keratins) and unknown contaminants. The corrected mass lists were used as peptide mass fingerprinting data for database searches. The Mascot search engine (Matrix Science, Tokyo, Japan) was used to identify protein spots by searching in entries under the *Homo sapience* category of SwissProt and NCBI-nr databases, based on the assumptions that peptides are monoisotopic, partially oxidized at methionine residues, and carbamidomethylated at cysteine residues. The window of error was set to up to 0.4 Da for monoisotopic data, allowing the peptide mass values to be matched. Proteins matched with a probability value of lower than 0.05, as indicated by probability-based MOWSE values, are reported in this study.

Results

Serum levels of antiendothelial cell antibody: anti-AECA in MCTD

We examined the serum AECA activity in 23 patients with MCTD using the HGEC cellular ELISA. As shown in Fig. 1, AECA titers in seven normal adults were determined. The mean OD_{490} was 0.289 ± 0.069. OD values exceeding 0.42, corresponding to the mean+2SD of normal controls, were defined as positive values. Sera from 12 patients (52%) were positive for anti-HGEC antibody activity. We also examined antibody activity against HGECs by immunofluorescence. Sera from normal controls were negative for anti-HGEC antibody activity (Fig. 2a). Among 23 patients, the sera from 16 patients (69.6%) showed distinct antibody activity for HGECs, as shown in the representative immunofluorescence picture (Fig. 2b).

Reactivity of serum AECA from MCTD patients with HGECs and HMCs in Western blotting

To identify the proteins responsible for the AECA positivity in the MCTD patient sera, we performed SDS gel electrophoresis and then Western blotting using HGECs and HMCs as antigens. As shown in Fig. 3, Western blotting showed a few positive bands for the normal control sera, but many of the positive bands from HGEC and HMC proteins reacted with sera from the MCTD patients. Representative patterns of immunoblotting proteins with sera from four MCTD patients revealed that the most prominent protein bands for MCTD patients were the 30, 36, and 66 kDa bands in the HGECs and HMCs. The ~30 kDa band in HGECs was positive in patients 1 and 2 but negative in patients 3 and 4. The 66 kDa band in both HGECs and HMCs was positive in patients 1, 2 and 4, but negative in patient 3.

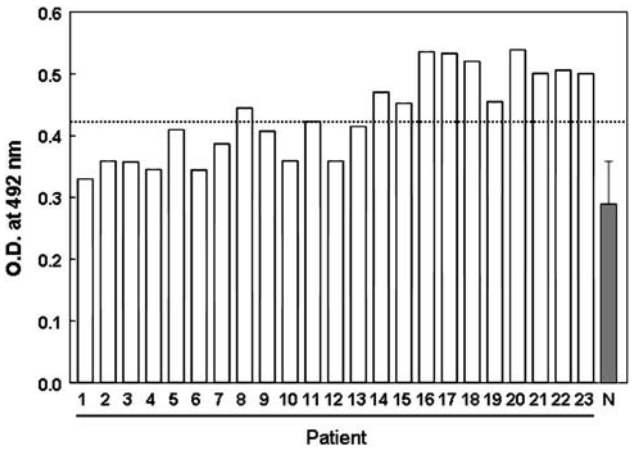


Fig. 1 Serum levels of antibody to HGECs in MCTD patients, as obtained by HGEC cellular ELISA: values of optical densities are expressed as means ± SD from four duplicates per serum. The value for sera from normal adults ($n = 7$) was 0.289 ± 0.069; values higher than 0.427 (mean + 2SD of normal adults) were considered to be positive

We have focused here on the protein with a MW of 36 kDa (hereafter abbreviated to "p36"), which was positive with HGECs in all four patients, but apparently negative for HMCs in patients 3 and 4. Although the data are not shown here, Western blotting using cultured human umbilical vein endothelial cells revealed that p36 was positive in patients 1 and 2 but negative in patients 3 and 4. Nineteen of the sera from the 23 (82.3%) MCTD patients exhibited reactivity against p36 (data not shown).

Characterization of the protein p36 that reacts with AECA from MCTD patients using the proteomics procedure

Two-dimensional gel electrophoresis of lysates of cultured HGECs allowed a much more precise pattern of protein spots to be obtained when presented on the 7-cm, linear pH 3–10 IPG strips after isoelectric focusing (Fig. 4a). Four protein spots with MWs of about 36 kDa were detected in two-dimensional immunoblotting using the MCTD patient sera (Fig. 4b). The protein spot image was overlaid with the immunoblotting image, which was then converted to a pseudocolored image to produce a merged gel image (Fig. 4c). One of the four immunostaining spots, with an IP of around 9, was identified by both silver staining and immunoblotting.

When we compared the merged images and the silver-stained gels, a spot with a MW of 36 kDa and an IP of around 9 was visualized (Fig. 5a), corresponding to the marked spot in the silver-stained gel image (Fig. 5c). In the merged image, with normal control serum, no spot was visible at the corresponding site (Fig. 5b). Furthermore, we confirmed that the targeted spot specifically reacted with anti-VDAC1 antibody when two-dimensional immunoblotting was used, as shown in Fig. 5d. This spot was excised and then processed for identification by mass spectrometry. Mass fingerprinting analysis using the MALDI-TOF mass spectrometer identified the protein spot as the voltage-dependent anion-selective channel 1 protein (VDAC-1, IP00216308) with 11 peptide matches: sequence coverage was 43% and no other significant hits were found (Fig. 6).

Discussion

AECA is a heterogeneous group of antibodies that are directed against phospholipids and/or proteins on

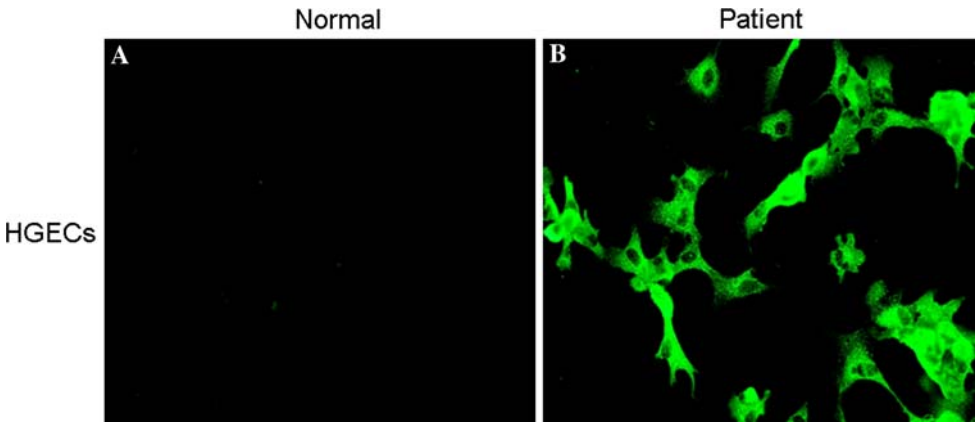


Fig. 2 Antibody reactivity in sera from MCTD patients, obtained by immunofluorescence using HGECs. **a** Indirect immunofluorescence studies showed that sera from normal controls were negative for anti-HGEC antibody activity. **b** Patient's serum showed positive cytoplasmic staining in a finely homogeneous pattern, in addition to weak membrane staining

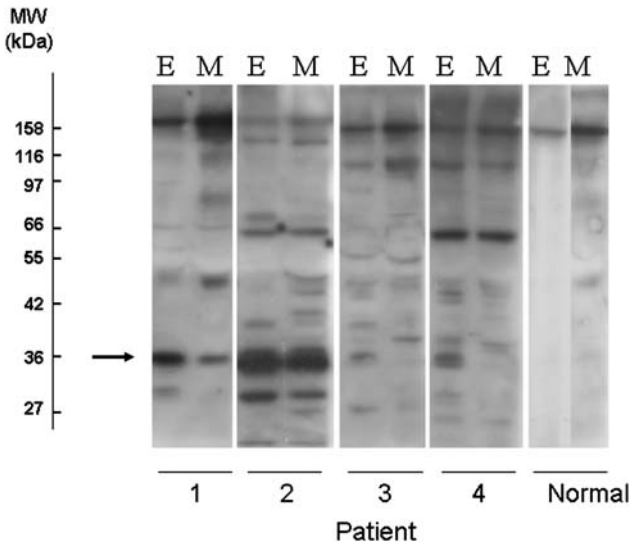


Fig. 3 Reactivity of AECA-positive sera to cell proteins from HGECs (*E*) and HMCs (*M*) on Western blotting; cell lysates from HGECs and HMCs were subjected to SDS-PAGE. Western blotting using AECA-positive sera from MCTD patients showed positive bands for HGECs and HMCs, particularly at 27, 36, and 66 kDa. All sera from four patients with MCTD were positive for the protein with 36 kDa obtained from HGECs, but sera from patients 3 and 4 were apparently negative for the protein with 36 kDa obtained from HMCs. Normal control sera were negative for this protein

endothelial cells [12–14]. The pathogenic role of AECA in endothelial cell injury is still unclear. In MCTD, deterioration of the vasculature may determine the disease course. Obliterative vasculopathy-associated endothelial cell proliferation is a characteristic complication of MCTD [2, 15–17]. There was a close association between serum AECA levels and plasma endothelin-1 levels in MCTD [2]. MCTD sera with AECA activity may show complement-dependent injury to the vascular endothelial cells [2, 12, 18, 19]. However, molecular characterization of the target antigen responsible for AECA has still not been done.

In this study, we first screened the AECA-positive sera of MCTD patients by cellular ELISA and immunofluorescence. AECA-positive sera were examined by immunoblotting, in which cell lysates from HGECs and HMCs were used as cell-associated antigens. Immunoblotting with sera from MCTD patients revealed that the representative protein bands that reacted with the AECAs were the 30, 36, and 66 kDa bands in HGECs and HMCs. For the 66 kDa band, sera from three of four patients were positive not only for HGECs but also for HMCs. In addition, the ~30 kDa band for HGECs was positive in only half of the MCTD patients. Therefore, we focused on a protein with a MW of 36 kDa that was obtained from the HGECs, which preferentially reacted with MCTD sera showing AECA activity but not with sera from healthy volunteers. In our preliminary examination, Western blotting showed that the sera from patients 1 and 2 but not those from patients 3 and 4 reacted with the 36 kDa protein band for cultured human umbilical vein endothelial cells (data not shown).

We prepared an HGEC-derived protein map using 2-D electrophoresis and an IPG strip. Immunoblotting analysis showed that AECA-positive sera from MCTD patients were reactive with four main spots of the 36 kDa proteins derived from HGECs that had isoelectric points ranging from about 5 to 9. Finally, state of the art proteomics technology using peptide mass fingerprinting revealed that one of these protein spots was human VDAC1 (MW 36 kDa and isoelectric point 8.9) given its high coincidence of 43% of the amino acid sequence. In addition, this protein reacted with the anti-VDAC1 antibody.

VDAC (voltage-dependent anion-selective channel), a protein approximately 35 kDa in size, was originally identified by Schein and colleagues in 1976 as being the pore-forming protein responsible for this characteristic permeability [20]. The pore formed through the outer mitochondrial membrane (OMM) by VDAC is almost

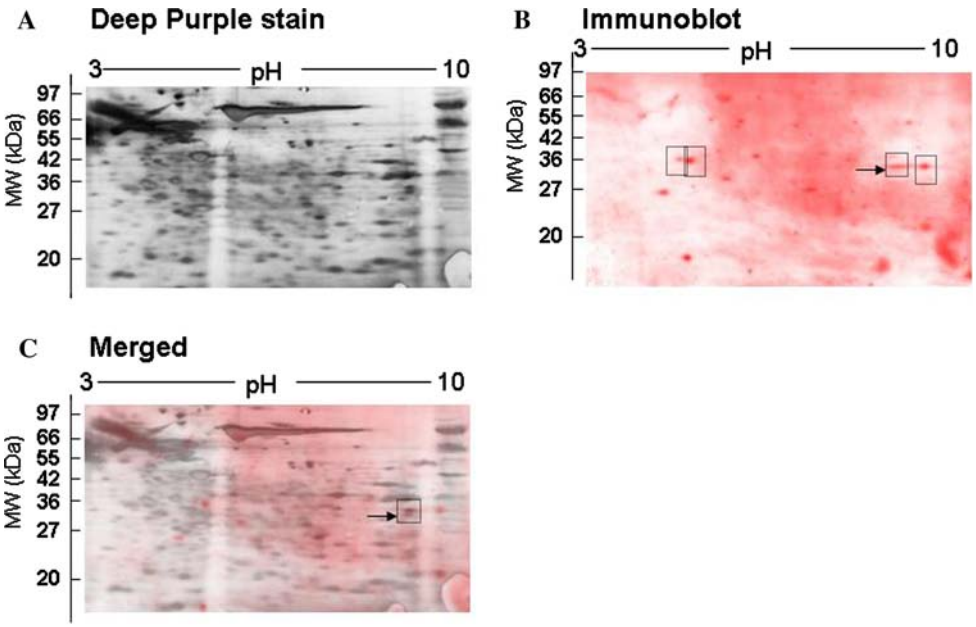


Fig. 4 Identification of the specific antigen reacting with AECA using the proteomics method. **a** Many protein spots were observed upon 2-D gel electrophoresis of the cultured HGECs after isoelectric focusing on the 7-cm, linear pH 3–10 IPG strips. **b** Two-dimensional immunoblotting with MCTD patient serum showed four protein spots at 36 kDa, with a large range of isoelectric points. **c** Overlaying the protein spots from **a** with the immunoblotting image from **b** revealed that one of four protein spots was a 36 kDa protein with a high IP

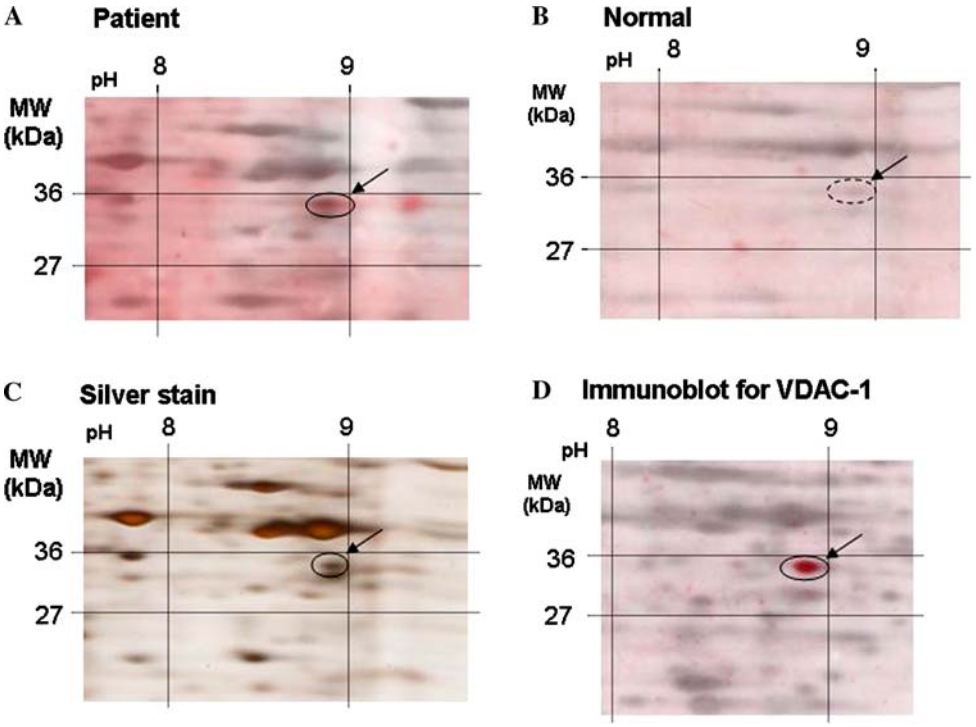
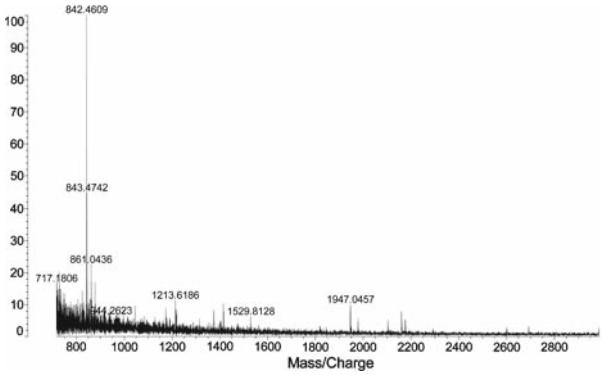


Fig. 5 Further purification of the target 36 kDa protein spot with high IP. **a** Merged image of the deep purple-stained PVDF membrane and the ECL-plus processed immunoblotting image for AECA-positive serum from a patient with MCTD. **b** A positively stained spot with a MW of 36 kDa and an IP of about nine was clearly demonstrated, while the spot was not observed with normal control serum. **c** A silver-stained gel image of the same sample separated under the same conditions. **d** Immunoblotting analysis clearly demonstrated that the anti-VDAC1 antibody reacted specifically with the protein spot that also reacted with AECA-positive serum from a MCTD patient

freely permeable to low molecular weight molecules [21]. VDAC1 (isoform 1) is also found in the plasma membrane and was shown to be identical to NADH-ferricyanide reductase, which may be involved in the maintenance of cellular redox homeostasis [22]. Regulating this enzyme will impact on cancer and autoimmune diseases, as well as neurodegenerative diseases from the viewpoint of apoptosis [21].

In this work we report for the first time that serum antibody to VDAC1 expressed on HGECs is found in MCTD patients. The modern proteomics technology used in this study was shown to be useful for identifying antigens that react with AECA in autoimmune diseases such as MCTD. Future studies will be needed to clarify the disease specificity and the pathogenic roles of VDAC1 itself and the autoantibody to it in MCTD.

A **Mass spectrum of 36-kDa protein with pI of around 9**



B **Identification of VDAC-1 by peptide mass fingerprinting**

Search item	Search result	Search item	Search result
Protein match at 1st rank	Volage-dependent anion-selective channel protein 1 (VDAC1)	Matched peptides	9
		Sequcence coverage	43 %
		Observed Mw	36-kDa
Accesssion #	IPI100216308	Observed pI	8.9
Mowse score of VDAC-1	114	Calculated Mw of VDAC-1	30.6-kDa
Mowse score of protein match at 2nd rank	32	Calculated pI of VDAC-1	8.63

Fig. 6 Analysis of the target protein spot by mass spectrometry. **a** The target spot shown in Fig. 5d was removed and examined by a peptide mass fingerprinting method with a MALDI-TOF MS. **b** Analysis of our protein mass data using the Mascot search engine showed that the protein spot possessed 43% identity with the amino acid sequence of VDAC1, which has a molecular weight of 36 kDa and an isoelectric point of 8.9

Acknowledgment The authors acknowledge the critical review and valuable comments provided by Dr. S. Batsford, Ph.D., Institute of Medical Microbiology and Hygiene, Freiburg University, Germany. This work was supported by research grants (B:15390266,C:12671032, to TO) from the Ministry of Education, Science, Sports and Culture, and a grant for The Promotion of Niigata University Research Projection.

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