sine myositis) as a distinctive subset within the idiopathic inflammatory dermatomyopathies spectrum of clinical illness? J Am Acad Dermatol 2002;46: 626–36.

11 Bohan A, Peter JB, Bowman RL, Pearson CM. Computer-assisted analysis of 153 patients with polymyositis and dermatomyositis. Medicine 1977;56:255–86.

12 Chinoy H, Fertig N, Oddis CV, Ollier WE, Cooper RG. The diagnostic utility of myositis autoantibody testing for predicting the risk of cancer-associated myositis. Ann Rheum Dis 2007;66:1345–9.

13 Watanabe A, Kodera M, Sugiura K et al. Anti-DFS70 antibodies in 597 healthy workers. Arthritis Rheum 2004; 50:892–900.

14 Ishikawa A, Muro Y, Sugiura K, Akiyama M. Development of an ELISA for detection of autoantibodies to nuclear matrix protein 2. Rheumatology 2012;51:1181–7.

15 Muro Y, Sugiura K, Hoshino K, Akiyama M. Disappearance of anti-MDA-5 autoantibodies in clinically amyopathic DM/interstitial lung disease during disease remission. Rheumatology 2012;51:800–4.

16 Shimono Y, Murakami H, Kawai K et al. Mi-2 beta associates with BRG1 and RET finger protein at the distinct regions with transcriptional activating and repressing abilities. J Biol Chem 2003;278:51638–45.

17 Moser JJ, Chan EK, Fritzler MJ. Optimization of immunoprecipitation-western blot analysis in detecting GW182-associated components of GW/P bodies. Nat Protoc 2009;4:674–85.

18 Gunawardena H, Betteridge ZE, McHugh NJ. Myositis-specific autoantibodies: their clinical and pathogenic significance in disease expression. Rheumatology 2009;48:607–12.

19 Nilasena DS, Trieu EP, Targoff IN. Analysis of the Mi-2 autoantigen of dermatomyositis. Arthritis Rheum 1995;38: 123–8.

20 Ramírez J, Hagman J. The Mi-2/NuRD complex: a critical epigenetic regulator of hematopoietic development, differentiation and cancer. Epigenetics 2009;4: 532–6.

21 Schultz DC, Friedman JR, Rauscher FJ 3rd. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes Dev 2001;15: 428–43.

often unnecessary as lesions typically develop rapidly and resolve spontaneously. Systemic steroids are the most helpful therapeutic modality. Reported pediatric dosages of prednisone therapy for Wells syndrome range from 1 to 2 mg/kg/d.[4]

To our knowledge only one report associates Wells syndrome to parvovirus B19 as a potential trigger factor.[5] This was an 8-year-old boy who developed firm, flesh-colored micropapular lesions of both arms and a slapped-cheek appearance on the face. Our patient could possibly be the first case with the association Wells syndrome and parvovirus B19 in a child younger than 24 months. Fortuitous associations cannot be excluded and we lacked the detection of the parvovirus B19 genome by polymerase chain reaction in the biopsy specimen to confirm the diagnosis.

Hugo Barreiros, MD, Diogo Matos, MD, Constança Furtado, MD, Henriqueta Cunha, MD, and Elvira Bártolo, MD

Department of Dermatology, Hospital Garcia de Orta, Almada, Portugal

Funding sources: None.

Conflicts of interest: None declared.

Correspondence to: Hugo Barreiros, MD, Department of Dermatology, Hospital Garcia de Orta, Avenida Torrado da Silva, Pragal, 2801-951 Almada, Portugal

E-mail: hbarreiros@gmail.com

REFERENCES

1. Wells GC. Recurrent granulomatous dermatitis with eosinophilia. Trans St Johns Hosp Dermatol Soc 1971;57:46-56.
2. Gilliam AE, Bruckner AL, Howard RM, Lee BP, Wu S, Frieden IJ. Bullous "cellulitis" with eosinophilia: case report and review of Wells' syndrome in childhood. Pediatrics 2005;116:149-55.
3. Moon HS, Park K, Lee JH, Son SJ. Eosinophilic cellulitis in an infant. Int J Dermatol 2010;49:592-3.
4. Anderson CR, Jenkins D, Tron V, Prendiville JS. Wells' syndrome in childhood: case report and review of the literature. J Am Acad Dermatol 1995;33:857-64.
5. Toulon A, Bourdon-Lanoy E, Hamel D, Fraitag S, Leruez-Ville M, de Prost Y, et al. Wells' syndrome after primoinfection by parvovirus B19 in a child. J Am Acad Dermatol 2007;56(Suppl):S50-1.

## Autoantibodies to nuclear matrix protein 2/MJ in adult-onset dermatomyositis with severe calcinosis

To the Editor: A 73-year-old woman presented with erythematous eruptions on the face, eyelids, and front of chest. The patient also showed multiple subcutaneous nodules on the lower aspect of her back, the buttocks (Fig 1), and the front of her right thigh. She had concurrent proximal weakness of muscles in the thigh. She did not have skin sclerosis anywhere on the body. Laboratory investigations revealed increased levels of serum creatine kinase and serum myoglobin, and the existence of circulating antinuclear antibodies. Circulating anticentromere antibodies were detected in the patient's serum by anti-centromeric protein-B enzyme-linked immunosorbent assay. Histopathological examinations of a biopsy sample from the right quadriceps femoris muscle revealed infiltration of lymphocytes in the muscle. Lung function tests revealed no apparent abnormalities. The patient underwent a series of investigations to exclude underlying malignancy, including computed tomography imaging of the chest and pelvis, and esophagogastroduodenoscopy. No internal malignancy was detected, although computed tomography confirmed marked soft-tissue calcification. Histopathologically, one of the calcification nodules showed infiltration of mixed inflammatory cells and focal calcification within the fat. The diagnosis was dermatomyositis (DM) with subcutaneous calcinosis. She was initially treated with oral betamethasone at 1.5 mg/d, which improved the DM symptoms dramatically.

Nuclear matrix protein 2 (NXP-2), also called MJ, is an autoantigen reported in juvenile DM (JDM).[1] We developed an immunoprecipitation assay for anti-NXP-2 autoantibodies using transcription and translation products of recombinant NXP-2. By this newly developed system, anti-NXP-2 autoantibodies were detected in our patient's serum (Fig 2).

In this study, the 73-year-old patient with adult-onset DM had anti-NXP-2 antibodies that had been thought to be JDM specific. Gunawardena et al[1] reported that sera from 37 (23%) of 162 patients with JDM were positive for anti-NXP-2 autoantibodies. To our knowledge, this is the first reported case of adult-onset DM with anti-NXP-2 antibodies.

Calcium deposits develop in 20% to 40% of children with JDM.[2] In contrast, calcinosis is a rare feature in adult-onset DM.[3,4] Anti-NXP-2 antibodies are significantly associated with calcinosis in patients with JDM.[1] Interestingly, the current patient with adult-onset DM and anti-NXP-2 antibodies also showed severe calcinosis, although calcinosis tends to be a rare feature in adult-onset DM, as mentioned above.

Gunawardena et al[1] reported that anti-NXP-2 antibodies were detected exclusively in patients with JDM and not in patients with JDM overlap syndrome or control subjects.[1] No patients who were anti-NXP-2 antibody positive had other myositis-specific autoantibodies.[1] In contrast, it is noteworthy
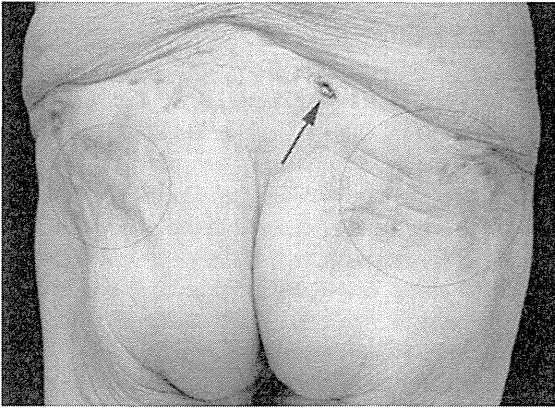
**Fig 1.** Dermatomyositis with calcinosis. Cutaneous findings of buttocks of patient: ulcers (*arrow*) and subcutaneous calcification (*circles*) are observed.
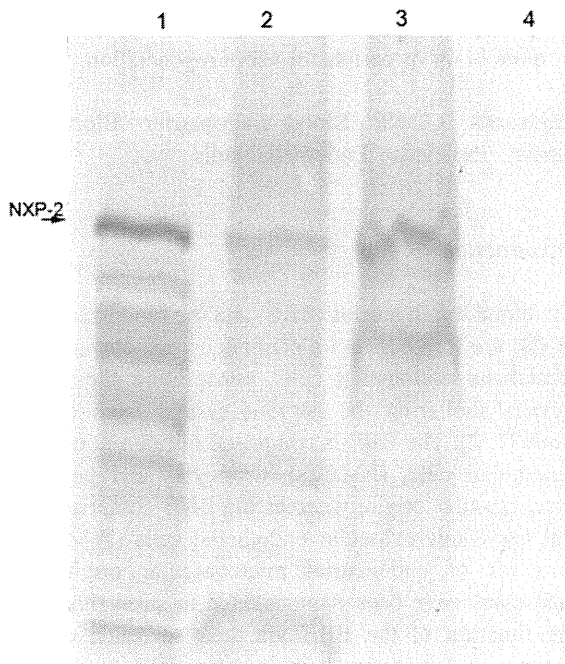


**Fig 2.** Immunoprecipitation assay for nuclear matrix protein 2 (*NXP-2*). Lane 1: recombinant NXP-2 used for assay; lane 2: positive control serum (our manuscript, in preparation); lane 3: patient's serum; lane 4: negative control serum.

that anticentromere antibodies, which are also associated with calcinosis, were concomitantly detected in the current patient. She had none of the other symptoms of CREST syndrome.

This case suggests that anti-NXP-2 antibodies might be a useful marker for calcinosis not only in JDM, but also in adult-onset DM, although we do not have any information on the prevalence of anti-NXP-2 autoantibodies in adult-onset DM to date.

Correlations between the anti-NXP-2 antibodies and calcinosis or other clinical manifestations, including interstitial pneumonia and internal malignancies, in DM should be clarified in the near future.

Kazumitsu Sugiura, MD, PhD, Yoshinao Muro, MD, PhD, and Masashi Akiyama, MD, PhD

Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Correspondence to: Kazumitsu Sugiura, MD, PhD, Department of Dermatology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan.

E-mail: kazusugi@med.nagoya-u.ac.jp

**REFERENCES**

1. Gunawardena H, Wedderburn LR, Chinoy H, Betteridge ZE, North J, Ollier WE, et al. Autoantibodies to a 140-kd protein in juvenile dermatomyositis are associated with calcinosis. Arthritis Rheum 2009;60:1807-14.
2. Pachman LM, Veis A, Stock S, Abbott K, Vicari F, Patel P, et al. Composition of calcifications in children with juvenile dermatomyositis: association with chronic cutaneous inflammation. Arthritis Rheum 2006;54:3345-50.
3. Weinel S, Callen JP. Calcinosis cutis complicating adult-onset dermatomyositis. Arch Dermatol 2004;140:365-6.
4. Kavala M, Sudogan S, Can B, Zindanci I, Kuru I, Beyhan S, et al. An extremely severe case of cutaneous calcinosis complicating adult dermatomyositis. Clin Exp Dermatol 2009;34:115-6.

## Giant nevus lipomatosus cutaneous superficialis with intramuscular lipomatosis

*To the Editor:* Nevus lipomatosus cutaneous superficialis (NLCS) was first described by Hoffmann and Zurhelle[1] in 1921. Clinically it is classified into two clinical subtypes: a multiple form and a solitary form. The lesions are usually found in the flank, buttocks, and upper part of the posterior aspect of the thigh, but they can occur on the abdomen, chest, and face.[2] We report a case of giant NLCS on the buttock associated with diffuse intramuscular lipomatosis within the entire gluteal muscle.

A 38-year-old man presented with an asymptomatic, slowly growing, huge mass on the right buttock

ORIGINAL PAPER

# Pericyte-derived Glial Cell Line-derived Neurotrophic Factor Increase the Expression of Claudin-5 in the Blood–brain Barrier and the Blood-nerve Barrier

Fumitaka Shimizu · Yasuteru Sano ·
Kazuyuki Saito · Masa-aki Abe · Toshihiko Maeda ·
Hiroyo Haruki · Takashi Kanda

**Abstract** The destruction of blood–brain barrier (BBB) and blood-nerve barrier (BNB) has been considered to be a key step in the disease process of a number of neurological disorders including cerebral ischemia, Alzheimer's disease, multiple sclerosis, and diabetic neuropathy. Although glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) facilitate neuronal or axonal regeneration in the brain or peripheral nerves, their action in the BBB and BNB remains unclear. The purpose of the present study was to elucidate whether these neurotrophic factors secreted from the brain or peripheral nerve pericytes increase the barrier function of the BBB or BNB, using our newly established human brain microvascular endothelial cell (BMEC) line or peripheral nerve microvascular endothelial cell (PnMEC) line. GDNF increased the expression of claudin-5 and the transendothelial electrical resistance (TEER) of BMECs and PnMECs, whereas BDNF did not have this effect. Furthermore, we herein demonstrate that the GDNF secreted from the brain and peripheral nerve pericytes was one of the key molecules responsible for the up-regulation of claudin-5 expression and the TEER value in the BBB and BNB. These results indicate that the regulation of GDNF secreted from pericytes may therefore be a novel therapeutic strategy to modify the BBB or BNB functions and promote brain or peripheral nerve regeneration.

## Introduction

The blood–brain barrier (BBB) and the blood-nerve barrier (BNB) are formed from a continuous monolayer of highly specialized endothelial cells, constituting the functional barriers sheltering the nervous system from circulating blood [1, 2]. The BBB is comprised of brain microvascular endothelial cells (BMECs), astrocytes and pericytes of microvascular origin, whereas the BNB comprises peripheral nerve microvascular endothelial cells (PnMECs) and pericytes of endoneurial microvascular origin [1, 3]. Astrocytes have been demonstrated to strengthen the barrier function of the BBB via their secretion of soluble factors, as demonstrated in in vitro BBB models [4, 5], although pericytes have also been shown to have this effect in in vitro BBB and BNB models [6, 7]).

The glia cell line-derived neutrophic factor (GDNF) is a member of the transforming factor-$\beta$ superfamily, and its neurotrophic action is mediated by a unique multi-component receptor system consisting of GDNF-family of receptors (GFR$\alpha$1-4) [8]. Several studies have demonstrated the exogenous administration of GDNF to support long-term neuronal survival, while it also protects and repairs dopaminergic neurons in Parkinson's disease [9, 10], and the motor neurons in amyotrophic lateral sclerosis (ALS) [11, 12]. On the other hand, brain-derived neurotrophic factor (BDNF) is a neurotrophin that promotes the

Special issue: In honor of Dr. Abel Lajtha.

F. Shimizu · Y. Sano · M. Abe · T. Maeda · H. Haruki ·
T. Kanda (✉)
Department of Neurology and Clinical Neuroscience,
Yamaguchi University Graduate School of Medicine,
1-1-1, Minamikogushi, Ube, Yamaguchi 7558505, Japan
e-mail: tkanda@yamaguchi-u.ac.jp

K. Saito
Department of Neurology and Clinical Neuroscience, Tokyo
Medical and Dental Graduate School of Medicine, Tokyo, Japan

🕭 Springer

survival and growth of developing neurons in vitro, and its effects are mediated by the tropomyosin receptor kinase family of receptors (Trk-B) [13].

Astrocytes, the most abundant glial cell type in the brain, have various physiological roles; for example, the maintenance of BBB function and the production of neurotrophic factors, including nerve growth factor (NGF), BDNF, and GDNF [14–16]. Some reports have indicated that the GDNF secreted from astrocytes modulates the barrier function of tight junctions in the BBB and blood-retinal barrier (BRB) [17, 18]. Although we have previously demonstrated that brain and peripheral nerve pericytes secrete several neurotrophic factors including NGF, GDNF and BDNF [7], it remains unclear whether these growth factors modulate the BBB and BNB function. In the present study, we examined the effects of GDNF and BDNF secreted from pericytes on an endothelial cell line which originated from the human BBB and BNB.

**Materials and Methods**

Reagents

The culture medium for pericytes and PnMECs consisted of Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) containing 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 25 ng/ml amphotericin B (Invitrogen, Grand Island, NY, U.S.A), 10% fetal bovine serum (FBS) (Sigma) and 2.5 mM D-glucose (Sigma). Polyclonal anti-claudin-5 and anti-occludin antibodies were purchased from Zymed (San Fransisco, CA, USA). The polyclonal anti-BDNF antibodies were purchased from Calbiochem (Darmstadt, Germany). Polyclonal anti-GDNF antibody was purchased from R&D Systems (Minneapolis, USA). The polyclonal anti-β-tubulin antibody, anti-GFRα antibodies, and anti-Trk-B antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Human astrocytes were purchased from Lonza (Walkersville, MD, USA).

Cell Culture and Treatment

The immortalized human BMECs and PnMECs, and brain and peripheral nerve pericyte cell lines were generated as described previously [7, 19, 20]. The study protocol for human tissue was approved by the ethics committee of the Medical Faculty of the University of Yamaguchi Graduate School and was conducted in accordance with the Declaration of Helsinki, as amended in Somerset West in 1996. Written informed consent was obtained from the families of the participants before they entered the study. The cell lines were isolated from human sciatic nerve and brain tissue, and retained the morphological characteristics of primary brain

and peripheral nerve endothelial cells, or brain and peripheral nerve pericytes, and expressed either endothelial or pericyte markers [7, 19, 20]. The cultures were maintained at 37°C in 5% $CO_2$, and the DMEM medium was replaced every 3 days.

Quantitative Real-time PCR Analysis

Total RNA was extracted from BMECs or PnMECs using an RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). Single-stranded cDNA was created from 40 ng of total RNA using the StrataScript First Strand Synthesis System (STRATAGENE, Cedar Greek, TX, USA). The sequence of each human primer pair and its reference used in the present studies were as follows; sense primer 5'-CTGTTTCCAT AGGCAGAGCG-3' and anti-sense primer 5'-AAGCAGA TTCTTAGCCTTCC-3' for claudin-5 [21]; sense primer 5'-TGGGAGTGAACCCAACTGCT-3' and anti-sense primer 5'-CTTCAGGAACCGGCGTGGAT-3' for occludin [22]; sense primer 5'-GTCAACGGAT TTGGTCTGTATT-3' and anti-sense primer 5'- AGTCT TCTGGGTGGCAGTG AT-3' for G3PDH [23]. The quantitative real-time PCR analyses were performed using a Stratagene Mx3005P instrument (STRATAGENE®, Cedar Greek, TX, USA) with FullVelocity® RSYBR® Green QPCR master mix (STRATAGENE®). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal standard. The samples were subjected to a PCR analysis using the following cycling parameters: 95°C for 10 min, 95°C for 15 s and 60°C for 1 min, for 40 cycles. The standard reaction curves were analyzed using the MxProTM (STRATAGENE®) software program, and the relative quantity according to the standard reaction curve (Rv) was calculated according to the formula Rv = RGene/RGAPDH on a computer.

Western Blot Analysis

The protein samples (10–20 μg) were separated by SDS–PAGE (Biorad) and transferred to nitrocellulose membranes (Amersham, Chalfont, UK). The membranes were treated with blocking buffer (5% skim milk in 25 mM Tris–HCl pH 7.6, with 125 nM NaCl and 0.5% Tween 20) for 1 h at room temperature and incubated with the relevant primary antibodies (dilution 1:100) for 2 h at room temperature. The membranes were then exposed to a peroxidase-conjugated secondary antibody (1:2000) at room temperature for 1 h. The membranes were visualized by enhanced chemiluminescence detection (ECL-plus, Amersham, UK), and recorded on a CCD camera (The Bio-Rad ChemiDoc XRS System. BIO-RAD, Hercules, CA). Quantification of the band intensity was performed using the Quantity One software program (BIO-RAD, Hercules, CA).

Transendothelial Electrical Resistance (TEER) Studies

Transwell inserts (pore size 0.4 μm, effective growth area 0.3 cm², BD Bioscience, Sparks, MD, USA) were coated with rat-tail collagen type-I (BD Bioscience). The TEER values of cell layers were measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM, World Precision Instruments, Sarasota, FL, USA). The BMECs were seeded (1 × 10⁶ cells/insert) on the upper compartment and incubated with each medium (non-conditioned medium used as a control, conditioned medium contained 10% patient sera) for 24 h.

Analysis of the Effects of GDNF or BDNF on the Expression of Tight Junction Molecules in BMECs or PnMECs

BMECs and PnMECs were either left untreated, or were treated with human GDNF (1, 10 ng/ml) or BDNF (1, 10 ng/ml) for 24 h. The total RNA was then extracted, or the TEER value was measured. Total proteins were obtained the next day.

GDNF Inhibitory Study

The conditioned media of brain pericytes (BPCT-CM), of peripheral nerve pericytes (PPCT-CM), or of astrocytes (AST-CM) were pre-treated with 2.0 μg/ml of a neutralizing antibody against GDNF or with normal rabbit IgG (control) for 12 h at 4°C. BMECs and PnMECs were cultured with the GDNF-neutralized- BPCT-CM, PPCT-CM or AST-CM at 37°C. The total proteins were extracted, and the TEER value was measured 2 days later.

Data Analysis

Unless otherwise indicated, all data represent the means ± SEM. An unpaired, two-tailed Student's $t$-test was used to determine the significance of differences between the means of two groups. A $P$ value of <0.01 was considered to be statistically significant.

## Results

The Effects of GDNF or BDNF on the Expression of Tight Junctional Molecules by BMECs and PnMECs

In order to determine the sensitivity of BMECs and PnMECs to GDNF or BDNF, we examined the expression of the GDNF receptor, GFR-α1, and BDNF receptor, Trk-B, in these cell lines using a Western blot analysis. The single band at 51 kDa for GFR-α1 was detected in BMECs, PnMECs, brain and peripheral nerve pericytes, and
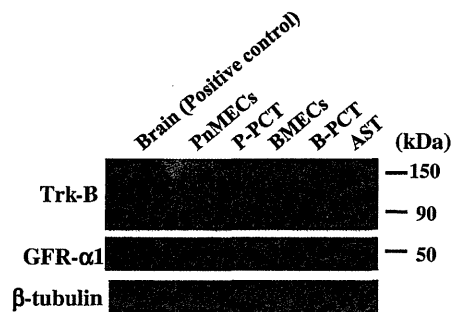


**Fig. 1** A Western blot analysis of GFR-α1 and Trk-B in BMECs, PnMECs, the brain and peripheral nerve pericytes, and astrocytes. Human brain tissues specimens were used as positive controls

astrocytes (Fig. 1). The bands at 145 kDa for Trk-B, corresponding to the full-length tyrosine kinase receptor, were also observed in BMECs, PnMECs, brain and peripheral nerve pericytes, and astrocytes. In contrast, the bands at 95 kDa for the truncated isoform of Trk-B were detected in brain and peripheral nerve pericytes, and astrocytes, although it was not observed in BMECs and PnMECs.

To determine the effects of GDNF or BDNF on the barrier function of the BBB and BNB, we also examined the changes in *claudin-5* or *occludin* expression by BMECs and PnMECs after exposure to GDNF or BDNF by means of relative quantification with a real-time RT–PCR analysis (Fig. 2a–h). The expression of *claudin-5* mRNA in BMECs and PnMECs significantly increased after incubation with GDNF (10 ng/ml) (Fig. 2a, c), whereas it was not affected by incubation with BDNF (Fig. 2b, d). In contrast, the expression of the *occludin* mRNA level in BMECs or PnMECs did not change following treatment with GDNF or BDNF (Fig. 2e–h).

Furthermore, the claudin-5 and occludin protein expression levels in BMECs and PnMECs after incubation with GDNF (1, 10 ng/ml) or BDNF (1, 10 ng/ml) were quantified using a Western blot analysis (Fig. 3a–j). The claudin-5 protein expression was increased after treatment with GDNF (10 ng/ml; Fig. 3c, e), whereas it was not changed by incubation with BDNF (Fig. 3d, e). On the other hand, the expression of the occludin protein expression did not significantly change after treatment with GDNF or BDNF (Fig. 3g–h).

The Changes in the TEER in BMECs and PnMECs after Incubation with GDNF or BDNF

The TEER across the monolayer of BMECs and PnMECs in response to treatment with GDNF or BDNF was measured to determine whether GDNF or BDNF enhances the barrier properties of BMECs and PnMECs (Fig. 4a–d). The TEER value of BMECs and PnMECs increased after
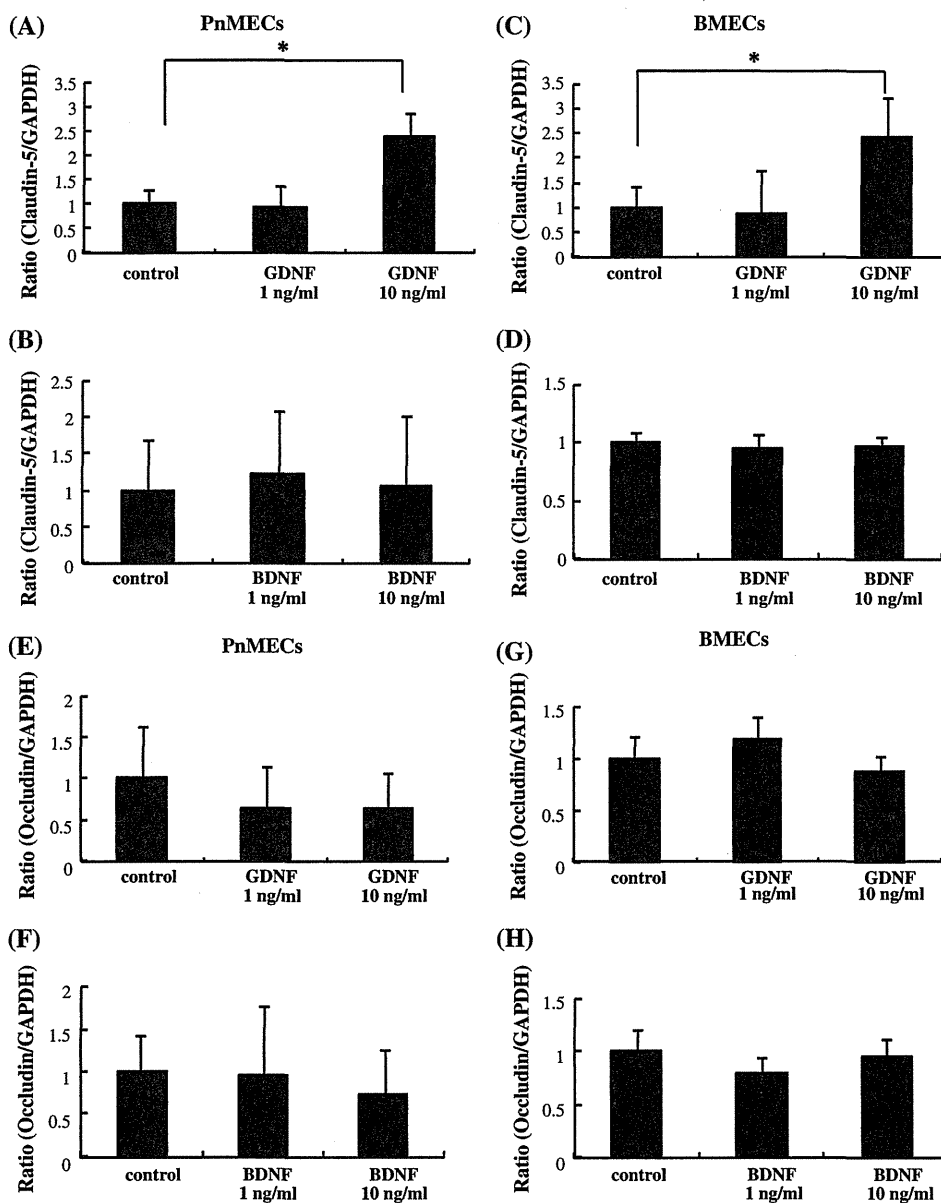
**(A) PnMECs**

**(B)**

**(C) BMECs**

**(D)**

**(E) PnMECs**

**(F)**

**(G) BMECs**

**(H)**

**Fig. 2** The *claudin-5* and *occludin* mRNA level after a 24 h application of GDNF or BDNF in PnMECs (**a, b, e, f**) and BMECs (**c, d, g, h**). The *claudin-5* and *occludin* mRNA levels in BMECs and

PnMECs were quantified by real-time RT–PCR and expressed as the ratio of target gene/GAPDH. Data are presented as the mean (±SEM) of six independent PCR runs

incubation with GDNF (10 ng/ml) (Fig. 4a, b), but there was no change after treatment with BDNF (Fig. 4c, d).

The Effects of the GDNF Neutralizing Antibody on the Induction of claudin-5 and TEER Changes Induced by Brain or Peripheral Nerve Pericyte-conditioned Media

To clarify the contribution of GDNF to the induction of claudin-5 in BMECs and PnMECs by the BPCT-CM, PPCT-CM or AST-CM, the GDNF activities were neutralized

using an anti-GDNF antibody (Figs. 5a–f). The expression of the claudin-5 protein in BMECs was decreased after incubation with BPCT-CM (Fig. 5d) or AST-CM (Fig. 5f) that were pretreated with the anti-GDNF antibody. In addition, the claudin-5 protein expression level was reduced after incubation with PPCT-CM pretreated with the anti-GDNF antibody (Fig. 5b). Furthermore, the TEER value of BMECs was significantly reduced following treatment with BPCT-CM (Fig. 5h) or AST-CM (Fig. 5i) that were pretreated with the anti-GDNF neutralizing antibody. The TEER value of PnMECs was also significantly decreased in PPCT-CM
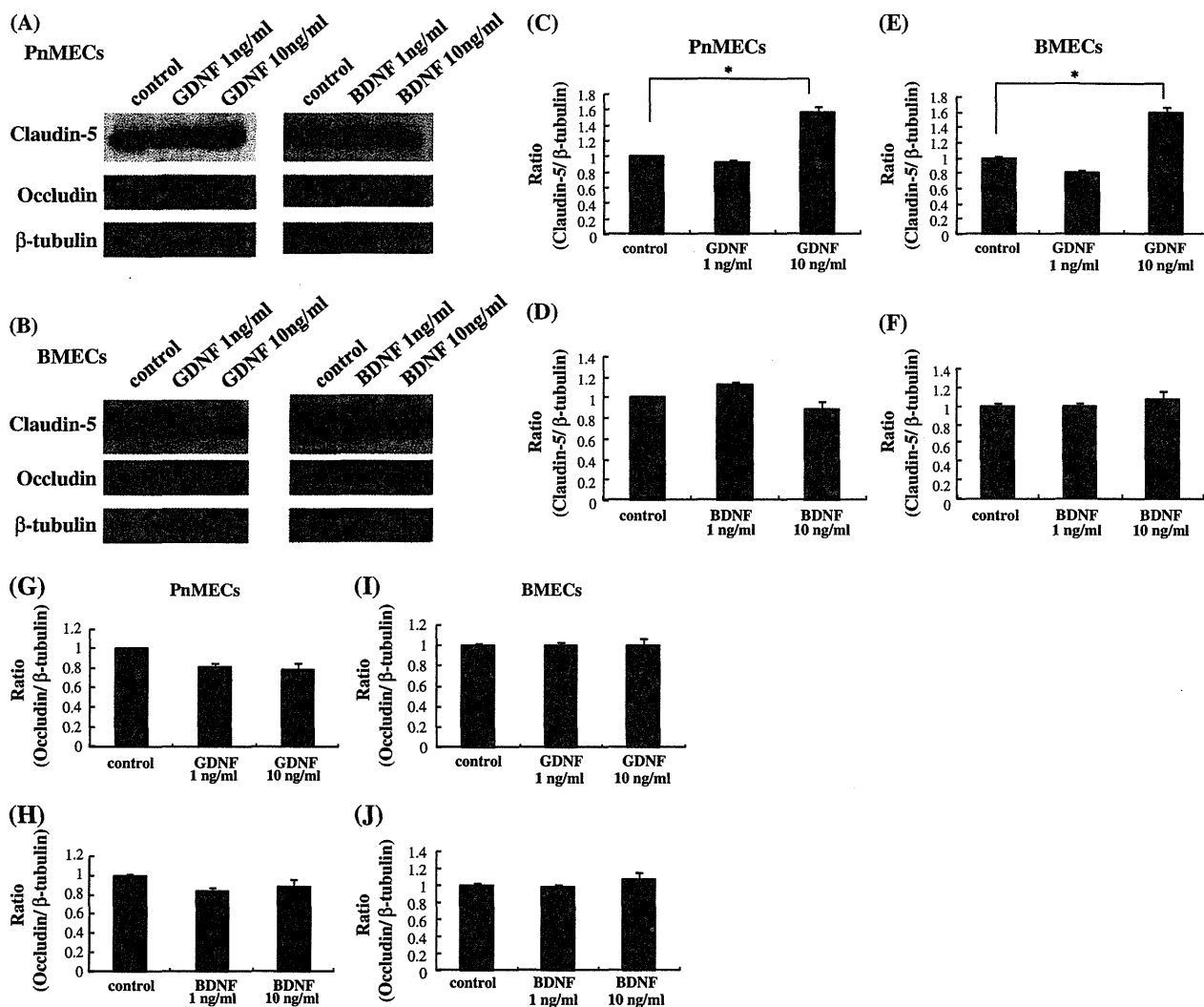
Fig. 3 a The effect of GDNF or BDNF on the claudin-5 or occludin protein expression in PnMECs after a 2-day treatment. b The effect of GDNF or BDNF on the claudin-5 or occludin protein expression in

BMECs. The expression of $\beta$-tubulin was used as an internal standard. The bar graph reflects the combined densitometry data from three independent experiments (mean ± SEM, $n = 3$, *$P < 0.01$)

pretreated with the anti-GDNF neutralizing antibody (Fig. 5g).

## Discussion

In this study, we examined whether GDNF and BDNF could alter the expression amount of tight junction proteins, including claudin-5 and occludin, in the BBB and BNB. Claudin-5 is now recognized as the most important component involved in maintaining BBB function [24] and was reported to be the most abundantly expressed subtype among the claudins in mouse brain capillary endothelial cells at the mRNA level [25]. Several reports have previously demonstrated that the expression of claudin-5 in

BMECs was increased by humoral factors such as adrenomedullin [26] and bFGF [27], and was reduced by VEGF [28]. We also previously demonstrated the expression of claudin-5 in PnMECs to increase by bFGF, while it decreased by VEGF or TGF-$\beta$ [7]. The current results demonstrated that the capillary endothelial cells forming the BBB and BNB express the GDNF receptor, GFR-$\alpha$1, and that GDNF could increase the expression of claudin-5 in BMECs and PnMECs at both the mRNA and protein levels, whereas the BDNF did not have such an effect. Furthermore, the TEER values of BMECs and PnMECs were increased after incubation with GDNF, suggesting that GDNF could enhance the barrier functions of the BBB and BNB. GDNF has been generally accepted as a neurotrophic factor that enhances the survival of dopaminergic

**Fig. 4** The effect of GDNF or BDNF on the TEER values across PnMECs (**a, c**) or BMECs (**b, d**) monolayer (mean ± SD, $n = 5$, *$P < 0.01$)
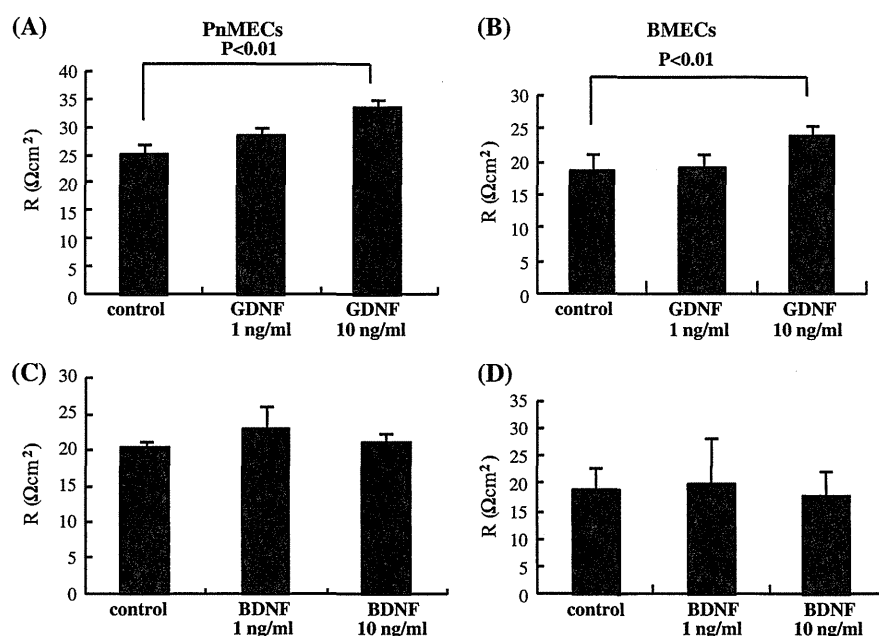


and motor neurons [8]. However, a few reports have recently demonstrated that the capillary endothelial cells forming the BBB and BRB expressed the GDNF receptor, GFR-α1, and that GDNF could modulate the barrier function of the BBB and BRB [17, 18, 29]. The present findings demonstrated that GDNF is a critical factor that enhances the barrier properties of the BBB/BNB.

Recently, the breakdown of the BBB has been considered to be a critical event in the development and progression of several disorders that affect the central nerve system (CNS), including cerebral infarction, Alzheimer's disease, and multiple sclerosis [30–32]. In addition, the breakdown of the BNB has also been reported to be a key initial step in many diseases of peripheral nerve system (PNS), such as Guillain-Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), and diabetic neuropathy [33–36]. This finding suggests that the modification of the integrity of tight junctions in the BBB and BNB may thus provide novel therapeutic avenues for many CNS and PNS diseases. The present study suggested that the intravenous administration of GDNF might thus have a therapeutic potential for repairing and modifying the barrier properties of the BBB and BNB in several CNS and PNS disorders.

Several studies have indicated that astrocytes strengthen the barrier function of BMECs via the secretion of soluble factors in the in vitro BBB model [4, 5], and that GDNF secreted from astrocytes has been reported to modulate the barrier function of BBB and blood-retinal barrier (BRB) [17, 18, 29]. We have previously demonstrated that brain and peripheral nerve pericytes produce GDNF, and that

soluble factors including bFGF secreted from brain and peripheral nerve pericytes strengthen the barrier function of the BBB and BNB by increasing the expression of claudin-5 [7]. We therefore hypothesized that GDNF secreted from brain and peripheral nerve pericytes may contribute to the enhancement of the BBB and BNB function by up-regulating the expression of claudin-5. Our present study demonstrated that the expression of claudin-5 and the TEER value decreased after adding a neutralizing anti-GDNF antibody to the conditioned medium of pericytes, thus indicating that the GDNF secreted from the brain and peripheral nerve pericytes was one of the key molecules responsible for the up-regulation of claudin-5 expression and the TEER value in the BBB and BNB. Several studies have indicated that the exogenous administration of GDNF helps to promote neuronal survival and axonal regeneration in animal models of neurodegeneration disorders, stroke, and peripheral nerve injury. However, neurotrophic factors such as GDNF and BDNF cannot be used for neuroprotection following intravenous administration because the BBB and BNB interrupt the entrance of neurotrophic factor into the CNS and PNS under normal conditions. Several studies have demonstrated various methods for delivering GDNF into the CNS and PNS in animal models, including direct tissue infusion, as well as adenoviral and lentiviral infection [37], however, these methods also have several shortcomings and concerns. On the other hand, the intraputamenal infusion of supraphysiological levels of GDNF have been shown to possibly have a toxic effect in an animal model of Parkinson's disease [38], thus suggesting the importance of physiologically relevant doses of

Fig. 5 Effects of GDNF neutralizing antibody on the induction of claudin-5 and TEER changes induced by brain- or peripheral nerve pericyte-conditioned media. a The PnMECs were cultured with PPCT-CM or PPCT-CM pre-treated with anti-GDNF antibody for 2 days. Claudin-5 protein was inhibited after pretreatment with anti-GDNF neutralizing antibody. b The bar graph reflects the combined densitometry data from thre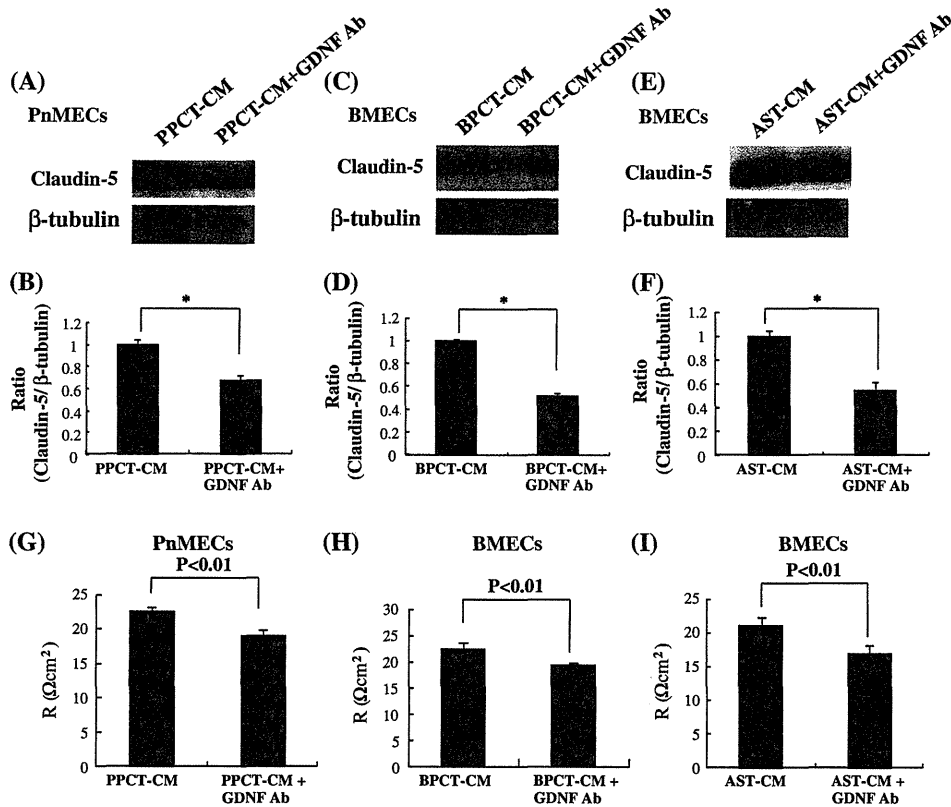e independent experiments (mean ± SEM, $n = 3$, *$P < 0.01$). c, e The BMECs were cultured with BPCT-CM or AST-CM pre-treated with anti-GDNF antibody for 2 days. The claudin-5 protein level decreased after pretreatment with anti-GDNF neutralizing antibody. d, f The bar graph reflects the combined densitometry data from three independent experiments

(mean ± SEM, $n = 3$, *: $P < 0.01$). g, h, i The effect of PPCT-CM, BPCT-CM, or AST-CM on the TEER values across PnMEC or BMECs monolayer. (mean ± SD, $n = 5$, *$P < 0.01$). BPCT-CM, conditioned medium of brain pericytes; PPCT-CM, conditioned medium of peripheral nerve pericytes; AST-CM, conditioned medium of astrocytes. PPCT-CM + GDNF Ab; conditioned medium of peripheral nerve pericytes pre-treated with GDNF neutralizing antibody. BPCT-CM + GDNF Ab; conditioned medium of brain pericytes pre-treated with GDNF neutralizing antibody. AST-CM + GDNF Ab; conditioned medium of astrocytes pre-treated with GDNF neutralizing antibody

GDNF to achieve both safety and efficacy. New studies for the development of better and safer viral vectors or engineered cell lines suitable for the CNS grafting and delivery have been launched [39–41]. Indeed, promising results have been obtained with cell grafts including astrocytes, which have been successfully engineered to deliver GDNF within therapeutic window [41–44]. Pericytes as well as astrocytes are also very promising candidates for new methods for carrying out GDNF delivery, and they may be easily engineered to produce and secrete the most beneficial dose of this neurotrophin in the BBB and BNB. Further investigation to establish the optimal methods to increase the production of endogenous GDNF from brain and peripheral nerve pericytes into the CNS and PNS space within the therapeutic window may therefore be useful for enhancing the barrier property in the BBB and BNB as well

as neuroprotection, and it may have a therapeutic potential for intractable disorders of both the CNS and PNS.

In conclusion, GDNF secreted from the brain or peripheral nerve pericytes strengthened the barrier function of the BBB or BNB by increasing the expression of claudin-5. Further research is therefore necessary to elucidate the molecular mechanisms by which pericytes regulate the BBB and the BNB function under both physiological and pathological conditions in order to establish new therapies for various neurological disorders of the CNS and PNS.

Research Committee) from the Ministry of Health, Labor and Welfare of Japan.

**Conflict of interest** The authors declare that there is no duality of interest associated with this manuscript.

# References

1. Poduslo JF, Curran GL, Berg CT (1994) Macromolecular permeability across the blood-nerve and blood-brain barriers. Proc Natl Acad Sci USA 91:5705–5709
2. Engelhardt B (2010) T cell migration into the central nervous system during health and disease: Different molecular keys allow access to different central nervous system compartments. Clin Exp Neuroimmunol 1:79–93
3. Sano Y, Shimizu F, Nakayama H et al (2008) Endothelial cells constituting blood-nerve barrier have highly specialized characteristics as barrier-forming cells. Cell Struct Funct 32:139–147
4. Hori S, Ohtsuki S, Hosoya K et al (2004) A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. J Neurochem 89:503–513
5. Kim JH, Kim JH, Park JA et al (2006) Blood-neural barrier: intercellular communication at glio-vascular interface. J Biochem Mol Biol 39:339–345
6. Shimizu F, Sano Y, Maeda T et al (2008) Peripheral nerve pericytes originating from the blood-nerve barrier expresses tight junctional molecules and transporters as barrier-forming cells. J Cell Physiol 217:388–399
7. Shimizu F, Sano Y, Abe MA et al (2011) Peripheral nerve pericytes modify the blood-nerve barrier function and tight junctional molecules through the secretion of various soluble factors. J Cell Physiol 226:255–266
8. Sariola H, Saarma M (2003) Novel functions and signalling pathways for GDNF. J Cell Sci 116:3855–3862
9. Gill SS, Patel NK, Hotton GR et al (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med 9:589–595
10. Sherer TB, Fiske BK, Svendsen CN et al (2006) Crossroads in GDNF therapy for Parkinson's disease. Mov Disord 21:136–141
11. Wang LJ, Lu YY, Muramatsu S et al (2002) Neuroprotective effects of glial cell line-derived neurotrophic factor mediated by an adeno-associated virus vector in a transgenic animal model of amyotrophic lateral sclerosis. J Neurosci 22:6920–6928
12. Klein SM, Behrstock S, McHugh J et al (2005) GDNF delivery using human neural progenitor cells in a rat model of ALS. Hum Gene Ther 16:509–521
13. Boyd JG, Gordon T (2003) Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo. Exp Neurol 183:610–619
14. Mizuta I, Ohta M, Ohta K et al (2001) Riluzole stimulates nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor synthesis in cultured mouse astrocytes. Neurosci Lett 310:117–120
15. Wang Y, Chang CF, Morales M et al (2002) Protective effects of glial cell line-derived neurotrophic factor in ischemic brain injury. Ann NY Acad Sci 962:423–437
16. Bruno V, Battaglia G, Copani A et al (2001) Metabotropic glutamate receptor subtypes as targets for neuroprotective drugs. J Cereb Blood Flow Metab 21:1013–1033

17. Utsumi H, Chiba H, Kamimura Y et al (2000) Expression of GFRalpha-1, receptor for GDNF, in rat brain capillary during postnatal development of the BBB. Am J Physiol Cell Physiol 279:361–368
18. Igarashi Y, Chiba H, Utsumi H et al (2000) Expression of receptors for glial cell line-derived neurotrophic factor (GDNF) and neurturin in the inner blood-retinal barrier of rats. Cell Struct Funct 25:237–241
19. Sano Y, Shimizu F, Abe M et al (2010) Establishment of a new conditionally immortalized human brain microvascular endothelial cell line retaining an in vivo blood-brain barrier function. J Cell Physiol 225:519–528
20. Kashiwamura Y, Sano Y, Abe M et al (2011) Hydrocortisone enhances the function of the blood-nerve barrier through the up-regulation of claudin-5. Neurochem Res 36:849–855
21. Varley CL, Garthwaite MA, Cross W et al (2006) PPARgamma-regulated tight junction development during human urothelial cytodifferentiation. J Cell Physiol 208:407–417
22. Ghassemifar MR, Eckert JJ, Houghton FD et al (2003) Gene expression regulating epithelial intercellular junction biogenesis during human blastocyst development in vitro. Mol Hum Reprod 9:245–252
23. Zhang ZL, Liu ZS, Sun Q (2006) Expression of angiopoietins, Tie2 and vascular endothelial growth factor in angiogenesis and progression of hepatocellular carcinoma. World J Gastroenterol 12:4241–4245
24. Nitta T, Hata M, Gotoh S et al (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161:653–660
25. Ohtsuki S, Yamaguchi H, Katsukura Y et al (2008) mRNA expression levels of tight junction protein genes in mouse brain capillary endothelial cells highly purified by magnetic cell sorting. J Neurochem 104:147–154
26. Honda M, Nakagawa S, Hayashi K et al (2006) Adrenomedullin improves the blood-brain barrier function through the expression of claudin-5. Cell Mol Neurobiol 26:109–118
27. Bendfeldt K, Radojevic V, Kapfhammer J et al (2007) Basic fibroblast growth factor modulates density of blood vessels and preserves tight junctions in organotypic cortical cultures of mice: a new in vitro model of the blood-brain barrier. J Neurosci 27:3260–3267
28. Argaw AT, Gurfein BT, Zhang Y et al (2009) VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc Natl Acad Sci USA 106:1977–1982
29. Nishikiori N, Osanai M, Chiba H et al (2007) Glial cell-derived cytokines attenuate the breakdown of vascular integrity in diabetic retinopathy. Diabetes 56:1333–1340
30. Kaur C, Ling EA (2008) Blood brain barrier in hypoxic-ischemic conditions. Curr Neurovasc Res 5:71–81
31. Cirrito JR, Deane R, Fagan AM et al (2005) P-glycoprotein deficiency at the blood-brain barrier increases amyloid-beta deposition in an Alzheimer disease mouse model. J Clin Invest 115:3285–3290
32. Holman DW, Klein RS, Ransohoff RM (2011) The blood-brain barrier, chemokines and multiple sclerosis. Biochim Biophys Acta 1812:220–230
33. Lach B, Rippstein P, Atack D et al (1993) Immunoelectron microscopic localization of monoclonal IgM antibodies in gammopathy associated with peripheral demyelinative neuropathy. Acta Neuropathol 85:298–307
34. Kanda T, Numata Y, Mizusawa H (2004) Chronic inflammatory demyelinating polyneuropathy: decreased claudin-5 and relocated ZO-1. J Neurol Neurosurg Psychiatry 75:765–769
35. Giannini C, Dyck PJ (1995) Basement membrane reduplication and pericyte degeneration precede development of diabetic

polyneuropathy and are associated with its severity. Ann Neurol 37:498–504

36. Shimizu F, Sano Y, Haruki H et al (2011) Advanced glycation end-products induce basement membrane hypertrophy in endo-neurial microvessels and disrupt the blood-nerve barrier by stimulating the release of TGF-$\beta$ and VEGF by pericytes. Diabetologia 54:1517–1526

37. Capowski EE, Schneider BL, Ebert AD et al (2007) Lentiviral vector-mediated genetic modification of human neural progenitor cells for ex vivo gene therapy. J Neurosci Methods 163:338–349

38. Hovland DN Jr, Boyd RB, Butt MT et al (2007) Six-month continuous intraputamenal infusion toxicity study of recombinant methionyl human glial cell line-derivedneurotrophic factor (r-metHuGDNF) in rhesus monkeys. Toxicol Pathol 35:1013–1029

39. Jakobsson J, Lundberg C (2006) Lentiviral vectors for use in the central nervous system. Mol Ther 13:484–493

40. Wong LF, Goodhead L, Prat C et al (2006) Lentivirus-mediated gene transfer to the central nervous system: therapeutic and research applications. Hum Gene Ther 17:1–9

41. Cunningham LA, Su C (2002) Astrocyte delivery of glial cell line-derived neurotrophic factor in a mouse model of Parkinson's disease. Exp Neurol 174:230–242

42. Pertusa M, García-Matas S, Mammeri H et al (2008) Expression of GDNF transgene in astrocytes improves cognitive deficits in aged rats. Neurobiol Aging 29:1366–1379

43. Shen LH, Li Y, Chopp M (2010) Astrocytic endogenous glial cell derived neurotrophic factor production is enhanced by bone marrow stromal cell transplantation in the ischemic boundary zone after stroke in adult rats. Glia 58:1074–1081

44. Laganiere J, Kells AP, Lai JT et al (2010) An engineered zinc finger protein activator of the endogenous glial cell line-derived neurotrophic factor gene provides functional neuroprotection in a rat model of Parkinson's disease. J Neurosci 30:16469–16474

# BRAIN

# Inflammatory myopathies associated with anti-mitochondrial antibodies

Meiko Hashimoto Maeda, Shoji Tsuji and Jun Shimizu

Department of Neurology, University of Tokyo, Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-8655, Japan

Correspondence to: Jun Shimizu, MD, PhD,
Department of Neurology,
University of Tokyo,
Graduate School of Medicine,·7-3-1 Hongo,
Bunkyo-Ku, Tokyo 113-8655,
Japan
E-mail: jshimizu-tky@umin.net

Anti-mitochondrial antibodies, the characteristic markers of primary biliary cirrhosis, have been detected in most patients with this disease. However, the prevalence of these antibodies in inflammatory myopathies and their clinical and histopathological significance has not been determined. Sera from 212 consecutive patients with inflammatory myopathies were screened for anti-mitochondrial antibodies by enzyme-linked immunosorbent assay. The clinical and histopathological features of anti-mitochondrial antibody-positive patients were analysed and statistically compared with those of anti-mitochondrial antibody-negative patients. Twenty-four patients positive for anti-mitochondrial antibodies (seven patients with and 17 patients without primary biliary cirrhosis) were identified (11.3%). Thirteen patients had a clinically chronic disease course of >12 months before their diagnosis at hospitals. Six of these 13 patients (four asymptomatic patients with increased creatine kinase levels and two patients with arrhythmia) had not been aware of muscle weakness, but all 13 patients had muscle atrophy at initial presentation. As complications, eight patients had cardiac involvement including arrhythmias (five patients with supra-ventricular tachycardia; two with ventricular tachycardia; and one patient with atrioventricular block), six patients had moder-ately decreased ejection fraction and six patients had decreased vital capacity, two of whom required respiratory support. Regarding muscle histopathological findings, in addition to inflammation, 13 patients had chronic myopathic changes and six had granulomatous lesions. Statistical analysis showed that the clinical features of a chronic disease course, cardiac involvement and muscle atrophy, and the histopathological features of chronic myopathic changes and granulomatous inflammation, were significantly more frequently observed in patients with anti-mitochondrial antibody-positive inflammatory myopathy than in patients who were negative for anti-mitochondrial antibodies. Except for cardiac involvement, which is more frequently observed in patients with primary biliary cirrhosis, no significant differences in clinical or histopathological features were found between patients with or without primary biliary cirrhosis. Our study revealed that inflammatory myopathies associated with anti-mitochondrial antibodies were frequently found in patients with the clinical features of a chronic disease course, muscle atrophy and cardiopulmonary involvement, and the characteristic histopathological feature of granulomatous inflammation. Our study suggests that inflammatory myopathies associated with anti-mitochondrial antibodies form a characteristic subgroup.

# Introduction

Inflammatory myopathies are a heterogeneous group of autoimmune diseases characterized by progressive muscle weakness and skeletal muscle inflammation. Among them, cases of inflammatory myopathy associated with serum autoantibodies exhibit characteristic clinical features (Love et al., 1991) in accordance with the type of autoantibody. Due to the close association between autoantibodies and characteristic clinical features, these autoantibodies are thought to be important not only as markers of subgroups of inflammatory myopathies, but also as factors involved in the mechanism underlying their pathogenesis (Love et al., 1991; Greenberg and Amato, 2004).

Primary biliary cirrhosis (PBC) is a chronic inflammatory autoimmune disease that mainly targets the cholangiocytes of interlobular bile ducts in the liver. Histopathologically, the hallmark of the disease is a loss of biliary epithelial cells and small intrahepatic bile ducts with the portal infiltration of T cells, B cells, macrophages, eosinophils and natural killer cells (Hohenester et al., 2009). The serological hallmark of the disease is the presence of circulating anti-mitochondrial antibodies (AMAs), which are found in 95% of cases with PBC (Van de Water et al., 1988, 1989; Mutimer et al., 1989; Miyakawa et al., 2001). They act against members of the 2-oxoacid dehydrogenase complexes existing in the inner membrane of mitochondria. Among them, the major autoantigen is the E2-subunit of the pyruvate dehydrogenase complex (PDC-E2). The reactivity of AMAs against other 2-oxoacid dehydrogenase complexes, namely, 2-oxo glutarate dehydrogenase (OGDC-E2) and the branched-chain 2-oxo acid dehydrogenase (BCOADC-E2) is also found at a low frequency (Selmi et al., 2011).

It has been reported that AMAs have a specificity of 98% for PBC when analysed with healthy controls (van de Water et al., 1989); however, there are several case reports showing the associations of PBC or AMA positivity with other autoimmune diseases including systemic sclerosis, Sjögren's syndrome, rheumatoid arthritis (Manthorpe et al., 1979; Berg et al., 1986; Skopouli et al., 1994) and sensory ataxic neuropathy (Charron et al., 1980; Illa et al., 1989; Dahlan et al., 2003; Talwalkar and Lindor, 2003). Thus, the prevalence and the significance of AMAs in autoimmune disease have not yet been studied systematically.

When it comes to the association of PBC with inflammatory myopathies, there have been 23 case reports (Uhl et al., 1974; Benoist et al., 1977; Epstein et al., 1981; Willson, 1981; Tsuchiya et al., 1985; Kumazawa et al., 1987; Saitoh et al., 1988; Ueda et al., 1988; Milosevic and Adams, 1990; Yasuda et al., 1990; Mader et al., 1991; Harada et al., 1992; Varga et al., 1993; Boki and Dourakis, 1995; Simpson and Nickl, 1995; Nakasho et al., 1996; Ono et al., 1996; Tsai et al., 1996; Matsui et al., 2000; Kasuga et al., 2004; Tanaka et al., 2007; Honma et al., 2008), since the first report by Uhl et al. (1974). In these case reports, clinical features including a chronic progressive course (Tsuchiya et al., 1985; Milosevic and Adams, 1990; Harada et al., 1992; Matsui et al., 2000; Kasuga et al., 2004; Tanaka et al., 2007), cardiac involvement (Uhl et al., 1974; Saitoh et al., 1988; Harada et al., 1992; Varga et al., 1993; Tsai et al.,

1996; Kasuga et al., 2004; Tanaka et al., 2007), respiratory muscle weakness (Varga et al., 1993; Matsui et al., 2000; Kasuga et al., 2004; Tanaka et al., 2007) and muscle atrophy (Tsuchiya et al., 1985; Kumazawa et al., 1987; Saitoh et al., 1988; Ueda et al., 1988; Varga et al., 1993; Tsai et al., 1996) were described. Although previous reports suggest some characteristic clinical features of inflammatory myopathies associated with PBC, because of the lack of large-scale and systemic clinical and histopathological studies, the prevalence of PBC in inflammatory myopathies is unknown, and the characteristic clinical and histopathological features of inflammatory myopathy-associated PBC have not yet been clarified.

In this study, we retrospectively reviewed 212 patients with inflammatory myopathies and found 24 patients with AMA-positive myositis (11.3%) (seven patients with and 17 patients without PBC). The analysis of clinical and histopathological features revealed that inflammatory myopathies associated with AMAs frequently include patients with a clinically chronic disease course, muscle atrophy, cardiopulmonary involvement and granulomatous inflammation, regardless of the presence or absence of PBC. Our study suggests that inflammatory myopathies associated with AMAs form a characteristic subgroup.

# Patients and methods

For the screening of patients with AMA-positive myositis, 212 consecutive patients with inflammatory myopathies referred to our department between November 1999 and April 2009 and whose serum samples were available were included in this study. The diagnosis of inflammatory myopathy was based on the criteria proposed by Bohan and Peter (1975a, b); in addition, one or two muscle biopsy findings, namely, inflammatory changes with necrotic and/or regenerating fibres and major histocompatibility complex (MHC) class I expression on non-necrotic muscle fibres (Bohan and Peter, 1975a, b; Hoogendijk et al., 2004) and exclusion of muscular dystrophy by immunohistochemistry were required. For the exclusion of inclusion body myositis, the criteria proposed by Griggs et al. (1995) were used, and sarcoid myopathy was excluded on the basis of clinical data including roentgen findings of the chest (Iannuzzi et al., 2007) and the titre of the angiotensin I-converting enzyme in serum.

The clinical records of the patients were reviewed to obtain clinical information. The clinical criteria established by the American Association for the Study of Liver Diseases (Lindor et al., 2009) were used for the diagnosis of PBC. The disease duration before diagnosis was defined as the duration between the time of the initial awareness of the symptoms and the time of muscle biopsy for histopathological diagnosis. In the assessment of neurological or laboratory studies, the findings at the time of muscle biopsy were used. Muscle power was evaluated using the Medical Research Council scale.

For the detection of AMAs, serum samples taken before the initiation of therapy and stored at −80°C were used. AMA titre was determined by enzyme-linked immunosorbent assay using a MESACUP-2 Test Mitochondria M2 (AMA-M2) kit (Medical and Biological Laboratories) (Kadokawa et al., 2003). In this method, recombinant PDC-E2, BCOADC-E2 and OGDC-E2 antigens are used as coating antigens, and peroxidase-conjugated anti-human immunoglobulin polyclonal antibody is used as a conjugate antibody to enable the capture the immunoglobulin G, M and A class autoantibodies against AMAs. Briefly, 100 μl of a patient's diluted serum was

added to each well of a microtitre plate precoated with recombinant PDC-E2, BCOADC-E2 and OGDC-E2 antigens, and incubated at room temperature for 60 min to allow anti-PDC-E2, -BCOADC-E2 and -OGDC-E2 antibodies to react with immobilized antigens. After washing, a peroxidase-conjugated goat anti-human immunoglobulin polyclonal antibody was dispensed into each well of the plate and incubated at room temperature for 60 min. Following another washing step, a peroxidase substrate was mixed with a chromogen and incubated at room temperature for 30 min. An acid solution ($H_2SO_4$) was then added to each well to terminate the enzyme reaction. The colour development was measured in a microplate reader at a frequency of 450 nm. 'Calibrator 1' (serum of 0 index) and 'Calibrator 2' (serum of 100 index) were also tested similarly. The index value was calculated using the following formula: (absorbance value of test serum − absorbance value of Calibrator 1)/(absorbance value of Calibrator 2 − absorbance value of Calibrator 1) × 100. Using this method, an index value of >7, which was determined using 168 normal control serum samples, was considered to indicate positivity for the antigens of interest with 90% sensitivity and 98% specificity (Takemura et al., 2001; Kadokawa et al., 2003). Myositis-specific/related autoantibodies including anti-Jo-1, -SRP, -Mi-2, -PL-7 and -PM/Scl100 antibodies were detected by the dot-blot method using recombinant Jo-1, SRP, Mi-2, PL-7 and PM/Scl100 (Diarect AG).

In all cases, biopsied muscle samples were processed for routine histochemistry and immunohistochemistry for the MHC class I, MHC class II, CD4, CD8, CD45, CD68 and C5b-9 antigens (DAKO).

During the analysis of clinical and histopathological features, differences between AMA-positive and -negative patients with myositis were compared. The differences were also analysed between AMA-positive myositis patients with PBC and those without PBC.

For the analysis of the correlation between the index AMA titre and clinical features (disease duration before diagnosis, modified Rankin scale and Medical Research Council scale), a simple linear regression was carried out. A two-tailed Mann–Whitney test was performed for different sets of continuous data of clinical features and a Fisher's exact test was used to compare categorical data. A P-value of <0.05 was considered significant.

# Results

## Patients with inflammatory myopathy and anti-mitochondrial antibodies

Twenty-four patients with AMA-positive myositis were identified among 212 consecutive patients with inflammatory myopathies referred to our department. Thus, the prevalence of AMAs in myositis was 11.3%. The clinical and histopathological findings of AMA-positive patients are shown in Table 1. Of the 24 patients, seven, including two with liver histopathological findings consistent with PBC, showed biochemical evidence of cholestasis with an increased alkaline phosphatase level and fulfilled the diagnostic criteria (Lindor et al., 2009) of PBC. Thus, these seven patients were diagnosed as having myositis with PBC and 17 patients were diagnosed as having myositis-associated AMAs without any clinical features of PBC. In the seven patients with PBC, PBC preceded the development of inflammatory myopathies in three patients, whereas PBC and inflammatory myopathies were diagnosed concurrently in four patients. With regard to coexisting diseases

other than PBC, seven patients had inflammatory myopathies associated with collagen diseases: (i) two patients with PBC: Sjögren's syndrome and systemic sclerosis, n = 1; ulcerative colitis, n = 1; (ii) 5 of 17 patients without PBC: systemic sclerosis, n = 2; systemic lupus erythematosus, n = 2; rheumatoid arthritis, n = 1; and (iii) three patients had inflammatory myopathies associated with malignancies: colon cancer, n = 1; tongue cancer, n = 1; stomach and colon cancer, n = 1, two within 1 year of admission and one concurrently.

Of the 24 myositis patients with AMA, nine were male and 15 were female. The average age at disease onset was 54 years (range 32–86 years).The average disease duration before diagnosis was 20 months (range 1–60 months) and the disease duration was >12 months in 13 patients (six patients with PBC and seven patients without PBC). The initial symptoms of the 24 patients were muscle weakness or atrophy in 18 patients, arrhythmia in two patients, and no subjective muscle symptoms except for an increased serum creatine kinase level found by chance during medical checkups in four patients.

Regarding neurological findings, all 24 patients, except for one with diffuse muscle weakness (Patient 17), showed muscle weakness with proximal dominance. Thirteen patients showed muscle atrophy (four patients with PBC and nine patients without PBC) and three patients with 24 or 60 months of disease duration showed lordotic posture (Patients 5, 7 and 23). Regarding laboratory findings, serum creatine kinase levels were elevated in all patients ranging from 232 to 15132 IU/l (2322 ± 3121 I/U).

The associated autoantibodies were detected in 10 of 24 patients with myositis with AMAs: (i) in six of seven patients with PBC: anti-nuclear antibody, n = 4; rheumatoid factor, n = 3; anti-Sjögren's syndrome-A antibody, n = 2; anti-Sjögren's syndrome-B antibody, n = 1; (ii) in 14 of 17 patients without PBC: anti-nuclear antibody, n = 12; rheumatoid factor, n = 7; anti-Sjögren's syndrome-A antibody, n = 1; anti-Sjögren's syndrome-B antibody, n = 1.

Muscle CT images were assessed in 13 patients, and 10 patients showed muscle atrophy with proximal dominance, and three patients (Patients 5, 7 and 23) with lordotic posture showed atrophy with fatty changes in paravertebral muscles (Fig. 1).

Regarding cardiac involvements, eight patients (five patients with PBC and three patients without PBC) showed arrhythmias (supraventricular tachycardia, n = 5; ventricular tachycardia, n = 2; atrioventricular block, n = 1), and six patients (four patients with PBC and two patients without PBC) showed a decreased ejection fraction (<50%). In the patients with arrhythmias, two (Patients 7 and 23) were treated by catheter ablation. With regard to respiratory involvements, six patients (two patients with PBC and four patients without PBC) had a vital capacity of <80%. Among them, two patients (Patients 3 and 7) required respiratory support.

Detailed information about the responses to treatments was available in 15 patients (observation period, 35.0 ± 34.8 months; range 3–96 months). Among them, 11 patients were treated with corticosteroids, one patient was treated with azathioprine and three patients refused treatment. In our series, bile acid therapy had been performed in only one patient (Patient 1, three years before the diagnosis of myositis). Thus, it was difficult to know

# Table 1 Baseline characteristics and treatment response for individual subjects

| Patient No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Onset age (years)/sex | 54/F | 72/F | 49/F | 48/M | 54/F | 39/F | 59/M | 67/F | 68/M | 39/M | 44/F | 33/F | 34/F | 86/M | 65/F | 58/F | 67/F | 57/M | 64/F | 57/M | 46/F | 53/M | 54/M | 32/F |
| Disease duration before diagnosis (months) | 11 | 12 | 24 | 24 | 24 | 24 | 60 | 1 | 1 | 2 | 2.5 | 3 | 3 | 5 | 6 | 6 | 10 | 12 | 17 | 24 | 36 | 48 | 60 | 60 |
| Initial symptoms | Mu | C | Mu | Mu | A | Mu | Mu | C | Mu | C | Mu | Mu | Mu | Mu | Mu | C | Mu | Mu | Mu | Mu | Mu | Mu | A | Mu |
| **Clinical signs and symptoms** | | | | | | | | | | | | | | | | | | | | | | | | |
| Weakness (MRC) | | | | | | | | | | | | | | | | | | | | | | | | |
| Neck (flexion) | NE | 3 | 4 | 3 | 4 | 4 | 4 | 4 | 5 | 5 | 4 | 3 | 4 | 5 | 3 | 3 | NE | 5 | 4 | 5 | 3 | 4 | 2 | 5 |
| UE (proximal/distal) | 4/5 | 4/5 | 4/5 | 3/5 | 4/5 | 4/5 | 3/4 | 5/5 | 4/5 | 4/5 | 3/4 | 4/4 | 4/4 | 5/5 | 4/4 | 4/5 | 3/3 | 3/4 | 5/5 | 5/5 | 4/5 | 3/5 | 4/4 | 5/5 |
| LE (proximal/distal) | 5/5 | 4/5 | 4/5 | 4/5 | 4/5 | 4/5 | 3/3 | 4/5 | 4/5 | 4/5 | 3/4 | 4/5 | 4/5 | 4/5 | 3/4 | 5/5 | 3/3 | 4/5 | 4/5 | 4/5 | 4/5 | 4/4 | 4/5 | 4/5 |
| Muscle atrophy | (−) | (−) | (+) | (+) | (+) | NE | (+) | NE | NE | NE | NE | (+) | (+) | NE | (+) | NE | NE | (+) | (+) | NE | (+) | (+) | (+) | (+) |
| Lordotic posture | (−) | (−) | (−) | (−) | (+) | (−) | (+) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (+) | (−) |
| Arrhythmia | AF | (−) | Af, PSVT | AV block | NSVT (PM) | (−) | AF (CA) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | Paf | (−) | (−) | (−) | (−) | Af | (−) | CRBBB, PVC (CA) | (−) |
| Cardiomyopathies[a] | (+) | (−) | (−) | (+) | (+) | (−) | (+) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (+) | (−) | (−) | (−) | (−) | (−) | (−) | (+) | (−) |
| Restrictive ventilatory impairment (vital capacity) | (−) | (−) | (+); NIPPV | (−) | (−) | (−) | (+) (45.8%); NIPPV | NE | (−) | NE | NE | (−) | (−) | NE | (−) | (+) (50.0%) | (+) (68.9%) | NE | (−) | (−) | (−) | (+) (56.0%) | (+) (77.5%) | NE |
| Modified Rankin scale | 1 | 1 | 2 | 2 | 1 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 3 | 2 | 3 | 1 | 1 | 2 | 2 | 1 |
| PBC preceding (duration: months) | (+) 84 | (−) | (−) | (−) | (+) 84 | (+) 10 | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) |
| Diagnosis type of PBC | Asymp. | Asymp. | Asymp. | Asymp. | Asymp. | Symp. | Asymp. | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) |
| **Associated disorders** | | | | | | | | | | | | | | | | | | | | | | | | |
| Collagen disease | Ulcerative colitis | SS, SSc | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | SSc | SLE | SLE | SSc | (−) | (−) | RA | (−) | (−) |
| Malignant disease | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | Colon Ca | (−) | Tongue Ca | (−) | (−) | Stomach Ca, Colon Ca | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) |
| **Laboratory data** | | | | | | | | | | | | | | | | | | | | | | | | |
| CK level (IU/l) | 1085 | 533 | 488 | 3620 | 619 | 1381 | 558 | 232 | 4230 | 15132 | 4000 | 1770 | 4332 | 692 | 1170 | 569 | 5147 | 1143 | 258 | 1844 | 2398 | 280 | 924 | 3325 |
| ESR (mm/h) | 96 | 49 | 104 | 81 | 36 | 71 | NE | 22 | 61 | NE | 42 | 33 | 22 | 34 | 23 | 67 | 83 | 39 | 19 | 61 | 38 | 68 | NE | 29 |
| CRP level (mg/dl) | 0.5 | 0.2 | 1 | 0.5 | 0.49 | 0.9 | 1.4 | 1.6 | 2.7 | 2.6 | 2.3 | 0.3 | 0 | 0.6 | 0.05 | 0.9 | 0 | 1.43 | 0.06 | 0.3 | <0.3 | 0.43 | 0.26 | 0 |
| ALP level(U/l)[b] | 1540* | 292* | 754* | 515* | 381* | 850* | 309* | 192* | 135* | 151* | NE | NE | 220* | NE | 253* | 36* | NE | NE | 122** | NE | 153* | 127* | 179** | NE |
| AMAs (index) | 95.5 | 100.3 | 114 | 117.9 | 119.5 | 25 | 116.2 | 15.8 | 7.4 | 37.6 | 18.1 | 90.8 | 13.4 | 109.4 | 36.7 | 8.6 | 12.8 | 20 | 67.6 | 124.3 | 57.8 | 27.9 | 85.1 | 85.3 |
| Anti-nuclear antibodies | X1280 (sp) | RNP | | | X40 (sp) | X160 (sp) | | X40 (sp, homo) | dsDNA | | | X80 (spe) | X5120 (sp, nucl), RNP | X40 (sp) | | X2560 (sp), Scl70 | dsDNA, RNP | X5120 (sp), Scl70, RNP | X320 (sp, nucl) | centromere | | X320 (sp, nucl), dsDNA | | dsDNA |
| Other autoantibodies | | SS-A, SS-B | | RF | | RF | SS-A, RF | | RF | RF | RF | | | | | | SS-A, SS-B, RF | RF | | | | RF | | RF |
| Myositis-specific/related autoantibodies | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | Jo-1 | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | (−) |
| Liver biopsy | CNSDC | NE | NE | CNSDC | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE | NE |
| **Treatment response** | | | | | | | | | | | | | | | | | | | | | | | | |
| Treatment | (−) | (−) | NI | CS | (−) | NI | AZP | NI | CS | CS | CS | CS | NI | NI | CS | CS | NI | NI | CS | NI | CS | CS | CS | NI |
| Follow-up period (months) | 96 | 36 | NI | 84 | 54 | NI | 12 | NI | 12 | 3 | 3 | 12 | NI | NI | 6 | 90 | NI | NI | 36 | NI | 84 | 12 | 12 | NI |
| Modified Rankin scale change | 1→1 | 1→1 | NI | 2→0 | 1→1 | NI | 3→2 | NI | 1→0 | 1→1 | 1→0 | 1→0 | NI | NI | 2→2 | 2→2 | NI | NI | 3→1 | NI | 1→1 | 2→2 | 2→2 | NI |
| Worsening of arrhythmia | (+) | (−) | NI | (+) | (+) | NI | (−) | NI | (−) | (−) | (−) | (−) | NI | NI | (−) | (−) | NI | NI | (−) | NI | (−) | (−) | (−) | NI |
| CK level (IU/l) after treatment | NE | w.n.l. | NI | w.n.l. | w.n.l. | NI | w.n.l. | NI | w.n.l. | w.n.l. | w.n.l. | w.n.l. | NI | NI | w.n.l. | w.n.l. | NI | NI | w.n.l. | NI | 934 | w.n.l. | w.n.l. | NI |

(continued)

**Table 1 Continued**

| Patient No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Histopathological findings** | | | | | | | | | | | | | | | | | | | | | | | | |
| Endomysial fibrosis | (−) | (±) | (−) | (+) | (++) | (+) | (++) | (−) | (−) | (−) | (−) | (++) | (+) | (+) | (+) | (+) | (+) | (−) | (++) | (−) | (+) | (+) | (++) | (−) |
| Inflammation[c] | (+)e | (±) | (±) | (+++)e | (+++)p | (+)p | (+++)p,e | (±) | (++)p | (−) | (+++)p | (++)e | (+++)e | (+) | (+++)p | (+++)p | (+++)p | (±)p | (++)p | (+)p | (+++)p | (+++)p | (+++)p | (+++)di |
| MHC class I[d] | (−) | (±)f | (++)di | (±)f | (±)f | (−) | (++)di | (±)f | (+++)di | (+++)di | (+++)di | (±)f | (+++)di | (±)f | (−) | (+++)di | (+++)di | (+++)di | (++)di | (+++)di | (±)f | (+++)di | (±)f | (++)di |
| Granulomatous inflammation | (−) | (−) | (−) | (+) CD4 | (+) | (−) | (+) CD4 | (−) | (−) | (−) | (−) | (−) | (+) CD4 | (−) | (−) | (+) | (−) | (−) | (−) | (−) | (−) | (−) | (+) CD4 | (−) |

a Cardiomyopathy was defined when ejection fraction decreased by <50%.
b The normal ranges are 80–260 U/l* and 100–325 U/l*.
c Density of inflammatory cells: −, 0; ±, 1 to <5; +, 5 to <10; ++, 10 to <20; +++, 20 to <100; ++++, ≥100.
d MHC class I antigen on the sarcolemma: −, 0%; ±, 1 to <5%; +, 5 to <50%; ++, 50 to <75%; +++, 75 to <100%.
A = arrhythmia; Af = atrial fibrillation; AF = atrial flutter; ALP = alkaline phosphatase; Asymp. = asymptomatic PBC; AV = atrioventricular; AZP = azathioprine; C = increased serum CK level; Ca = cancer; CA = catheter ablation; CD4 = CD4-positive T cell predominance in granulomatous lesions; CK = creatine kinase; CNSDC = chronic non-suppurative destructive cholangitis; CRBBB = complete right bundle branch block; CRP = C-reactive protein; CS = corticosteroids;
di = diffuse; dsDNA = anti-double-stranded DNA antibody; e = endomysium; ESR = erythrocyte sedimentation rate; f = focal; F = female; homo = homogeneous staining pattern; LE = lower extremities; M = male; MRC = Medical Research Council; Mu = muscle symptoms (weakness or atrophy); NE = not examined; NI = no information; NIPPV = non-invasive positive-pressure ventilation; NSVT = non-sustained ventricular tachycardia; nucl = nucleolar staining pattern;
p = perimysium; Paf = paroxysmal atrial fibrillation; PM = pacemaker; PSVT = paroxysmal supraventricular tachycardia; PVC = premature ventricular contraction; RA = rheumatoid arthritis; RF = rheumatoid factor; RNP = anti-RNP antibody;
Scl70 = anti-Scl70 antibody; SLE = systemic lupus erythematosus; sp = speckled staining pattern; SS = Sjögren syndrome; SS-A = anti-SS-A antibody; SS-B = anti-SS-B antibody; SSc = systemic sclerosis; Symp. = symptomatic PBC;
UE = upper extremities; w.n.l. = within normal limit.

whether bile acid therapy had some association with any change in muscle pathology/function.

Of the 12 patients treated, all except one patient, who was treated with low-dose corticosteroids in accordance with her wishes, showed normalization of creatine kinase levels within 3 months. Improvement of muscle power was observed in six patients and it remained the same in the others. One patient, whose index of AMAs was followed up after initiation of treatment, showed a mild decrease in index, from 85.1 to 21.0 in Patient 23, but two patients did not show a decrease.

In three non-treated patients (Patients 1, 2 and 5), despite no worsening of muscle weakness during the observation period (62.0 ± 30.8; range 36–96 months), two patients (Patients 1 and 5) developed arrhythmia, one of whom (Patient 5) required an implantable pacemaker.

## Histopathological features of patients with inflammatory myopathy that have anti-mitochondrial antibodies

With regard to histopathological studies, in addition to the findings of inflammatory myopathies (necrotic and/or regenerating fibres, n = 23; inflammatory changes, n = 23; positive staining of the sarcolemma with MHC class I, n = 21; variation of muscle fibre size, n = 18), endomysial fibrosis, which suggests a chronic myopathic process, was found in 15 patients. No perifascicular atrophy was observed, and the invasion of non-necrotic muscle fibres by mononuclear cells was observed in one patient (Patient 22). Intriguingly, six patients (three patients with PBC and three patients without PBC) had granulomatous inflammatory lesions and four of them showed CD4-positive T cell predominance over CD8-positive T cells in the lesions (Fig. 2).

## Comparison of clinical and histopathological features between anti-mitochondrial antibody-positive and anti-mitochondrial antibody-negative patients

Table 2 shows a comparison of clinical and histopathological features between AMA-positive and AMA-negative patients, and between AMA-positive patients with myositis with and without PBC.

Regarding clinical features, the AMA-positive patients with myositis had a longer disease duration before diagnosis (P < 0.0005), more frequently showed muscle atrophy at the initial presentation (P < 0.005), showed cardiac involvement (P < 0.005) and less frequently showed skin rash (P < 0.05) than the AMA-negative patients. Regarding histopathological findings, the AMA-positive patients with myopathy more frequently showed variation in muscle fibre size (P < 0.005), endomysial fibrosis (P < 0.01) and granulomatous inflammation (P < 0.01) than the AMA-negative patients. In the AMA-positive patients with myositis, no significant difference was found between the patients with PBC and those without PBC, except that cardiac involvement was more frequently observed in patients with PBC.
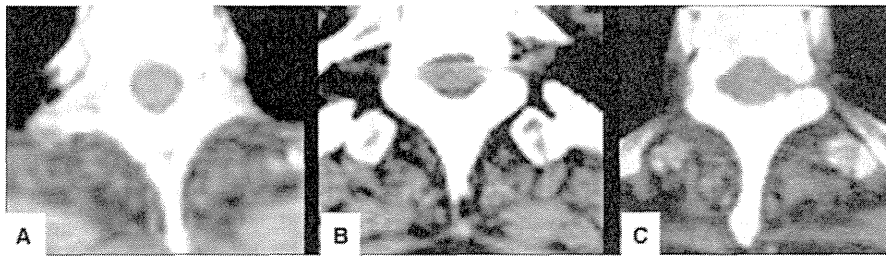
**Figure 1** Muscle CT images of AMA-positive patients with the symptoms of weakness in neck flexion and lordotic posture. Paravertebral muscles at the upper thoracic level showed muscle atrophy and fatty changes in Patient 5 (**A**), Patient 7 (**B**) and Patient 23 (**C**).
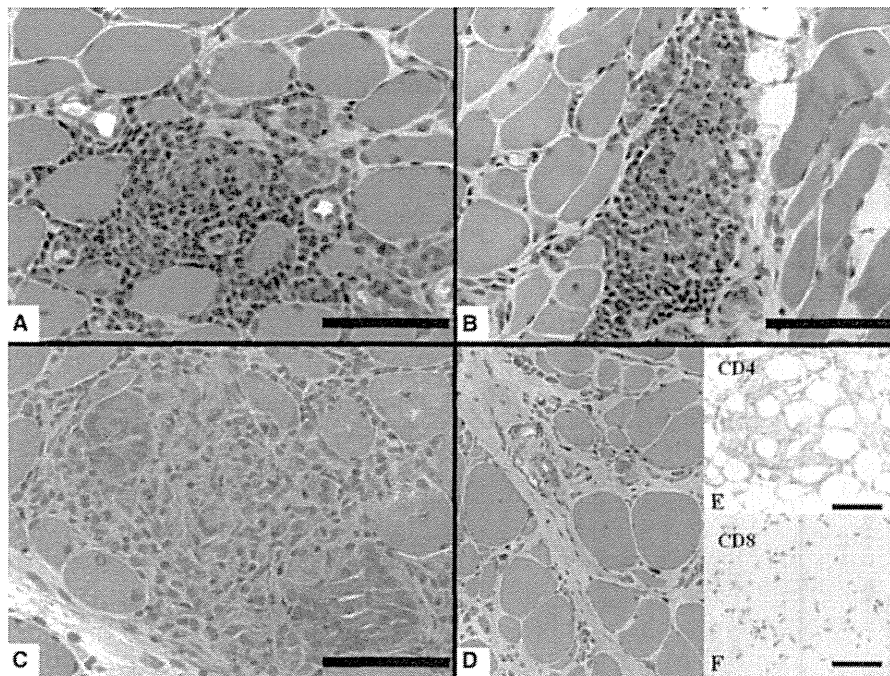


**Figure 2** Muscle histopathology of AMA-positive patients with granulomatous inflammation (Patients 4, 5 and 7). Granulomatous inflammatory changes in the endomysial space are replacing muscle fibres in Patient 4 (**A**), Patient 5 (**B**) and Patient 7 (**C**). Marked fibre variation in size and increased volume of connective tissue in endomysium are also observed in Patient 7 (**D**). Surface marker analysis of infiltrating lymphocytes shows the predominance of CD4-positive lymphocytes compared with CD8-positive lymphocytes in Patient 7 (**E** and **F**). Scale bar = 100 μm.

## Titre of anti-mitochondrial antibodies and clinical features

The indices of the 24 AMA-positive patients ranged from 7.4 to 124.3 (62.8 ± 43.2 index). In 13 patients with a disease duration of 12 months or longer, the index increased significantly (82.0 ± 38.6) in comparison with that of the 11 patients with a disease duration of <12 months (40.5 ± 38.8; $P < 0.05$).

A comparison of clinical and histopathological features between the patients who have high (>80) and low (≤80) indices of AMAs showed that the patients with high indices tend to have cardiomyopathies, arrhythmias and granulomatous inflammation more frequently, but differences between the two groups were not significant. However, five out of six patients with cardiomyopathies, six out of eight patients with arrhythmia and four out of six patients with the histopathological finding of granulomatous inflammation had high indices of AMAs.

In the correlation between the index of AMAs and clinical features, although the disease duration before diagnosis significantly correlated with the index of AMAs ($P < 0.05$), disease severity (modified Rankin and Medical Research Council scales) did not (Fig. 3). There was no correlation between the index of AMAs and severity or activity of PBC.

Table 2 Comparison of clinical and histopathological features between AMA-positive patients versus AMA-negative patients with myositis and AMA-positive patients with PBC versus AMA-positive patients without PBC

| Clinical and histopathological findings | AMA-positive myositis patients (N = 24) | AMA-negative myositis patients (N = 188) | Significance (AMA-positive versus AMA-negative) | AMA-positive myositis patients with PBC (N = 7) | AMA-positive myositis patients without PBC (N = 17) | Significance (PBC[+] versus PBC[−]) |
|---|---|---|---|---|---|---|
| Sex (M:F) | 9:15 | 58:130 | NS | 5:2 | 4:13 | NS |
| Age at disease onset (years) | 54 ± 13 | 55 ± 15 | NS | 54 ± 10 | 54 ± 15 | NS |
| Disease duration before diagnosis (months) | 20 ± 20 | 12 ± 36 | $P < 0.0005$ | 26 ± 17 | 17 ± 21 | NS |
| Clinical signs and symptoms, n/N (%) | | | | | | |
| Severe limb muscle weakness (≤MRC3) | 7/24 (29) | 55/177 (32) | NS | 2/7 (29) | 5/17 (29) | NS |
| Severe neck muscle weakness (≤MRC3) | 7/22 (32) | 51/137 (37) | NS | 2/6 (33) | 5/16 (31) | NS |
| Myalgia | 7/23 (30) | 95/163 (58) | $P < 0.05$ | 2/6 (33) | 5/17 (29) | NS |
| Muscle atrophy | 13/24 (54) | 40/188 (21) | $P < 0.005$ | 4/7 (57) | 9/17 (53) | NS |
| Skin rash | 2/24 (8) | 60/188 (32) | $P < 0.05$ | 0/7 (0) | 2/17 (12) | NS |
| Cardiac involvement[a] | 8/24 (33) | 17/188 (9) | $P < 0.005$ | 5/7 (71) | 3/17 (18) | $P < 0.05$ |
| Restrictive ventilatory impairment[b] | 6/19 (32) | 41/126 (33) | NS | 2/7 (29) | 4/12 (33) | NS |
| Dysphagia | 4/21 (19) | 30/121 (25) | NS | 2/7 (29) | 2/14 (14) | NS |
| Associated disorders, n/N (%) | | | | | | |
| Collagen disease | 7/24 (29) | 43/188 (23) | NS | 2/7 (29) | 5/17 (29) | NS |
| Malignancies | 3/24 (13) | 28/188 (15) | NS | 0/7 (0) | 3/17 (18) | NS |
| Interstitial lung disease | 6/24 (25) | 75/188 (40) | NS | 1/7 (14) | 5/17 (29) | NS |
| Laboratory data | | | | | | |
| CK level (U/l) | 2322 ± 3121 | 3160 ± 8627 | NS | 1183 ± 1126 | 2791 ± 3567 | NS |
| ESR (mm/h) | 51 ± 26 | 41 ± 30 | $P < 0.05$ | 73 ± 26 | 43 ± 20 | $P < 0.05$ |
| CRP level (mg/dl) | 0.8 ± 0.8 | 1.5 ± 4.1 | NS | 0.7 ± 0.4 | 0.8 ± 0.9 | NS |
| AMAs (index) | 62.8 ± 43.2 | 2.4 ± 1.5 | $P < 0.0001$ | 98.3 ± 33.6 | 48.2 ± 38.5 | $P < 0.01$ |
| Histopathological findings, n/N (%) | | | | | | |
| Variation in muscle fibre size | 18/24 (75) | 75/188 (40) | $P < 0.005$ | 7/7 (100) | 11/17 (65) | NS |
| Disruption of myofibrillar architecture | 6/24 (25) | 72/188 (39) | NS | 1/7 (14) | 5/17 (29) | NS |
| Internal nuclei[c] | 9/24 (38) | 59/188 (32) | NS | 5/7 (71) | 4/17 (24) | NS |
| Necrotic and/or regenerating fibres | 23/24 (96) | 168/188 (89) | NS | 7/7 (100) | 16/17 (94) | NS |
| Endomysial fibrosis | 15/24 (63) | 46/188 (25) | $P < 0.01$ | 5/7 (71) | 10/17 (59) | NS |
| Perifascicular atrophy | 0/24 (0) | 20/188 (11) | NS | 0/7 (0) | 0/17 (0) | − |
| Inflammatory cell infiltration[d] | 23/24 (96) | 173/188 (92) | NS | 7/7 (100) | 16/17 (94) | NS |
| Mononuclear cells invading non-necrotic muscle fibres | 1/24 (4) | 8/188 (4) | NS | 0/7 (0) | 1/17 (6) | NS |
| Positive staining of the sarcolemma with MHC class I | 21/24 (88) | 174/188 (93) | NS | 5/7 (71) | 16/17 (94) | NS |
| Granulomatous inflammation | 6/24 (25) | 11/188 (6) | $P < 0.01$ | 3/7 (43) | 3/17 (18) | NS |
| CD4-positive lymphocyte predominance | 4/24 (17) | 10/139 (7) | NS | 2/7 (29) | 2/17 (12) | NS |

a Cardiac involvement was defined as a condition when ejection fraction was decreased by <50% or presence of arrhythmia.
b Restrictive ventilatory involvement was defined as a condition when the vital capacity was <80%.
c Positive finding of internal nuclei was defined as a finding of >5% of muscle fibres associated with internal nuclei.
d Positive inflammatory change was defined as a change when more than five inflammatory cells infiltrated the perivascular or endomysium in the muscle tissues.
CK = creatine kinase; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; F = female; M = male; MRC = Medical Research Council; NS = not significant.
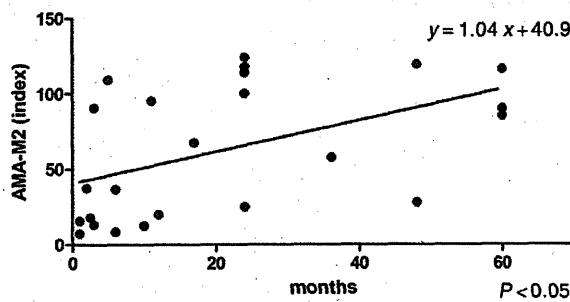
**Figure 3** Correlation between titre of AMAs and disease duration before diagnosis. The index of AMA-M2 significantly correlated with the disease duration before diagnosis ($P < 0.05$).

## Discussion

The association of PBC with inflammatory myopathies has been reported mainly as case reports, and comprehensive studies of the prevalence and the clinical and histopathological features of patients with inflammatory myopathies and AMAs or PBC have not been conducted thus far. In this study, we retrospectively reviewed 212 patients with inflammatory myopathies, and found 24 patients with AMAs (11.3%) and seven patients with PBC (3.3%). By analysing the clinical and histopathological features, we found that AMA-positive patients with inflammatory myopathies frequently include patients with a clinically chronic disease course, muscle atrophy, cardiac involvement and granulomatous inflammation regardless of the presence or absence of PBC. Although the comparative analysis did not show a significant difference, in our 24 patients with AMAs, two patients required respiratory support because of restrictive ventilator impairment, which is unusual for typical cases of inflammatory myopathies.

Considering the characteristic clinical features and the significant correlation between the index of AMAs and the disease duration before diagnosis, we believe that these suggest the importance of AMAs not only as markers but also as factors involved in pathogenic mechanisms. Of the 24 AMA-positive patients, despite the characteristic features of the whole group, some patients showed a subacute or acute clinical course, no muscle atrophy, and no association with cardiopulmonary involvements, indicating some variation in clinical features. Further study should be carried out to clarify the roles of AMAs in the pathogenesis of AMA-positive myositis.

Presently, the mechanisms underlying PBC pathogenesis remain unknown. In the initiation of the mechanism underlying PBC pathogenesis, the mimotopes of the vulnerable epitope of the PDC-E2 autoantigen are considered to become the autoantigens in PBC (Lleo et al., 2008). In the production of the mimotopes, the modification of native proteins following the exposure to infectious microorganisms, environmental xenobiotics/chemical compounds or apoptotic biliary epithelial cells has been suggested (Selmi et al., 2011). In the presence of altered regulation of self-tolerance and genetic background, it is suggested that AMAs are produced by PDC-E2 autoantigen-specific B cells and

PDC-E2 autoantigen-specific T cells present in sera (Selmi et al., 2011). Regarding the pathogenesis of liver damage in PBC, it has been revealed that the autoreactive CD4-positive and CD8-positive T cells infiltrating the liver in PBC recognize PDC-E2 (Hohenester et al., 2009), which supports the hypothesis that T cell responses contribute to bile duct injury in PBC.

On the other hand, although AMAs are highly specific for PBC, the pathogenic role for them in this disease is uncertain since in contrast to other autoimmune diseases, PBC responds poorly to immunosuppressive agents and changes in autoantibody titre do not seem to correlate with disease severity (Selmi et al., 2011).

Recently, it has been suggested that the pathogenic immune attack in PBC may be directed not only against the proteins of the 2-oxo acid dehydrogenase family (M2 antigen) but also against other antigens that become exposed during apoptosis and proliferation of biliary epithelial cells. Among these antigens, nuclear antigens, neuroendocrine compartments such as the acetylcholine receptor muscarinic M3 receptor, the $\alpha1$ adrenergic receptor and proteins of the Bcl-2 family have been suggested (Berg, 2011).

Indeed, it has been known that antinuclear antibodies are detected in ~50% of serum samples from patients with PBC (Selmi et al., 2011). Among them, an autoantibody against glycoprotein 201 was reported to correlate with severity of PBC.

Furthermore it was recently shown that sera from patients with PBC have functionally active anti-acetylcholine receptor muscarinic M3 autoantibodies and the author suggested the possibility of receptor desensitization due to repeated interactions of the receptor with the autoantibodies (Berg, 2011). Nicotinic but not muscarinic anti-acetylcholine receptor autoantibodies were detected in AMA-positive patients with PBC in early studies (Sundewall et al., 1987; Kyriatsoulis et al., 1988). Considering a previous report describing the relationships between autoimmunity against the $\beta1$-adrenergic receptor autoantibody or muscarinic acetylcholine receptor and dilated cardiomyopathy (Jahns et al., 2004), in addition to autoreactive CD4-positive and CD8-positive T cells, autoantibodies against antigens other than AMAs may have some pathogenic role in patients with PBC. Further studies are necessary to reveal the mechanism of skeletal muscle damage in patients with PBC.

In our series, eight patients showed myositis associated with arrhythmia. Among them, two patients required treatment by catheter ablation and one patient required an implantable pacemaker. Furthermore, in three patients who refused any treatment, two developed arrhythmia, one of whom required an implantable pacemaker.

In previous case reports, as far as we searched, cardiac involvement in myositis in the presence of AMAs has been described in eight patients (Uhl et al., 1974; Saitoh et al., 1988; Harada et al., 1992; Varga et al., 1993; Tsai et al., 1996; Kasuga et al., 2004; Tanaka et al., 2007). Among them, five patients had a chronic disease course (2 years, $n = 2$; 5 years, $n = 3$), and all eight patients, including one patient who required an implantable pacemaker, showed arrhythmia (supraventricular tachycardia, $n = 5$; ventricular tachycardia, $n = 2$; atrioventricular block, $n = 1$). Seven patients, including three patients who received treatment for dilated cardiomyopathies, had a decreased ejection fraction.