

Autoantibodies against HSP70 family proteins were detected in the cerebrospinal fluid from patients with multiple sclerosis

Susumu Chiba ^{a,*}, Shin-ichi Yokota ^b, Kazumi Yonekura ^a, Shingo Tanaka ^a,
Hiroyasu Furuyama ^a, Hiroshi Kubota ^c, Nobuhiro Fujii ^b, Hiroyuki Matsumoto ^a

^a Department of Neurology, Sapporo Medical University School of Medicine, Minami 1-Jo Nishi 16 chome, Chuo-ku, Sapporo 060-8543, Japan

^b Department of Microbiology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

^c Department of Molecular and Cellular Biology, Institute for Frontier Medical Science, Kyoto University, Sakyo-ku, Kyoto 606-9397, Japan

Received 18 July 2005; received in revised form 28 August 2005; accepted 18 October 2005

Available online 21 November 2005

Abstract

We evaluated the specific IgG antibodies against heat shock proteins (HSPs) in cerebrospinal fluids (CSF) from patients with multiple sclerosis (MS). ELISA was employed to examine IgG antibodies against ten HSPs (HSP27, α A and α B crystallins, HSP60, CCT, *Mycobacterium bovis* HSP65, *Escherichia coli* GroEL, HSP70, HSC70 and HSP90) in CSF from 30 patients with MS, and 25 patients with motor neuron diseases (MND). Significantly higher antibody titers against HSP70 and HSC70 proteins were found in CSF obtained from patients with MS as compared with MND independent of CSF total protein, IgG concentrations and IgG indices, respectively. The antibody titers against HSP70 were indicated to be significantly higher in the progressive cases than in cases of remission. The results suggest that IgG antibodies against specific types of HSPs especially HSP70 family proteins (HSP70 and HSC70) in CSF may play an important role in the pathophysiology of MS through the modification of immune response and cytoprotective functions of molecular chaperons.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Anti-heat shock protein antibody; Cerebrospinal fluids; Multiple sclerosis; ELISA

1. Introduction

Heat shock proteins (HSPs) are called stress proteins or molecular chaperons that assist cell rescue through the folding of synthesized or stress-denatured proteins. HSPs are also believed to be causative factors in various autoimmune diseases, whose etiologies are considered to spring from immune responses against HSPs as a target molecule. For instance, elevated antibody titers to HSP60 family proteins are reported in cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) and other neurological diseases [1–3]. Recently, we found the presence of antibodies against HSP60 homologue and vacuolating cytotoxin

of *Helicobacter pylori* in CSF from patients with Guillain–Barre syndrome (GBS) [4]. In addition, antibody titers against various types of HSPs including HSP60 and HSP70 families are found to be significantly high in CSF obtained from patients with GBS [5]. Furthermore, recent reports indicated that soluble HSP60 and HSP70 should influence the innate immune system as ligands of the toll-like receptors and subsequently induce inflammatory responses [6,7].

In the present study, we examined antibody titers against various types of HSPs in CSF of patients with MS and motor neuron diseases (MND). HSPs examined here are HSP60 (chaperonin) family [mammal mitochondria HSP60 (mtHSP60) and cytosolic chaperonin CCT, *Mycobacterium bovis* HSP65, and *Escherichia coli* GroEL], HSP27 (small HSP) family [mammal HSP27, α A crystallin and α B crystallin], HSP70 family [mammal HSP70 and cognate of HSP70 (HSC70)] and HSP90.

* Corresponding author. Tel.: +81 11 611 2111x3822; fax: +81 11 622 7668.

E-mail address: chiba@sapmed.ac.jp (S. Chiba).

2. Materials and methods

2.1. Patients and cerebrospinal fluids

CSFs were donated by 30 patients with clinically definite MS (9 men and 21 women; mean age, 39.1 years), and 25 patients with MND (14 men and 11 women; mean age, 62.1 years) as disease controls. The clinical diagnosis of MS was made according to the disease criterion described by Poser et al. [8]. According to the last 3 months clinical observation, patients with MS were divided into 8 cases of progressive, 15 cases of relapsing/remitting and 7 cases of stable phase (consisting of 6, 12 and 3 under steroid therapy, respectively). In order to examine the correlation between antibody titers to HSP proteins and other laboratory factors of CSF, total protein, IgG concentrations and IgG Indices were evaluated in patients with MS. Patients with MND consisted of 20 patients with amyotrophic lateral sclerosis, 4 patients with spinal progressive muscular atrophy, and a patient with progressive bulbar palsy. Informed consent was obtained from each patient with MS and MND.

2.2. Antigens

Human HSP27, bovine α A crystallin, bovine α B crystallin, human mtHSP60, *M. bovis* HSP65, *E. coli* GroEL, human HSP70, bovine HSC70 and human HSP90

were purchased from Stressgen (Victoria, British Columbia, Canada). Human cytosolic chaperonin CCT was purified from lysate of BALL-1 cells and the purity of CCT preparation was described previously [9]. According to the manufacturer's specifications, these commercial protein preparations are more than 90% pure as determined by SDS-PAGE. We confirmed that no other band was observed by SDS-PAGE with Coomassie brilliant blue staining (data not shown).

2.3. ELISA

ELISA was basically performed as described elsewhere [9,10]. Antigen was diluted in 50 mM sodium carbonate buffer (pH 9.6) to a final concentration of 1 μ g/ml and dispensed to 96-well multiplates (50 μ l per well). After incubation at 4 $^{\circ}$ C overnight, the wells were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature for 2 h. Human CSFs were diluted in PBS containing 0.05% Tween 20 (PBST) and 2% BSA at 10-fold. The dilution buffer without CSF was used as a background control. The samples were applied to the multiplate coated with each antigen, and then incubated at room temperature for 90 min. After washing, specific binding of CSF IgG or IgM was detected by subsequent incubation of horseradish peroxidase-conjugated goat antibodies specific for the γ -chain of human IgG or μ chain of human IgM (BioSource International, Carmelito, CA) as a

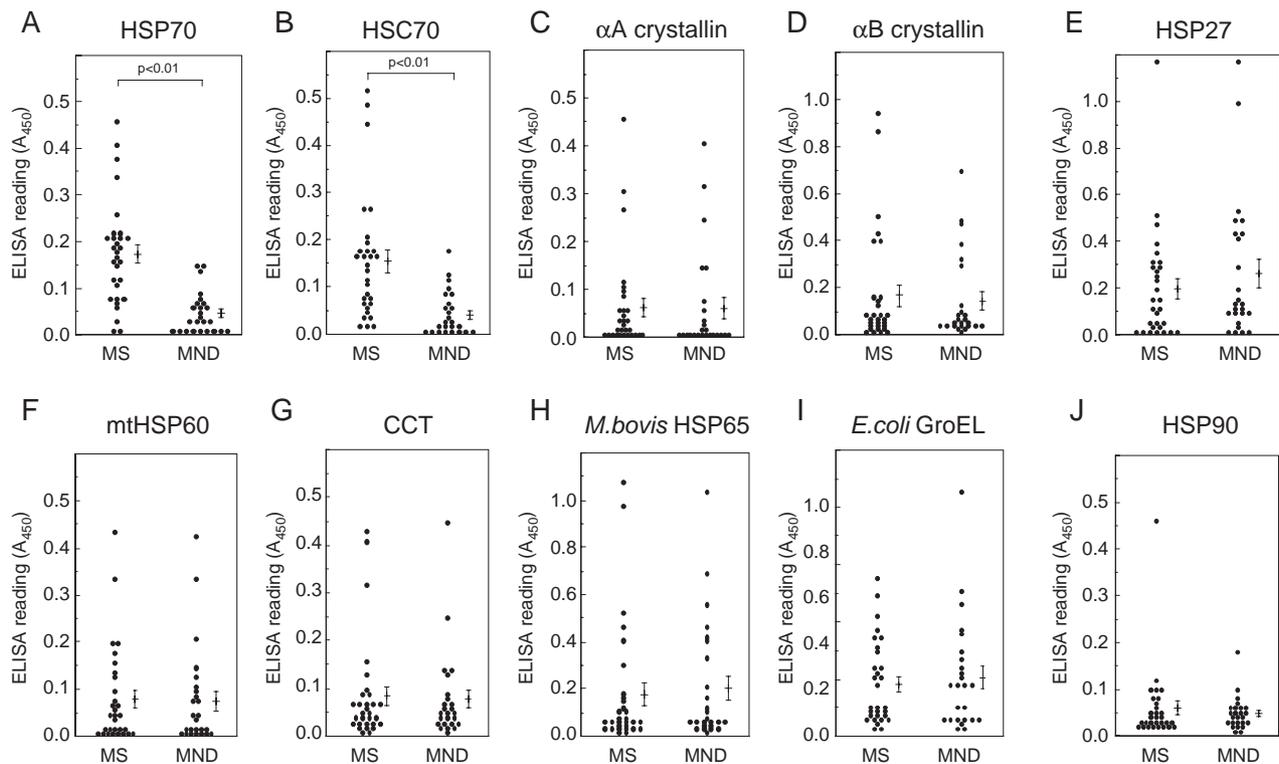


Fig. 1. Antibody titers against various types of HSPs in the cerebrospinal fluids from patients with MS and MND. IgG antibodies against HSP70 (A), HSC70 (B), α A crystallin (C), α B crystallin (D), HSP27 (E), HSP60 (F), CCT (G), *M. bovis* HSP65 (H), *E. coli* GroEL (I) and HSP90 (J). Antibody titers are expressed as absorbance at 450 nm.

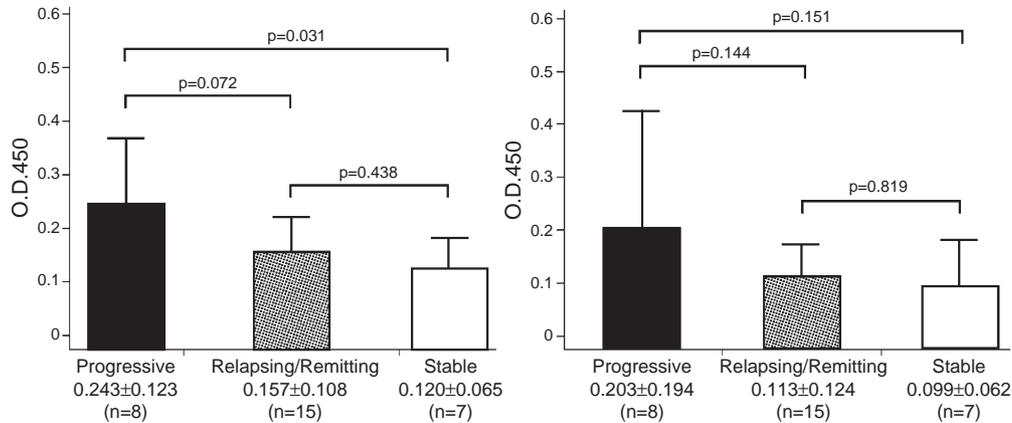


Fig. 2. Correlations between the clinical features of MS and the IgG antibody titers against HSP70 and HSC70. The antibody titers to HSP70 and HSC70 tended to be higher as disease activity increased. Antibody titers to HSP70 were significantly higher in the progressive cases as compared with in the stable cases.

secondary antibody, respectively, and 3,3',5,5'-tetramethylbenzidine solution as a substrate for developing. After terminating the reaction with an equal volume of 1 M phosphoric acid, absorbance at 450 nm was measured and taken as the antibody titer.

Value of antibody titer in each group is expressed as mean±S.E. in Fig. 1 and mean±S.D. in Fig. 2. A significant difference between two mean values was examined by unpaired *t*-test with Bonferroni's correction.

3. Results

We examined antibodies against various HSPs in CSF obtained from patients with MS and MND (Fig. 1). From the results of ELISA at various dilution of CSF, 10-fold dilution was found to be suitable for detecting the titer of antibody against HSPs eliminating non-specific binding (data not shown). The titers of IgG antibody against HSP27, α A crystallin and α B crystallin in CSF showed no difference between patients with MS and those with MND. As regards CSF antibodies against chaperonin family proteins, such as human mtHSP60, human CCT, *E. coli* GroEL and *M. bovis* HSP65, no significant difference was disclosed between MS and MND patients. However, IgG antibody titers against HSP70 and HSC70 in CSF obtained from patients with MS were significantly higher than those in CSF from MND patients. We additionally confirmed that specific bindings of the antibodies to HSP70 or HSC70 in CSF from MS patients were examined by Western-blotting using HSP70 or HSC70 protein (data not shown). IgG antibody titers against HSP90 in CSF obtained from patients with MS tended to be higher than those in CSF from MND patients, but not to the point of statistical significance. We also examined the titers of IgM antibodies against these HSPs. In ELISA using all examined HSPs except for GroEL, almost all CSF showed that the ELISA readings (A_{450}) were less than 0.1 at 10-fold dilution. Significantly positive IgM was found in a few cases, although the positive

cases were observed in both MS and MND patient groups (data not shown). IgM against GroEL in CSF was detected in many CSF samples, however significant difference between MS and MND was not observed [means±SE were 0.126 ± 0.08 (MS) and 0.117 ± 0.016 (MND)]. Consequently, significant IgM antibody titers against the HSPs were not found in CSF from both MS and MND patients.

Concerning the correlations between the clinical features of MS and the IgG antibody titers against HSP70 and HSC70, we found not only that antibody titers to HSP70 and HSC70 tended to be higher according to disease activity but also that antibody titers to HSP70 were significantly high in the progressive cases as compared with in the stable cases (Fig. 2). On the other hand, we failed to disclose the significant correlation between high antibody titers to both HSP70 and HSC70, and the values of CSF total protein, IgG concentrations and IgG indices, respectively.

4. Discussion

An etiological relationship between some neurological diseases and HSP has occasionally been described. Increased HSP27 and reduced HSC70 expressions were found in plaque legion and myelin of MS patients [11]. α B crystallin, which is a member of small HSP family, is also proposed to be a candidate for T-cell autoantigen in MS [12,13]. However, we failed to find a significant difference in IgG antibodies against small HSP family proteins including HSP27, α A crystallin and α B crystallin in CSF between patients with MS and those with MND. A similar observation was made in anti-chaperonin family proteins. We have previously reported that the IgG antibody titers against various types of HSPs including small HSP, HSP60, HSP70 families and HSP90 are found to be significantly higher in CSF obtained from patients with GBS as compared to those from patients with MND [5]. However, the IgG antibody titers to all chaperonin family proteins tested in CSF showed no significant difference between MS

and MND patients. Interestingly, CSF IgG antibodies against HSP70 family proteins, namely HSP70 and HSC70, were significantly elevated in patients with MS without any correlation not only with the concentrations of CSF total protein and IgG but also with IgG indices. Several laboratories have shown an elevated T cell response to HSP60 and HSP70 family proteins in patients with MS [14]. However, IgG autoantibodies against HSP70 family proteins in neurological diseases have not been reported. In contrast, no significant IgM antibody titers against the HSPs were found in CSF from both MS and MND patients.

The pathophysiological role of these antibodies against various types of HSPs in CSF is still unknown. Furthermore, the profile of CSF antibody against bacterial antigens was quite different from that of serum antibody derived from the same individual [4]. HSPs are abundantly and essentially expressed in almost all types of cells residing in the nervous system. Generally, HSPs are considered to be expressed inside the cells, such as in cytosol, mitochondria, endoplasmic reticulum and nucleus and to participate in protein folding as molecular chaperones. How anti-HSP antibodies work pathogenically in CSF remains unclear. However cell surface expression of HSPs has been reported [15,16] and soluble mtHSP60 and HSP70 can act as cytokines to activate NF- κ B and subsequently induce inflammatory responses [6,7]. These HSPs existing in special circumstances may be a target for antibodies. The HSPs expressing on the cell surface may be attacked by the specific antibodies. HSPs can associate with a broad range of peptides as molecular chaperones. Among them, HSP70, HSP90 and gp96 are involved in antigen presentation [17]. Recent reports suggest that HSP70 promotes antigen presentation of autoantigen, namely myelin basic protein (MBP) and proteolipid protein (PLP), by MHC class II [18]. In addition, HSP70 specifically associates with MBP and PLP in brain tissue derived from MS patients [19]. MBP and PLP are considered to be possible target autoantigens in MS. Recently, receptors specific for HSPs, such as CD40 and a scavenger receptor LOX-1, have been proven to exist on antigen presenting cells [20,21]. Extracellular HSP70 associated with some peptides, such as cancer antigenic peptide fragments, are indicated to be taken in by endocytosis via the receptors, and the peptides associated with HSP70 are carried into the antigen presenting system. In fact, antigen presenting cells take in MBP forming complex with HSP70 more efficiently than MBP only [19]. Judging from these observations, we consider that anti-HSP70 antibodies may modify the HSP70-mediated antigen presentation [6,7]. On the other hand, the induction of inflammatory responses by soluble HSPs may be modified, either inhibited or enhanced, by the anti-HSP antibodies.

In this study, we additionally found that IgG antibody titers to HSP70 and HSC70 tended to increase according to the disease activity, and IgG antibody titers to HSP70 were significantly higher in the progressive cases than in the stable cases.

Judging from the above results, the specific IgG antibodies against HSP70 and HSC70 in CSF are thought to be an indirect marker of disease activity and to play an important role in the development of MS, through modifications not only of focal immune responses but also of the cytoprotective functions of HSPs as molecular chaperones. Nevertheless, the pathophysiological role of the specific CSF IgG antibodies to HSP70 and HSC70 remains unresolved.

References

- [1] Gao YL, Raine CS, Brosnan CF. Humoral response to hsp 65 in multiple sclerosis and other neurologic conditions. *Neurology* 1994; 44:941–6.
- [2] Prabhakar S, Kurien E, Gupta RS, Zielinski S, Freedman MS. Heat shock protein immunoreactivity in CSF: correlation with oligoclonal banding and demyelinating disease. *Neurology* 1994;44:1644–8.
- [3] Tasci B, Direskeneli H, Serdaroglu P, Akman-Demir G, Eraksoy M, Saruhan-Direskeneli G. Humoral immune response to mycobacterial heat shock protein (hsp) 65 in the cerebrospinal fluid of neuro-Behcet patients. *Clin Exp Immunol* 1998;113:100–4.
- [4] Chiba S, Sugiyama T, Matsumoto H, Hisano K, Awakawa T, Hiura K, et al. Antibodies against *Helicobacter pylori* were detected in the cerebrospinal fluid obtained from patients with Guillain–Barre syndrome. *Ann Neurol* 1998;44:686–8.
- [5] Yonekura K, Yokota S, Tanaka S, Kubota H, Fujii N, Matsumoto H, et al. Prevalence of anti-heat shock protein in cerebrospinal fluids of patients with Guillain–Barre syndrome. *J Neuroimmunol* 2004; 156:204–9.
- [6] Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 2002; 277:15028–34.
- [7] Vabulas RM, Wagner H, Schild H. Heat shock proteins as ligands of toll-like receptors. *Curr Top Microbiol Immunol* 2002;270:169–84.
- [8] Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983;13:227–31.
- [9] Yokota S, Hirata D, Minota S, Higashiyama T, Kurimoto M, Yanagi H, et al. Autoantibodies against chaperonin CCT in human sera with rheumatic autoimmune diseases: comparison with antibodies against other Hsp60 family proteins. *Cell Stress Chaperones* 2000;5:337–46.
- [10] Yokota S, Kubota H, Matsuoka Y, Higashiyama T, Kurimoto M, Yanagi H, et al. Prevalence of HSP47 antigen and autoantibodies to HSP47 in the sera of patients with mixed connective tissue disease. *Biochem Biophys Res Commun* 2003;303:413–8.
- [11] Aquino DA, Capello E, Weisstein J, Sanders V, Lopez C, Tourtellotte WW, et al. Multiple sclerosis: altered expression of 70- and 27-kDa heat shock proteins in lesions and myelin. *J Neuropathol Exp Neurol* 1997;56:664–72.
- [12] van Noort JM, van Sechel AC, Bajramovic JJ, el Ouagmiri M, Polman CH, Lassmann H, et al. The small heat-shock protein alpha B-crystallin as candidate autoantigen in multiple sclerosis. *Nature* 1995;375:798–801.
- [13] van Noort JM. Multiple sclerosis: an altered immune response or an altered stress response? *J Mol Med* 1996;74:285–96.
- [14] Brosnan CF, Battistini L, Gao Y-L, Raine CS, Aquino DA. Heat shock proteins and multiple sclerosis: a review. *J Neuropathol Exp Neurol* 1996;55:102–389.
- [15] Schett G, Xu Q, Amberger A, Kuppper M, Ahrens B, Scharner A, et al. Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. *J Clin Invest* 1995;96:2569–77.

- [16] Milani V, Noessner E, Ghose S, Kuppner M, Ahrens B, Scharner A, et al. Heat shock protein 70: role in antigen presentation and immune stimulation. *Int J Hyperthermia* 2002;18:563–75.
- [17] Srivastava P. Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu Rev Immunol* 2002;20:395–425.
- [18] Mycko MP, Cwiklinska H, Szymanski J, Szymanska B, Kudla G, Kilianek L, et al. Inducible heat shock protein 70 promotes myelin autoantigen presentation by the HLA class. *J Immunol* 2004; 172:202–13.
- [19] Cwiklinska H, Mycko MP, Luvsannorov O, Walkowiak B, Brosnan CS, Raine CS, et al. Heat shock protein 70 associations with myelin basic protein and proteolipid protein in multiple sclerosis brains. *Int Immunol* 2003;15:241–9.
- [20] Delneste Y, Magistrelli G, Gauchat J, Haeuw J, Aubry J, Nakamura K, et al. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 2002;17:353–62.
- [21] Becker T, Hartl FU, Wieland F. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol* 2002; 158:1277–85.