Titolo rivista/libro: Journal of neuropathology and experimental neurology

Titolo articolo/sezione: Positive or negative involvement of heat shock proteins in multiple sclerosispathogenesis: an overview

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ISSN: 0022-3069

DOI: 10.1097/NEN.0000000000000136

Anno: 2014

Volume: 73

Fascicolo: 12

Editore:

Pag. iniziale: 1092

Pag. finale: 1106

J Neuropathol Exp Neurol

Vol. 73, No. 12
December 2014
pp. 1092–1106

REVIEW ARTICLE

Positive or Negative Involvement of Heat Shock Proteins in Multiple Sclerosis Pathogenesis: An Overview

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Abstract

Multiple sclerosis (MS) is the most diffuse chronic inflammatory disease of the central nervous system. Both immune-mediated and neurodegenerative processes apparently play roles in the pathogenesis of this disease. Heat shock proteins (HSPs) are a family of highly evolutionarily conserved proteins; their expression in the nervous system is induced in a variety of pathologic states, including cerebral ischemia, neurodegenerative diseases, epilepsy, and trauma. To date, investigators have observed protective effects of HSPs in a variety of brain disease models (e.g. of Alzheimer disease and Parkinson disease). In contrast, unequivocal data have been obtained for their roles in MS that depend on the HSP family and particularly on their localization (i.e. intracellular or extracellular). This article reviews our current understanding of the involvement of the principal HSP families in MS.

Key Words: Heat shock proteins, Innate immunity, Multiple sclerosis, Myelin antigens, Toll-like receptors.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a complex disease that is influenced by genetic, epigenetic, and environmental factors, including gender, sex hormones, ethnic origin, latitude of early life residence, smoking, pathogen exposure, and vitamin D levels (1–5). Recent epidemiologic data suggest a genetically determined susceptibility and indicate that the incidence of MS correlates with environmental factors that occur during childhood, which, after several years of latency, determine the onset of MS (6–8). Therefore, the clinical, pathologic, and immunologic phenotypes of MS are highly heterogeneous, indicating that it may better be defined as a syndrome rather than a single disease. Multiple sclerosis is the most common chronic inflammatory central nervous system (CNS) disease of likely autoimmune etiology. It is thought to be caused by an inappropriate immune T cell–mediated response, that is, T-helper Type 1 and T-helper Type 17 (Th1, Th17), against CNS myelin or other antigens (9). This representation is called the *outside-in* model. However, a recent reinterpretation of the available experimental data suggested another hypothesis called the *inside-out* model (10). According to this model, MS is a primary neurodegenerative disease, and the inflammatory response is an epiphenomenon caused by the host's aberrant immune response. Indeed, laboratory and clinical observations have shown some inconsistencies in the ''outside-in'' model, particularly in the initial stages of the disease during which the largest myelin abnormalities sometimes begin at the inner myelin sheath, which is not accessible to antibody- or immune cell–mediated attack (10). In autopsy material obtained from patients in early active stages of MS, no infiltration of T and B cells was observed in areas of demyelination and oligodendrocyte loss; only macrophage infiltration and microglial activation, markers of the innate immune response activation, were detected (11,12).

Recent results from clinical trials in MS have confirmed that immunomodulatory drugs significantly attenuate the course of the disease (13). Demyelinating lesions are predominantly located in the white matter and contain clonally expanded CD8-positive/CD4-positive T cells (14–17), γδ T cells (18), and monocytes (19,20). It has additionally been demonstrated that the gray matter structures of the brain are also affected (21). Clinical symptoms and signs vary based on the site of the lesions. As a consequence of myelin sheath destruction, nerve action potentials are disrupted, resulting in neurologic disability. Pathologic hallmarks of MS include areas of focal demyelination characterized by gliosis and neuron and oligodendrocyte loss that are particularly common in the brain, spinal cord, and optic nerves (22). The majority of patients (nearly 85%–90%) experience a sudden onset of symptoms, with subsequent episodes of acute attacks followed by partial or complete recovery and variable periods of remission. In the remaining 10% to 15% of patients, the course of MS is progressive from the onset, that is, primary progressive (PP) MS. Most patients with a relapsing-remitting (RR) disease course at onset eventually experience a change in the disease course to

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This work was supported by University funding FFR STEBICEF R2FFRAD15 + PNFK, progetto di ricerca per la sclerosi multipla R4D15 + P118 MERC. No competing financial interests exist.

become progressive, that is, secondary progressive (SP) MS (23). Pathogenic studies have clearly indicated that axonal injury is a key feature of MS pathogenesis; the extent of axonal damage is also correlated to the degree of inflammation in the relapsing phases of the disease. A close relationship between inflammation and degeneration has also been described for all disease stages of MS. Nevertheless, the specific mechanisms of the interdependence between focal inflammation, diffuse inflammation, and neurodegeneration remain unclear.

Unlike other neurologic diseases in which it is possible to define high-affinity antibodies that recognize self-antigens (24–26), it is difficult to identify a single antigen specificity in MS patients that is responsible for the autoreactive response. The general idea is that, in MS pathogenesis, not one but several antigens are involved in the disease. It is likely that the initial autoreactivity is specific for a particular antigen but, in a second step of the disease, a process of epitope or antigen spreading may increase the pool of activated immune cells.

Previously, myelin antigens were believed to be targets of pathogenic T cells, including the following: myelin basic protein (MBP), one of the most immunogenic proteins of the CNS (and which is synthesized in the CNS only by oligodendrocytes); myelin proteolipid protein (PLP), the most abundant component of CNS myelin and one of the major targets of the autoimmune response (27); myelin oligodendrocyte glycoprotein (MOG); and myelin-associated glycoprotein. Other potential immunogenic proteins have included nonmyelin antigens such as αB-crystallin (HSPB5), transaldolase, and CNPase (28–35).

When considering T-cell autoreactivity, however, it is crucial to remember that not all MS patients show elevated levels of autoimmune responses to myelin antigens because T-cell responses are typically transient. In addition, T-cell responses can change with time from the onset of disease as well as during fluctuations. Despite the general perception of MS and its changing pathogenesis, it is now understood that, although specific myelin antigens are targets for T cells during MS, T-cell activation is not specific to a single antigen. Recent reports have identified a large series of CNS-specific proteins that are hidden from the immune system during its development and maturation (36). Currently, it is impossible to prove whether T cells specific for PLP or other myelin antigens are pathogenic in patients with MS or whether they are produced secondarily to the release of myelin by demyelinating processes.

T-cell activation induces the secretion of inflammatory cytokines, including interferon (IFN)-γ, tumor necrosis factor, interleukin (IL)-1β, and IL-6 (37). The classic paradigm in which activated T cells breach the blood-brain barrier and then migrate into the CNS has begun to be questioned.

At present, the most widely accepted hypothesis regarding T-cell activation suggests that T cells are initially activated in peripheral lymphoid organs and thereby acquire the capability to cross the blood-brain barrier, thereby inducing CNS damage. After the initiation of CNS inflammation, the so-called *epitope-spreading* mechanism occurs, in which antigen-presenting cells process cell debris resulting from axonal and tissue damage and then migrate into the circulating peripheral blood, where they induce autoreactive T-cell growth toward these new autoantigens (38,39). It has been shown that naive T lymphocytes are also able to penetrate the CNS during the course of an acute inflammatory process; they are activated directly by antigen-presenting cells in the CNS and bypass the peripheral activation mechanism. This additional type of activation makes T cells the main actors in the epitope-spreading process. Cumulative data indicate that, after the CNS is damaged, sensitization to other antigens may also arise, contributing to the development of chronic disease.

It is not currently possible to prove whether T cells specific for myelin antigens are truly pathogenic in patients with MS or whether they are produced secondarily to the release of myelin as a consequence of the demyelinating process. The development of an adequate animal model would be highly useful to prove the disease relevance of T-cell responses directed against the candidate autoantigens.

To date, many results have been obtained using an experimental animal model of autoimmune encephalomyelitis (EAE), a T cell–mediated autoimmune disease of the CNS (40–45). Experimental autoimmune encephalomyelitis can be induced in rodents and primates via the administration of myelin antigens (e.g. MOG, PLP, and MBP), usually with adjuvants (35,46,47). Experimental autoimmune encephalomyelitis can reproduce many of the clinical neuropathologic and immunologic aspects of MS (48). Nevertheless, there is increasing support for the idea that EAE is no longer an optimal model for MS.

There are important differences between MS and EAE. The most evident is that MS is a spontaneous disease, whereas EAE is induced. For this reason, one of the problems associated with animal models is that they tend to elucidate mechanisms that are deliberately selected a priori for perturbation. Moreover, the inducing antigens in EAE are known, whereas, in MS, there is no unique antigen responsible for the disease. Therefore, important differences between the animal model and MS may result from the way autoreactive T cells are primed and activated. Recently, spontaneous models of EAE have been developed, but they require transgenic approaches (49–51). Another important difference between MS and EAE is that the latter is mainly studied in inbred animals or in a genetically homogeneous population, whereas, in MS, the specific response depends on the specific genetic background of the individual. In conclusion, because MS has different hallmarks depending on the typology of the disease (e.g. RR vs PP), the complexity of the pathology can be reflected in EAE only when a broad spectrum of models induced in different species by different sensitization methods are studied (52).

Almost all the therapies used in MS treatment were initially tested in EAE models with different results. For some drugs (e.g. IFN-β and natalizumab), there was a correlation between EAE and MS therapeutic success. In contrast, for other drugs, treatment successes have been obtained for EAE that have not been translated into successful treatments of MS patients (53). It remains an open question as to whether autoimmune reactivity against myelin antigens causes MS.

In addition to CNS antigens, non–CNS-specific antigens expressed in response to or as a consequence of the inflammatory insult to the CNS may also be involved in MS

progression. One of these immunogenic factors may be represented by heat shock proteins (HSPs).

HEAT SHOCK PROTEINS

Heat shock proteins are molecular chaperones that assist in the proper folding of newly synthesized proteins and of proteins subjected to stress-induced denaturation. Heat shock proteins also exhibit a variety of cytoprotective (54,55) and cytostimulatory functions (56). A molecular chaperone is a protein that, by the controlled binding and release of the substrate protein or peptide, facilitates its correct fate in vivo; this fate may include folding, oligomeric assembly, transport to the appropriate subcellular compartment, or switching between active/inactive conformations (57). Molecular chaperones also exhibit a variety of cytoprotective functions (54,55). In addition to their role as chaperones, HSPs inhibit the apoptotic cascade, increasing cell survival (58).

Heat shock proteins are classified into different families based on their molecular mass, that is, Hsp110, Hsp90, Hsp70, Hsp60, Hsp40, and the small HSP families. In 2009, Kampinga et al. (59) proposed new guidelines for the nomenclature of human HSP families as well as for the human chaperonin families (Table 1). The HSPs in the high-molecular-weight group (i.e. the HSPC, HSPA, and HSPD1 families) are adenosine triphosphate (ATP)–dependent chaperones, and they are stabilized in their ATP- or adenosine diphosphate–bound forms by the so-called *co-chaperones* (e.g. DNAJB1). In contrast, the small HSPs (HSBPs) are ATP independent. It is possible that their activation is regulated by their phosphorylation status (60,61). The most studied members of this family are HSPB1 and HSPB5 (62).

One of the most conserved subsets of HSPs is the HSPA family (63). Almost all HSP families have a constitutively expressed member that plays a housekeeping role and a stress-induced member that plays a crucial role in recovery after cellular stress. The feature common to both constitutive and inducible HSPs is that they bind solvent-exposed hydrophobic segments of non-native polypeptides, permitting folding, transport, and assembly of the polypeptide through a cycle of binding and release (64–66).

The transcription factor responsible for HSP transcriptional activation is heat shock transcription factor 1 (HSF1) (67–69). According to the chaperone-based model, HSF1 in unstressed cells is maintained in an inactive complex with HSPC, DNAJB1, and HSPA1A. When elevated HSP levels are required in response to cellular stresses, HSF1 is released from the complex and migrates to the nucleus. The active homotrimeric hyperphosphorylated HSF1 binds heat shock elements in the promoter of HSP genes, leading to their upregulation (68,70). Heat shock proteins are present not only as intracellular but also as extracellular proteins (71,72).

TABLE 1. Heat Shock Protein Family Nomenclature

Old Names	Molecular Mass, kDa	New Names
Small Hsps	34 or lower	HSPB
Hsp27		HSPB1
αA-crystallin		HSPB4
αB-crystallin		HSPB5
Hsp40	35–54	DNAJ
Hsp40		DNAJB1
Hsp60	55–64	HSPD1
Hsp70	65–80	HSPA
Hsp72		HSPA1A
Hsc70		HSPA8
Grp75		HSPA9
Grp78		HSPA5
Hsp90	81–99	HSPC
Hsp90		HSPC1
Hsp90β		HSPC3
Hsp100	100 or higher	HSPH

Grp, glucose-related protein; Hsp, heat shock protein.

Extracellular HSP Roles and Their Receptors

It is now widely accepted that almost all HSPs are released into the extracellular environment. One emerging question concerns how these proteins, which lack any exocytosis signal, can exit from cells. It was initially believed that HSP release was caused by cellular necrosis, but it is now known that HSPs are released through exosomes (73,74), extracellular vesicles that originate from the fusion of multivesicular bodies with the plasma membrane. HSPA1A, HSPA8, HSPD1, and HSPC1 have also been shown to be released through membrane vesicles, extracellular vesicles that originate directly from the plasma membrane (Tinnirello, unpublished data).

Extracellular HSPs have different roles from their intracellular counterparts because they are involved in the induction of the innate immune response via interactions with macrophages or dendritic cells (75). Moreover, they are also involved in enhancing adaptive immunity. In both cases, HSPs interact with target cells through receptors that can be grouped into 2 categories: Toll-like receptors (TLRs) and scavenger receptors (75).

The TLR family includes both extracellular and intracellular members, and they are responsible for lymphocyte activation and also mediate responses to autologous components (e.g. HSPA1A, HSPC1, and HSPD1). Two of these receptors, TLR2 and TLR4, are involved in neurodegeneration (76–81) and may also be involved in MS pathogenesis (82–86). HSPA1A, HSPC1, and HSPD1 can be recognized by TLR2 and TLR4 (87–89). Indeed, activation of TLR2 and TLR4 stimulates the synthesis of several cytokines thought to be responsible for CNS autoimmunity and neurodegenerative diseases. Hasheminia et al (90) demonstrated that, in peripheral blood mononuclear cells obtained from MS patients, there was an increase in the levels of TLR2/4 compared with healthy donors. In particular, TLR2 overexpression was correlated with the Expanded Disability Status Scale. Increasing levels of TLR2/4 were also observed in mononuclear cells from the cerebrospinal fluid (CSF) (91). Elevated expression of TLR2 was detected in oligodendrocytes in MS lesions, and a specific agonist inhibited the maturation of oligodendrocyte precursor cells (OPCs), a progression that inhibits the remyelination of OPCs (92).

The potential role of TLRs in MS pathogenesis was demonstrated in an induced EAE model. Toll-like receptor

4 knockout increased the severity of clinical signs because of increased activity of Th17 cells (82,84,93). Similarly, TLR2 expression increased during $MOG_{35–55}$–induced EAE in several CNS regions (94). Toll-like receptor agonists have also been shown to promote the differentiation of mouse Th17 cells (84,95).

HSPs in the CNS

Defining the role of HSPs in normal and pathologic CNS is complicated by the large number of cell types present, and their differences preclude extrapolation of the results from one cell type to another. Heat shock protein expression has been detected in multiple CNS cell types, including neurons, glia, and endothelial cells (96). Heat shock proteins are also induced in a variety of pathologic states, including cerebral ischemia, neurodegenerative disease, epilepsy, and trauma (97). They are thought to exert 2 neuroprotective roles, that is, they prevent protein aggregation and misfolding through their chaperone activity and they induce antiapoptotic mechanisms by inhibiting multiple steps in apoptosis in both the intrinsic and the extrinsic pathways (58,98–100).

As previously described, HSPs are also present as extracellular proteins that are released both through physiologic secretory mechanisms and during necrotic cell death (101). Heat shock proteins in the extracellular milieu can increase stress resistance as a consequence of binding to stress-sensitive recipient cells such as neurons (56). For example, glial cells produce and release HSPs, including HSPA8 and HSPA1A (102), which are rapidly captured by neurons. In contrast, neurons express high HSPA8 levels, but they are not able to induce HSPA1A under stress conditions (103). Therefore, the supply of exogenous HSPs in the CNS, or its pharmacologic induction, can reduce neuronal death in neurodegenerative diseases (104).

Extracellular HSPs can also signal danger to inflammatory cells and aid in immunosurveillance by transporting intracellular peptides to immune cells (72). Two characteristics of HSPs confer the ability to initiate or perpetuate autoimmune diseases: 1) their phylogenetic conservation (e.g. immune responses to bacterial HSPs cross-react with mammalian HSPs [105]) and 2) their ability to evoke strong immune responses.

Table 2 summarizes the currently known functions of HSPs in the CNS.

TABLE 2. Intracellular and Extracellular Heat Shock Protein Functions in the CNS

Location	Functions
Intracellular	Cytoprotection
	Chaperone function
	Apoptosis inhibition
Extracellular	Immune response mediator
	Antigenenic adjuvant
	Antigen-presenting cell maturation and innate immune response induction

THE HSPA FAMILY

Unlike other HSPs (e.g. HSPC), the expression pattern of HSPA proteins extends to almost all intracellular compartments as well as secretion into the extracellular milieu and surrounding cells. In humans, the HSPA multigene family includes the cytosolic and nuclear-localized HSPA8 and HSPA1A, the endoplasmic reticulum–localized HSPA5 and the mitochondrial HSPA9. However, many of these proteins are capable of shuttling between various compartments.

HSPA8, HSPA5, and HSPA9 are abundantly expressed during normal growth conditions and form critical compartment-specific protein-folding machinery. HSPA family members contain several fairly well-conserved domains: the ATPase domain in the N-terminus, a substrate-binding domain (also referred to as the ‘‘chaperone function’’) and a C-terminal region that regulates the release of the substrate on nucleotide exchange. In normal cellular environments, these HSPAs function in concert with specific binding partners, particularly the chaperones of the DNAJ family, and with specific nucleotide exchange factors. In contrast, HSPA1A levels are regulated by growth (106,107) and are induced in response to a variety of stressful stimuli (e.g. hyperthermia, oxidative stress, heavy metals, amino acid analogs, and mechanical stress) in all living organisms. However, in addition to protein folding, HSPA family members recognize and bind exposed hydrophobic residues of misfolded or denatured proteins. These proteins are often held for ubiquitination and subsequent targeting to the proteasome for degradation. HSPA family members are also responsible for the recognition of proteins containing a KFERQ-like pentapeptide. These proteins are then transferred into lysosomes by HSPA1A proteins via chaperone-mediated autophagy (108). HSPA family members do not bind normal active proteins, with the exceptions of clathrin and σ32 (109,110).

HSPA1A and Autoimmune Diseases: A Negative Role?

Immune activation within the CNS is a characteristic feature of ischemia, neurodegenerative diseases, immune-mediated disorders, infections, and trauma, and it often contributes to neuronal damage. Because of their evolutionary conservation and high immunogenicity, HSPs can act as potential autoantigens to amplify and/or modify autoimmune responses. It has been demonstrated that extracellular HSPs can induce innate immunity through their interactions with cell surface receptors, including TLRs, leading to the expression of proinflammatory cytokines (111,112) and chemokines (113,114) and to the activation of dendritic cells (115,116). However, in acquired immunity, extracellular HSPs enhance the antigen presentation of bound polypeptides. To confirm their immunogenic roles, increased expression of HSPs has been observed in autoimmune forms of arthritis and diabetes, and HSP-reactive T-cell lines have been demonstrated in patients with these diseases. Such T cells are also able to induce arthritis in animal models (117–122). The principal HSP implicated in the formation of the immunogenic complex is HSPA1A (123–128).

In many neurodegenerative diseases (i.e. so-called *misfolding diseases* such as Parkinson disease, Alzheimer disease, and polyglutamine diseases), both intracellular and extracellular HSPs have neuroprotective roles in the CNS because they reduce misfolded proteins. A similar role does not apply to MS, in which extracellular HSPA1A exacerbates

the immune response by acting as an adjuvant for myelin peptides and as a proinflammatory cytokine (129).

As previously described, MS is a multifactorial disease that, in many patients, is characterized by an inappropriate immune T cell–mediated response to CNS myelin antigens. The activation of T cells requires accessory molecules represented by either class I or class II components of the major histocompatibility complex (MHC). Numerous studies have reported that HSPA1A enhances antigen presentation through the MHC I antigen presentation pathway. In addition, Mycko and coworkers (130) demonstrated that HSPA1A is also able to promote antigen presentation via an MHC class II–dependent pathway.

Under normal conditions, HSPA8 was observed to act as a chaperone for MBP, one of the 2 major myelin proteins of the myelin sheath (131). In contrast, PLP, the other main component of the myelin sheath, is not likely to require chaperoning by HSPA8. It is conceivable that HSPA8 should be similarly required for remyelination during the process of lesion repair in the remitting phase of MS. During this phase, association of the chaperone with myelin proteins on the cell membrane may function as an additional target of the immune response. Remyelination may also be impaired by a reduction in cellular HSPA8 content. In fact, the HSPA8 content in MS lesions from autopsy tissue has been found to be 30% to 50% below that in normal brain tissue, with chronic lesions showing the lowest expression (40,41). This reduction might be responsible for the permanent loss of myelin from the lesions (131).

In MS, however, the immune response in the CNS leads to an inflammatory and oxidative condition that is responsible for the overexpression of most HSPs, including HSPA1A, both within the lesion and at the lesion edge. This overexpression was observed both in MS patients and in EAE (40–45) and could be interpreted as an activation of endogenous neuroprotective mechanisms (41,44,132). In contrast, Cwiklinska et al detected HSPA1A complexes with either MBP or PLP in human MS lesions. Both complexes were highly immunogenic and, in an EAE model, they were able to induce a specific T-cell response (130,132,133). In contrast, no coimmunoprecipitation was observed in human control brain tissues, confirming the specificity of the complex in MS. A similar result was observed in mouse models of EAE (132). In addition, Cwiklinska et al demonstrated that the addition of HSPA1A to MBP in vitro could enhance its uptake by antigen-presenting cells and its presentation by MHC II and, via an adjuvant-like mechanism, could enhance immune responses to myelin antigens (130,132).

Chiba et al (134) examined antibody titers against various types of HSPs in the CSF of patients with either MS or motor neuron diseases. These authors observed higher levels of IgG antibodies against both HSPA8 and HSPA1A but no autoantibodies against other HSPs, including HSPB1, HSPD1, or HSPC1 (134,135). In addition, significantly higher anti-HSPA1A levels are found in patients with progressive MS than in patients in a stable state. Yokota et al (136) demonstrated that CSF obtained from patients with high anti-HSPA1A titers displayed elevated HSPA1A-induced IL-8 production in monocytic THP-1 cells, resulting in enhanced extracellular HSPA1A-induced inflammatory responses. In early active and chronic active lesions, HSPA1A immunoreactivity was strongly positive on reactive astrocytes and some macrophages at the leading edge (84). HSPA1A upregulation was also observed in inflammatory lesions in the CNS of animals with EAE (131).

In contrast, other studies report that there are no differences between the serum of MS patients and that of healthy controls (130,133,137). Consistent with these data, Cwiklinska et al (138) demonstrated that HSPA1A was not overexpressed in ex vivo peripheral blood mononuclear cells from MS patients, whereas on cell stress, HSPA1A was significantly overexpressed compared with healthy controls. This overexpression was caused by an increase in HSF1 nuclear translocation, which was dependent on the A group of PKC isoenzymes. In contrast to previous studies, Mansilla et al (139) recently reported upregulation of HSPA1A in peripheral blood samples of MS patients compared with healthy donors. They also demonstrated that, in MS CD4-positive T lymphocytes after heat shock, there was only a moderate increase in HSPA1A levels compared with healthy controls. This result could be explained by a chronic induction of the protein. A similar result was obtained in CD8-positive lymphocytes and macrophages from MS patients (139).

Demyelinated brain lesions of RR MS patients have been demonstrated to contain a subpopulation of clonally expanded γδ T cells that respond to HSPA1A (138,140–142). These cells produce large amounts of IL-17 (143), a potent proinflammatory cytokine that is involved in MS pathogenesis and EAE, as well as in other autoimmune diseases (144,145). Based on these data, we hypothesize that deregulated HSPA1A expression is involved in the pathogenesis of MS by contributing to the chronic inflammation of the environment and/or by facilitating myelin autoantigen presentation. Moreover, Lund et al (133) demonstrated that HSPA1A was associated with MBP peptides in normal-appearing white matter (NAWM) in both MS and normal human brain. These authors also found an adjuvant-like effect of HSPA1A-associated MBP-derived peptides. Based on these results, the authors hypothesized that a small dose of HSPA1A-MBP peptide secreted by stressed oligodendrocytes stimulated an in vivo adaptive immune response specific for the associated autoantigen. In addition, in vivo experiments demonstrated that HSPA1A was involved in EAE resistance. Indeed, hsp70.1$^{-/-}$ mice were found to be resistant to EAE after immunization with $MOG_{35–55}$ peptide; HSPA1A was essential for the induction of the autoimmune response to this peptide (128).

These data demonstrate that HSPA1A is overexpressed intracellularly in the CNS of MS patients, and that this overexpression may have a neuroprotective function in neurons and oligodendrocytes in an inflammatory environment. Nonetheless, intracellular HSPA1A is released into the extracellular milieu, where it is responsible for the induction or exacerbation of an immunologic response depending on its cytokine-like properties as well as its capacity as a myelin-peptide adjuvant.

Conflicting results were obtained by Galazka et al (146) who demonstrated that the subsequent induction of EAE was reduced in mice immunized with an HSPA1A fraction associated with peptide complexes isolated from animals with EAE. In contrast, the disease was not induced using HSPA1A-peptide

complexes isolated from healthy controls. These divergent results suggest substantial differences in the peptide that binds HSPA1A in normal versus pathologic CNS. In contrast, pharmacologic induction of HSPA1A (e.g. with geldanamycin [GA]) suppressed the glial inflammatory response and ameliorated the pathology of EAE (147). Other possible drugs capable of suppressing EAE by inducing HSPA1A are triptolide (148) and its less toxic derivatives (5R)-5-hydroxy-triptolide (149) and celastrol. HSPA1A is responsible for nuclear factor-κB inhibition, which attenuates the proinflammatory response (148–150). In fact, nuclear factor-κB is responsible for the transcription of various cytokines that are relevant to MS pathogenesis, and increased activity of this factor has been observed in microglia and in invading macrophages associated with active MS lesions of MS patients (151).

THE HSPC FAMILY

Like other chaperones, HSPC1 exhibits potent protective capacities such as the prevention of nonspecific aggregation of non-native proteins (152). However, HSPC1 seems to be more selective than many other chaperones, interacting only with specific subsets of the proteome (153). An additional feature of HSPC1 is its ability to induce conformational changes in folded native-like proteins, resulting in their activation and/or stabilization (154). In its active configuration, HSPC1 is a dimer, and its monomer contains an ATP-binding pocket (155). Unlike other chaperones, ATP hydrolysis by HSPC is relatively slow (156), and this ATP hydrolysis is responsible for conformational changes that are required for reaching or maintaining an activated state of substrate protein. In general, several cofactors interact sequentially with HSPC1 to assemble the chaperone machinery (157,158). Therefore, HSPC1 is regulated at several levels, that is, ATPase activity, cofactor interactions, and posttranslational modifications (e.g. acetylation, S-nitrosylation, and phosphorylation) (159–163). HSPC1 substrates generally belong to 2 classes: transcription factors such as p53 and signaling kinases. The proteins in this family appear to play important roles in the etiology of autoimmune diseases such as rheumatoid arthritis (164), systemic lupus erythematosus (165), and Type I diabetes (166).

HSPC1 AND NEURODEGENERATIVE DISEASES: FOCUS ON MULTIPLE SCLEROSIS

In several neurodegenerative disorders associated with protein aggregation, including Alzheimer disease and Parkinson disease, HSPC1 maintains the functional stability of aberrant neuronal proteins, thus sustaining the accumulation of toxic aggregates (167,168). Oligodendrocyte precursor cells retain the characteristics of multipotent CNS stem cells (169) and have been found both in adult rodent brains (170) and in the adult human CNS (171–173). These cells are involved in remyelination (171). Remyelination fails in MS, however, suggesting that OPCs are ineffective. Repair of demyelinated plaques is possible only during the initial phases of the disease. When MS becomes chronic, this capacity is lost and no CNS remyelination occurs (174). Cid et al (175,176) identified antibodies in MS patient CSF (particularly in patients who are in remission) that recognize antigens on OPCs in culture conditions. They demonstrated that the antibodies recognize the β isoform of HSPC (HSPC3), a protein that is expressed or overexpressed specifically on the OPC surface (177). These antibodies did not recognize cytosolic HSPC3 and were not found in control subjects or in patients with other inflammatory diseases (175,177). These authors further demonstrated that the recognition between antibodies in the CSF and HSPC3 on OPCs is responsible for complement fixation, which causes complement-mediated OPC death (175). Taken together, these findings provide a potential explanation for OPC death and explain the significant decrease in OPCs with the duration of the disease (175,177). Numerous reports have demonstrated that HSPC1 inhibition by GA blocks the release of cytokines from activated monocytic cells (178–180). Furthermore, Murphy et al (181) observed that GA reduced the expression and activity of nitric oxide synthase 2 in astrocytes and also reduced both the incidence and severity of EAE, but the therapeutic potential of GA is limited by its toxicity (182). Consequently, Dello Russo et al (147) studied the effect of the less toxic GA derivative 17-(allylamino)-17-demethoxygeldanamycin (17AAG) on glial cell activation in vitro. In addition, they tested the in vivo effects of a novel formulation of 17AAG called *EC72*. In vitro experiments with 17AAG confirmed that there was a reduction in astrocyte responses, but only minor inhibitory effects on microglial activation were observed. In vivo treatment with EC72 significantly reduced the incidence of EAE when administered before the appearance of clinical signs and induced clinical recovery when administered to mice that were already ill. No significant reduction was observed in T-cell activation. A similar result was obtained in vitro with the application of 17AAG to T cells during restimulation with MOG. No reduction in IFN-γ production was observed. By contrast, an inhibitory effect on IL-2 production was observed, suggesting that there was a selective effect on T cell–derived cytokines (147). Taken together, these results suggest that HSPC1 inhibition may reduce or delay the clinical development of demyelinating disease.

HSPD1

The HSPD1 stress protein belongs to a subgroup of molecular chaperones called *chaperonins*. They are distinguished from other chaperones by their special architecture and are subdivided into Type I and Type II chaperonins. Type I chaperonins, including HSPD1, consist of rings formed from 7 subunits; they collaborate with the co-chaperonin Hsp10, which functions as a type of lid to close the chaperonin cavity. Whether chaperonins assist protein folding by isolating target proteins from the crowded environment or simply by accelerating the folding process remains under debate. Biochemical and electron microscopy analyses have indicated that HSPD1 exists in a dynamic equilibrium between monomers, single-ring heptamers, and double-ring dodecamers (183). HSPD1 is present in both the cytosol and the nucleus, as well as in mitochondria (184).

Molecular chaperones in the mitochondrial matrix are involved in almost all of the major steps of mitochondrial biogenesis, including translocation, refolding, and assembly of both imported and mitochondrially encoded proteins. A

subset of imported proteins requires more folding assistance and must be transferred from HSPA9 to chaperonin HSPD1, which requires ATP hydrolysis and regulation by Hsp10 (185).

HSPD1 and the Immune System

HSPD1, like other HSPs, can be secreted into the extracellular environment from a variety of cell types under normal physiologic conditions. HSPD1, both foreign and self, is an antigen for B and T cells (186). Autoantibodies to self-HSPD1 have been found in several autoimmune and inflammatory diseases, including Type I diabetes (187,188), rheumatoid arthritis (189,190), and MS (29,191,192). It has also been shown that HSPD1 regulates immune responses in animal models of MS (43,193). This HSP may have both inflammatory and anti-inflammatory properties. The former activity is carried out through a signal via monocytes, B cells, and effector T cells. In contrast, the latter activity depends on B cells, regulatory T cells, and anti-ergotypic T cells (186).

HSPD1 in MS and EAE

As previously described, EAE represents an animal model of MS. Depending on the species used and the age at the time of sensitization, EAE may manifest as an acute episode or may develop into a more chronic syndrome with periods of exacerbation and remission. Gao et al (43) tested the hypothesis that inflammation in the CNS is associated with an altered expression of HSPs, which may be targets for the development of chronic disease. The CNS of animals with acute EAE displayed lesions of white matter with increased immunoreactivity for HSPD1, predominantly in infiltrating macrophages, with most of the staining at nonmitochondrial sites. In contrast, normal mice showed HSPD1 immunoreactivity exclusively in the mitochondria (43). However, during the chronic phase of EAE, both astrocytes and oligodendrocytes were immunoreactive. There was also a small increase in HSPD1 levels in the spinal cords of animals with chronic disease (43).

It has been demonstrated that, in early MS lesions, myelin degradation is not always associated with the depletion of oligodendrocytes, the cells involved in myelin formation. In fact, oligodendrocyte proliferation has been observed at borders of demyelinating plaques (194,195). This proliferation is responsible for some remyelination of axons. Nevertheless, this process remains incomplete and, with time, oligodendrocytes are depleted. Studies of MS patients have demonstrated HSPD1 reactivity in immature oligodendrocytes. No staining was present in interfascicular oligodendrocytes or in other cell types from MS patient tissues (29,191).

In vitro experiments confirmed the presence of HSPD1 in oligodendrocytes (191,196,197), but not in astrocytes, which preferentially express members of the HSPA family (198). Reactive oligodendrocytes are present at the margins of chronic lesions in areas of demyelination containing TCR γδ lymphocytes (29,141,199), which are also present in the CSF of MS patients (200). Because γδ T cells are present in the brains of MS patients and in the brains and CSF of patients with other neurologic diseases (200,201), their presence per se is not disease specific. However, both the T cells and HSPD1 expression were found in MS plaques and were not detected in the CNS of patients with other non-MS inflammatory diseases (18,29). Coexpression of HSPD1 and TCR γδ cells in the same portion of the MS lesion might imply that reactive oligodendrocytes involved in myelin repair become targeted by TCR γδ cells, which enter the CNS with other inflammatory cells. Activation of TCR γδ cells by HSPD1-positive oligodendrocytes might explain their selective depletion in MS.

SMALL HSPs

Small HSPs (HSPBs) have molecular weights between 12 and 43 kDa, which distinguish them in size from large HSPs (202,203). There are 10 human HSPBs (204). All of the proteins in this family contain the so-called *α-crystallin domain*, a region composed of 90 residues that is homologous to the corresponding region in the primary structure of the main lens proteins HSBP4 and HSPB5 (205). This domain is considered to be an important hallmark of small HSPs, independent of their origin and nature (206). In addition, HSPBs have the capacity to form oligomers (207). As chaperone proteins, HSPBs bind misfolded proteins and prevent them from aggregating, similarly to high-molecular-weight chaperones. However, HSPBs are unable to actively refold the protein themselves because of their lack of ATPase activity. Instead, they sequester the misfolded proteins within the cell to prevent aggregation until a large HSP can assist in refolding (208). Under physiologic conditions, most HSPBs form multisubunit oligomers via their α-crystallin domains (209).

HSPBs in the Nervous System

Almost all HSPBs are constitutively expressed at low levels in the brain (210). Only 3 members of this family, including HSPB1 and HSPB5, are induced in response to cellular stress (211). HSPB1 is induced during development and stressful conditions such as heat stress (68). In addition, it has been reported that HSPBs have antiapoptotic functions (212,213). Although the role of HSPBs has not been established, HSPB1 and HSPB5 have been implicated in several neurologic disorders. HSPBs are frequently released extracellularly in the CNS, as well as in pathologic conditions such as Alzheimer disease (214,215). Both HSPB1 and HSPB5 can be secreted through exosomes (44,216,217), suggesting that they may have additional roles outside the cell.

A general function of extracellular HSPBs is the activation of macrophages or macrophage-like cells during inflammation (12). As with other HSPs, it is likely that this action is mediated by TLRs or other scavenger receptors (72,75).

HSPBs in MS

Several studies have suggested that both HSPB5 and HSPB1 are present in demyelinating plaques in the brains of MS patients (218). Ce et al (219) evaluated HSPB1 blood levels during both relapse and remission phases in acute MS patients. The authors observed a striking increase in HSBP1 levels during MS relapse. In contrast, serum HSPB1 levels in MS patients were only slightly increased during the remission period. The authors hypothesized that the overexpression of HSPB1 during MS might exert a protective role by

inhibiting the misfolding of proteins and the aggregation of toxic substances. They conceded that their study design cannot explain the exact role of the HSPB1 elevation during MS, however.

Van Noort et al (220) first demonstrated the involvement of HSPB5 in MS pathogenesis by showing that this molecule was the most immunodominant myelin T-cell antigen in this disease. Multiple sclerosis–affected brain tissue is in a state of persistent oxidative stress and diffuse mild inflammation (221–227). This state is associated with the widespread enhanced expression (up to 20-fold) of the glial stress protein HSPB5 (44,228–232). HSPB5 is selectively induced in glial cells by oxidative stress but not in astrocytes or axons in so-called *preactive MS lesions* in MS NAWM (232). Moreover, HSPB5 acts as an intracellular signaling factor. In fact, HSPB5 is the major target of CD4-positive T-cell immunity, particularly when it accumulates to relatively high levels (34,228,232).

The hypothesis of van Noort and colleagues was based on the reactivity of peripheral blood mononuclear cells from both MS patients and healthy subjects to proliferate in response to the myelin fraction containing HSPB5 obtained from MS brains. These findings suggested that HSPB5 may be an autoantigen in MS and that immune cells attacked endogenous HSPB5 as part of the pathogenetic mechanisms in MS patients. This hypothesis was also supported by data showing high levels of HSPB5 in astrocytes and oligodendrocytes in MS lesions (233,234); as demonstrated later, this HSPB was the most abundant transcript in MS lesions when compared with the brain tissue of healthy controls (42). Additional studies have validated the initial reports that HSPB5 is elevated in the brains of MS patients (229,235,236) and in the blood of MS patients (237). It was recently demonstrated that HSPB5 accumulates in the cytosol of CNS oligodendrocytes but not in astrocytes or axons in ''preactive lesions'' in NAWM (232). These lesions are defined as clusters of activated microglia that appear in the absence of any obvious blood-brain barrier impairment, leukocyte infiltration, or demyelination (11,238–241). In particular, HSPB5 is also found at the interface between oligodendrocytes and microglia, as well as between the layers of the myelin sheath and axons, often in granule-like patterns of expression. In this way, oligodendrocytes may facilitate the survival of the other cell types by supplying them with HSPB5 released by exosomes (217,242). The existence of ''preactive lesions'' has been confirmed using several in vivo imaging techniques (243–246). According to some researchers, MS patients displayed abnormal immunity because of the migration of peripheral activated T cells into the CNS and the tissue specificity of the inflammatory process. In contrast, van Noort et al (247) proposed that these observations could also be caused by the interaction of IFN-γ and HSPB5. Interferon-γ promotes the activation of microglia and macrophages, thereby enhancing tissue destruction. In addition, IFN-γ kills OPCs, preventing the process of remyelination (248). As demonstrated by van Noort et al (247), HSPB5 accumulates in oligodendrocytes and myelin in the MS brain because of neurodegeneration. These authors hypothesized a mechanism of interaction between IFN-γ and HSPB5 to explain the development of an MS lesion. According to this model, an abnormal immune system is not required for the development of MS. The large amount of HSPB5 in the CNS of MS patients is presented by perivascular antigen-presenting cells. This event triggers a response by HSPB5-reactive memory T cells, which release IFN-γ. Thus, IFN-γ modifies the originally protective effects of HSPB5, which then become proinflammatory through TLR2 signaling. This process initiates a positive feedback loop that increases myelin destruction. In conclusion, in this model, no abnormal autoimmune reactions are needed to trigger MS lesions (249).

Nevertheless, attempts to induce EAE using HSPB5 as an antigen rather than using a myelin antigen have been unsuccessful (250, 251). Although HSPB5 is upregulated during the course of MS, its role might be protective rather than pathologic. In 2007, a study by Ousman et al (252) demonstrated that mice deficient in HSPB5 developed more severe EAE than wild-type mice (especially in clinical paralysis), and that treatment with exogenous HSPB5 ameliorated the signs. More severe EAE was caused by an elevated inflammatory state of immune cells, a higher level of immune cell infiltration (i.e. CD4-positive lymphocytes and macrophages) into the brain and increased demyelination in the brain and spinal cord in both the acute and progressive phases of the disease. HSPB5 is a negative regulator of inflammation in EAE and in the brains of MS patients and is a potent modulator of glial apoptosis (253). In particular, HSPBs, especially HSPB5, have been shown to exert protective roles after their release into the extracellular environment. In fact, exogenous administration of HSPB5 in deficient mice decreases immune infiltration into the brain and shifts the phenotype of these immune cells to an anti-inflammatory state. However, cessation of protein therapy resulted in the return of paralytic signs, similar to the effects of the biologic inhibitor (237). Experimental data demonstrated that the level of circulating HSPB5 was lower in normal plasma than in plasma from MS patients. In particular, an increased level of HSPB5 is observed in inflammatory sites in mice with EAE because of apoptotic cell release or to direct release through exosomes. Similarly, healthy donors displayed small numbers of inflamed loci that stimulated the synthesis of HSPBs because of their increased temperature. The inability of HSPB5 to modulate the proliferation of T or B cells in in vitro experiments, along with its inability to ameliorate clinical EAE when induced directly by myelin-specific Th17 transfer, suggests that the inhibition of inflammation is not caused by the modulation of the adaptive response but rather to the functions of the HSPB chaperone (237,254). In particular, the extracellular chaperone HSPB5 binds to partially unfolded proteins present in the plasma, such as proinflammatory cytokines. This binding ability increased as a function of the temperature (209), which increased at sites of inflammation. Kurnellas et al (255) confirmed that the chaperone activity of HSPBs was responsible for their therapeutic efficacy in EAE. They also demonstrated that bacterial HSPBs (e.g. of *Mycobacterium tuberculosis*) can modulate disease severity in a mouse model and identified the active peptides obtained from HSPB5, which showed activity equivalent to that of the entire protein. In contrast, proteins and peptides that did not exhibit chaperone activity did not have therapeutic effects on EAE.

The conflicting results between MS patients and EAE models confirm that there are relevant differences between species and that EAE is not completely equivalent to MS.

Differential HSP Expression in Chronic Active Versus Inactive MS Plaques and in Different Areas of the Active Lesion

One of the most common types of MS plaques is the chronic-active type in which lesion activity is restricted to the lesion edge (22,256,257). In such lesions, the center lacks inflammatory activity and is composed of a demyelinated parenchyma, reactive astrocytes, and glial scarring (22,257,258). Lesion activity is not always restricted to the marginal zone and may extend into adjacent NAWM.

Mycko and et al (258,259) reported the first use of a differential gene expression analysis of material obtained from different MS lesions (chronic-active and chronic-inactive) and from regions of the lesions with different activity (margin vs center) together with the adjacent white matter. The chronic active lesions displayed significant differential gene expression between the center and margin of the lesion. Silent lesions showed less evidence of differences between the 2 regions. As expected, significant differences were observed between the marginal zones of active and silent lesions (258,259). A detailed analysis of the changes in HSP genes has revealed a distinct pattern of upregulation of HSP in both the margin and the center of chronic active lesions compared with NAWM. Heat shock proteins, particularly HSPC1 and HSPA1A, were also enriched at the lesion margin of the chronic active plaques compared with the central region, which could be attributed to the heterogeneity of the pathologic processes in different regions of MS lesions. The upregulation of one of the heat shock transcription factors, HSF4, was also observed at both the margin and the center of chronic-active lesions compared with NAWM. This result suggests that HSF4 may be a major factor driving HSP activation in active lesions (260).

In addition to differential gene expression analysis, Quintana et al (192) conducted antigen microarrays to identify self-antigens in different clinical subtypes of MS and demonstrated that unique autoantibodies of the HSP signature characterize the RR, SP, and PP subtypes of MS. Strikingly, antibody responses to HSP were decreased in both SP MS and PP MS, consistent with the less inflammatory nature of progressive MS.

SUMMARY

In this review, we have considered the association of HSPs with pathogenetic mechanisms in MS. Considerable experimental data have shown that non–CNS-specific antigens may be involved in MS progression, and HSPs may be among these immunogenic factors. Many reports have demonstrated the involvement of HSPs in CNS diseases, particularly those linked with the presence and accumulation of misfolded proteins, and because HSPs are found in protein aggregates, along with disease proteins, ubiquitin or other cellular molecules, we hypothesize that both intracellular and extracellular HSPs reverse the effect of the mutant gene or refold the misfolded proteins. In contrast, conditions in which CNS immune activation is a prominent feature, such as ischemia, neurodegenerative diseases, immune-mediated disorders, infections, and trauma, may involve extracellular HSPs because of both their ability to induce the innate and adaptive immune systems and their phylogenetic conservation. Through their interaction with cell surface receptors, extracellular HSPs are responsible for the expression of proinflammatory cytokines and chemokines and the activation of dendritic cells. The principal HSP implicated in the immune response is HSPA1A, and anti-HSPA1A autoantibodies were found to be significantly higher in the CSF of MS patients than in that of healthy controls. Moreover, the highest levels of autoantibodies were detected in patients with progressive MS, in contrast to patients with a stable disease. A higher level of autoantibodies against HSPA8 was also observed in patients with progressive MS. HSPA1A was also found in and around MS lesions, and it may be involved in the induction or exacerbation of the immunologic response because of its ability to act as a proinflammatory cytokine. Moreover, higher levels of anti-HSPA1A antibodies are always detected in stable or progressive MS than in healthy controls. This increase corresponds to the elevated production of IL-8 in THP-1 monocytes with the consequent higher levels of inflammation. In addition, there is physical contact between HSPA1A and MBP or PLP, as demonstrated by immunoprecipitation. In contrast, in EAE, pharmacologically induced overexpression of HSPA1A has a protective role because it attenuates the inflammatory response and ameliorates clinical signs.

Completely different roles were observed for intracellular HSPA members. In fact, the intracellular expression of HSPA1 and HSPA8 may be neuroprotective. In particular, HSPA8 acts as a chaperone for MBP under physiologic conditions. According to this model, HSPA8 may be required for remyelination during lesion repair in the remitting phase of MS. Indeed, there is some evidence that damage to the myelin sheath in MS patients exposes MBP to an aqueous extracellular environment that is responsible for its unfolding (261). The reduced levels of MBP in MS lesions may be responsible for the permanent myelin loss observed in these areas. In contrast, HSPA1A is responsible for inhibiting nuclear factor-κB, a transcription factor involved in the activation of various cytokines that are relevant to MS pathogenesis. Mansilla et al (262) recently studied the role of HSPA1A both in vitro and in EAE and demonstrated that, in the MOG-induced EAE model, HSPA1A promotes T-cell responses against autoantigens, and this ability is much more relevant than its capability to protect CNS cells from apoptosis induced by inflammatory injury.

Another HSP that has a positive effect on MS progression is HSPC3; in the CSF, antibodies with the ability to recognize HSPC3 induce OPC complement–mediated cell death. Experiments in mice with EAE confirmed that the inhibition of HSPC1 could reduce EAE symptoms. In addition, the chaperonin HSPD1 is responsible for oligodendrocyte depletion in MS patients. However, conflicting results have been obtained for HSPBs. For example, HSPB5 is present at levels up to 20-fold higher in glial cells in MS-affected brain samples than in normal controls, and the timing of its

expression is interesting. HSPB5 accumulates in oligodendrocytes not only during later stages of inflammation but also before any peripheral blood cells have entered the tissue—in so-called *preactive MS lesions*. Thus, HSPB5 initially induces innate immune responses that are neuroprotective, whereas its accumulation in response to neurodegeneration induces an adaptive immune response that results in tissue damage. Notably, the increase in HSPB5 levels in MS plaques can modulate inflammation depending on its chaperone role. Unlike monoclonal antibodies, which have a single target, HSPB chaperone proteins are able to bind to a broad spectrum of ligands. Therefore, they may represent a unique therapeutic reagent. Thus, it will be interesting to investigate drug treatments that cause HSP overexpression or inhibition.

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