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Presence of heat shock protein 70 in secondary lymphoid tissue correlates with stroke prognosis

Manuel Gómez-Choco [a], Cedric Doucerain [b,c], Xabier Urra [a,c], Anna M. Planas [b,c], Ángel Chamorro [a,c,d,*]

[a] Functional Unit of Cerebrovascular Diseases, Hospital Clínic, Barcelona, Spain
[b] Department of Brain Ischemia and Neurodegeneration, Institute for Biomedical Research of Barcelona (IIBB), Spanish Research Council (CSIC), Barcelona, Spain
[c] August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain
[d] School of Medicine, University of Barcelona, Spain

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ABSTRACT

Heat shock protein 70 (Hsp-70) can act as a danger signal and activate immune responses. We studied the presence of Hsp-70 in lymphoid tissue and plasma of acute stroke patients and asymptomatic controls free of neurological disease. Immunofluorescence, Western blotting, qRT-PCR and flow cytometry studies were performed. Plasma Hsp-70 concentration at day 7 was similar in patients and controls, whereas patients disclosed stronger immunoreactivity to Hsp-70 in lymphoid tissue than controls. Most Hsp-70 + cells were antigen presenting cells located in T cell zones. Stronger immunoreactivity to Hsp-70 was associated with smaller infarctions and better functional outcome.

1. Introduction

Dead cells can behave like damage-associated molecular patterns (DAMPs) and activate the innate and adaptive arms of the immune system (Kono and Rock, 2008). Moreover, DAMPs are produced after brain ischemia and may affect the clinical course and outcome of patients with acute stroke (Iadecola and Anrather, 2011; Chamorro et al., 2012). Brain-derived antigens may leave the central nervous system (CNS) *via* the blood stream (Hochmeister et al., 2010) or *via* the bulk flow of intracranial fluids along cranial nerves and perivascular spaces of brain cortical arteries and arterioles (Bradbury et al., 1981; Weller et al., 2008). After stroke, brain-derived antigens and antigen presenting cells (APCs) can reach the brain-draining secondary lymph nodes, such as the cervical lymph nodes (CLN) (Harling-Berg et al., 1999; deVos et al., 2002; van Zwam et al., 2008) and the palatine tonsils (PT) (Planas et al., 2012). At these lymph nodes, brain derived-antigens loaded onto MHC molecules could be presented to T cells, and subsequently activate them (von Andrian and Memple, 2003). Interestingly, it has been argued that immune responses originating in the CLN have a Th2 phenotype to facilitate tolerogenic mechanisms against further inflammation and brain injury (Harling-Berg et al., 1999).

Heat shock protein 70 (Hsp-70) is a molecular chaperone that may behave as a danger signal under certain conditions (Basu et al., 2000). While Hsp-70 expression is virtually undetectable in the brain under physiological conditions, it is strongly induced in neurons following brain ischemia (Nowak et al., 1990; Kinouchi et al., 1993; Sharp et al., 1993; Planas et al., 1997; de la Rosa et al., 2013). Robust experimental evidence supports the hypothesis that Hsp-70 induction is protective in brain ischemia (Nowak and Jacewicz, 1994; Yenari et al., 1998, 1999; Yenari, 2002), and that these neuroprotective effects may be due to various mechanisms including a more efficient formation, folding and assembly of nascent proteins, a better refolding and stabilization of damaged peptides, and some anti-apoptotic effects (Yenari et al., 1999; Hartl and Hayer-Hartl, 2002; Yenari et al., 2005). Likewise, Hsp70.1 knockout mice show larger infarct volumes after transient focal ischemia (Lee et al., 2001a), while transgenic mice constitutively expressing the human (Plumier et al., 1997; Rajdev et al., 2000) or rat (Tsuchiya et al., 2003) inducible Hsp-70, and mice overexpressing Hsp-70 in astrocytes (Xu et al., 2010), are protected against ischemic brain damage. Hsp-70 overexpression, however, was not protective in certain models of permanent focal (Lee et al., 2001b) or global (Olsson et al., 2004) ischemia. In ischemia/reperfusion, virally delivered Hsp-70 (Badin et al., 2006) or intravenous administration of cell penetrating recombinant

* Corresponding author at: Hospital Clínic, Villarroel, 170, 08036 Barcelona, Spain. Tel.: +34 93 227 54 14; fax: +34 93 227 57 83.
E-mail address: achamorro@ub.edu (Á. Chamorro).

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Hsp-70 (Doeppner et al., 2009; Zhan et al., 2010) exerts neuroprotective actions mediated, at least in part, by anti-apoptotic effects. Recently, anti-inflammatory effects of TAT-Hsp70 involving downregulation of nuclear factor kappa B (NF-κB) activation have been reported after ischemia/reperfusion (Doeppner et al., 2013).

In contrast to these findings, stimulation of macrophages with Hsp-70 increased the secretion of nitric oxide, proinflammatory cytokines and chemokines, and enhanced the maturation and migration of APCs to the draining lymph nodes (Basu et al., 2000; Saito et al., 2005). Indeed, Hsp-70 has important immunoregulatory functions that include binding to peptides derived from necrotic cells to facilitate their uptake and presentation to lymphocytes, and the activation of antigen-specific T cells (Binder and Srivastava, 2005). Recently, Hsp-70 purified from a non-bacterial origin to exclude the possibility of microbial contamination, was shown to reduce the stimulatory capacity of dendritic cells and T cell responses *in vitro*, indicating that Hsp-70 moderated immune mediated inflammatory reactions (Stocki et al., 2012). Therefore, in addition to anti-apoptotic (Yenari et al., 2005) and anti-inflammatory (Zheng et al., 2008) actions on brain cells after ischemia, Hsp-70 may also attenuate immune responses.

In this study, we sought to identify the presence of Hsp-70 in brain draining lymph nodes of patients with acute stroke, as well as to assess whether the expression levels of Hsp-70 were associated with the main clinical outcomes in these patients.

2. Materials and methods

2.1. Patients

Forty-six patients with acute stroke were enrolled in this study after excluding patients with a history of infection, and an intake of antibiotics, steroids, or immunosuppressants within the preceding 3 months. Neurological impairment was assessed with the National Institutes of Health Stroke Scale (NIHSS), and functional outcome was measured at 3 months with the modified Rankin Scale (mRS). The volume of brain infarction was measured at day 7 ± 2 (mean ± SD) after stroke, using diffusion-weighted imaging (DWI) MRI (MRIcro software, Chris Roden, University of Nottingham, Nottingham, UK). Controls free of neurological symptoms (n = 16) were recruited from patients' relatives, or from obstructive sleep apnea syndrome clinics. The study was approved by the local ethics committee and participants or their relatives gave their written informed consent.

2.2. Blood and tissue samples

Blood samples were collected in 46 patients at day 7 of stroke and in 11 controls to measure the plasmatic levels of Hsp-70 protein using a commercial ELISA kit (#ADI-EKS-715, ENZO Life Sciences). PT biopsies were obtained without complications in 12 stroke patients at 85 ± 36 (mean ± SD) hours from stroke onset, and in 7 controls. Biopsy samples were divided into 1, 2 or 3 parts depending on the size of the specimens obtained; that on average were approximately $5 \times 3 \times 2$ mm^3 in size. Post mortem CLN and/or PT samples of 15 additional patients with malignant brain infarction were obtained from the Tumour Bank, Hospital Clínic-IDIBAPS Biobank at a median of 7 days after stroke onset (ranging from 1 to 21 days).

2.3. Flow cytometry

Lymphocyte subsets were analyzed in fresh tissue from PT biopsies of patients (n = 7) and controls (n = 6) using flow cytometry immediately after tissue extraction by investigators blinded to the clinical data. A cellular suspension of the PT was obtained after repetitive squirting with RPMI culture medium using a fine needle, obtaining a total of around 10 to 15×10^5 cells. A volume of 100 μL of the cell suspension, containing at least 2×10^5 cells, was used for each test, which allowed for the quantification of different lymphocyte subtypes and the use of appropriate isotype controls. The following monoclonal antibodies were used to identify T lymphocytes, T helper lymphocytes, cytotoxic T lymphocytes, natural killer cells and B lymphocytes using flow cytometry: CD25 and CD19 conjugated to fluorescein isothiocyanate, CD3, CD4 and CD56 conjugated to phycoeritrin, CD45, CD8 and CD4 conjugated to peridinin chlorophyll, and CD3 conjugated to allophycocyanin (BD Biosciences). Apoptotic lymphocytes were identified using the Annexin V FITC kit (Bender MedSystems, Vienna, Austria). Each lymphocyte subset was recognized using CD3, CD4, CD8, CD19 and CD56 antibodies conjugated to PE (all from BD Biosciences). The proportion of Annexin-V positive cells was calculated for each lymphocyte subset.

2.4. Immunofluorescence

Immunofluorescence for Hsp-70 was performed in the PT biopsies of 6 patients and 7 controls. PT biopsies and post mortem PT and CLN samples were snapped frozen in optimum cutting temperature (OCT) compound and stored at −80 °C. Cryostat sections were fixed in acetone, blocked with corresponding goat or rabbit serum and incubated overnight at 4 °C with primary antibodies followed by corresponding fluorescent secondary antibodies (Table 1). Immunofluorescence controls were carried out by omission of the primary antibody to verify the absence of non-specific staining. Samples were counterstained with Hoechst. The number of Hsp-70 positive cells in PT samples was counted under the fluorescence microscope by an investigator blinded to the clinical data. The mean value obtained in three areas per sample was taken as representative of the sample value. Double immunohistochemistry was carried out to identify the location and nature of the Hsp-70 immunoreactive cells within the lymphoid tissue. The two primary antibodies were obtained in different species to avoid cross-reactions (see Table 1).

2.5. Western blotting

Western blotting was carried out in PT biopsy tissue (weighing around 30 mg) from stroke patients (n = 9) and controls (n = 5). After extraction, tissue was immediately frozen and kept at −80 °C. Protein extraction was carried out using radioimmunoprecipitation (RIPA) buffer. Ten μg of protein was loaded in 10% polyacrilamide gels, run under denaturing conditions and proteins were transferred to a PVDC membrane. Primary antibodies used for Western blotting were a mouse monoclonal antibody against Hsp-70 (#HSP01 Millipore, diluted 1:1,000) and a rabbit polyclonal antibody against N-methyl-D-aspartate (NMDA) receptor subunit NR-2A (#AB155, Chemicon; 1:100). Mouse monoclonal Abs against GAPDH (#CSA-335, Stressgen; 1:500), or β-tubulin (#T4020, Sigma; 1:5000), were used as protein gel loading controls. Control and patient samples were run in parallel in the gels. The optical density of the bands was measured in a densitometer. After correcting for protein loading with the density of the reference band, relative band intensity was expressed as percentage of control.

2.6. RT-PCR

PT biopsy tissue from patients (n = 12) and controls (n = 5) was immediately frozen, RNA was extracted, and gene expression analysis was carried out. Quantitative real-time PCR (qRT-PCR) was performed using FAM-labeled Taqman® Gene Expression Assay probes for Hsp72 (Hs00358147_s1) and β-actin (Hs03023943_g1) (Applied Biosystems, Foster City, CA, USA). The amplification conditions were: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. CT values were analyzed using the $2^{-\Delta\Delta C_T}$ method.

2.7. Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normal distribution of the data, and the Fisher's exact test, Student's *t* test, Mann–