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Effects of heat shock protein 72 (Hsp72) on evolution of astrocyte activation following stroke in the mouse

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ABSTRACT

Astrocyte activation is a hallmark of the response to brain ischemia consisting of changes in gene expression and morphology. Heat shock protein 72 (Hsp72) protects from cerebral ischemia, and although several protective mechanisms have been investigated, effects on astrocyte activation have not been studied. To identify potential mechanisms of protection, microarray analysis was used to assess gene expression in the ischemic hemispheres of wild-type (WT) and Hsp72-overexpressing (Hsp72Tg) mice 24 h after middle cerebral artery occlusion or sham surgery. After stroke both genotypes exhibited changes in genes related to apoptosis, inflammation, and stress, with more downregulated genes in Hsp72Tg and more inflammation-related genes increased in WT mice. Genes indicative of astrocyte activation were also upregulated in both genotypes. To measure the extent and time course of astrocyte activation after stroke, detailed histological and morphological analyses were performed in the cortical penumbra. We observed a marked and persistent increase in glial fibrillary acidic protein (GFAP) and a transient increase in vimentin. No change in overall astrocyte number was observed based on glutamine synthetase immunoreactivity. Hsp72Tg and WT mice were compared for density of astrocytes expressing activation markers and astrocytic morphology. In animals with comparable infarct size, overexpression of Hsp72 reduced the density of GFAP- and vimentin-expressing cells, and decreased astrocyte morphological complexity 72 h following stroke. However, by 30 days astrocyte activation was similar between genotypes. These data indicate that early modulation of astrocyte activation provides an additional novel mechanism associated with Hsp72 overexpression in the setting of ischemia.

Introduction

Astrocytes are dynamically involved in synaptic transmission (Perea and Araque, 2007; Perea et al., 2009), metabolic and ionic homeostasis (Dienel and Hertz, 2005), inflammation, and maintenance of the blood brain barrier (Ballabh et al., 2004). Within the central nervous system astrocytes respond to insults including infection, trauma, neurodegenerative disease, and ischemia by activation, or reactive astrogliosis, which involves changes in gene expression and morphology, and may lead to glial scar formation and cell proliferation (Correa-Cerro and Mandell, 2007; Eddleston and Mucke, 1993; Maragakis and Rothstein, 2006; Pekny and Nilsson, 2005; Sofroniew, 2005, 2009). Astrocyte activation includes the morphological changes of hypertrophy of cellular processes and hyperplasia of the cell body, and gene expression changes including induction of glial fibrillary acidic protein (GFAP) and re-expression of the radial glial markers vimentin (Vim), nestin, and somewhat delayed expression of brain lipid binding protein (BLBP) (White and Jakeman, 2008). The intermediate filament network is very prominent in the main processes and soma of activated astrocytes (Bushong et al., 2002, 2004).

Ischemia and subsequent reperfusion cause increased production of reactive oxygen species (ROS) and oxidative stress (Niizuma et al., 2009), one of the triggers of astrocyte activation (Pekny and Nilsson, 2005). Hsp72, a member of the 70 kDa class of molecular chaperones or heat shock proteins (Hsps) regulates both apoptotic and necrotic cell death (Giffard et al., 2004; Parcellier et al., 2003; Yaglom et al., 2003) and when overexpressed is associated with reduced oxidative stress (Xu et al., 2009; Zheng et al., 2008). Previous studies showed that overexpressing Hsp72 protects from cerebral ischemia (Giffard et al., 2008; Hoehn et al., 2001; Lee et al., 2006; Plumier et al., 1997; Rajdev et al., 2000; van der Weerd et al., 2005; Xu et al., 2009; Yenari et al., 1998; Zheng et al., 2008), but the effects of Hsp72 overexpression on overall gene expression and astrocyte activation following focal ischemia are not well understood.

To further assess the neuroprotective effects of Hsp72 overexpression, gene expression profiling was performed in wild-type (WT) and Hsp72 overexpressing (Hsp72Tg) mice. This analysis showed that many genes,

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including genes associated with astrocyte activation, are differentially expressed after ischemia, even when harvesting all cell types in the injured hemisphere. Among the differentially regulated genes in response to ischemia were 8 astrocyte genes largely involved in inflammation. The effect of Hsp72 overexpression on astrocyte activation in response to focal ischemia was then quantitated by cell counting and morphological analysis of astrocytes in the ischemic penumbra at different time points after stroke. This showed that astrocytes express a time-dependent activation profile, and astrocytes in Hsp72Tg mice have a significantly less-activated phenotype, independent of lesion size. These data demonstrate that astrocyte activation changes in the lesion penumbra over time, indicative of different astrocyte functions or phenotypes over a time course extending from hours to 30 days. Reduced early astrocyte activation in Hsp72Tg mice may contribute to the observed neuroprotection.

Materials and methods

Hsp72Tg mice

Adult male mice expressing a chimeric transgene of the rat-inducible Hsp72 gene under control of the chicken-β-actin promoter and human cytomegalovirus enhancer (Hsp72Tg) were originally produced by Dillmann and colleagues (Marber et al., 1995) and back bred into the C57BL/6 background. The strain is maintained as a heterozygous transgenic (Tg) line. Tg and wild type littermates were used for experiments after genotyping by polymerase chain reaction (PCR) analysis of tail DNA. C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA).

Focal cerebral ischemia

Transient ischemia was induced using the suture occlusion technique, as previously described (Han et al., 2009), with slight modifications. Male mice 25 ± 5 g aged 2–4 months were anesthetized, and the left carotid artery bifurcation was exposed. A 6–0 monofilament nylon suture (Doccol Co., Redlands, CA, USA) was inserted from the common carotid artery (CCA) into the internal carotid artery to occlude the left middle cerebral artery (MCA) at its origin. After 60 min the suture was removed for reperfusion, the CCA ligated, and the wound closed. Sham-operated animals underwent identical procedures as far as isolation of the carotid bifurcation but without opening the artery or suture insertion. Rectal temperature was maintained at 37 °C ± 0.5 °C controlled by a Homeothermic blanket control unit (Harvard Apparatus, Holliston, MA, USA). Temperature and respiratory rate were monitored during the surgery, the duration of which was always 20 min. Infarct volumes are generally 35–45% of the hemisphere (Xiong et al., 2011).

Preparation of microarray samples

C57BL/6 and Hsp72Tg mice were deeply anesthetized with isofluorane and sacrificed 24 h after MCAO or sham surgery. Brains were rapidly removed, divided into hemispheres and RNA was extracted from the ischemic hemisphere or for sham animals the ipsilateral hemisphere using Qiagen RNeasy® Midi kit (Alameda, CA, USA). Total RNA samples were processed at the Stanford Protein and Nucleic Acid Biotechnology Facility by one-cycle target preparation, labeling, and hybridization to Affymetrix 430_2® (Affymetrix, Santa Clara, CA) whole genome mouse arrays, according to the manufacturer's protocol. Three animals were analyzed for each genotype at 24 h perfusion after MCAO, and two animals of each genotype for sham.

Microarray data analysis

Raw image files were processed using Affymetrix GCOS software. The Significance Analysis of Microarrays (SAM) method (Tusher et al., 2001) was used to determine genes that were significantly differentially expressed between WT and Hsp72Tg and those differentially expressed between sham and MCAO conditions. For SAM analysis (Tusher et al., 2001) initial chip processing and signal calling was done with R (http://rss.acs.unt.edu/Rdoc/library/siggenes/html/sam.html) and Bioconductor (Gentleman et al., 2004) using the Affymetrix package (Gautier et al., 2004). The RMA background correction method, quantile normalization and the median polish probe set summary method were employed. Differentially expressed genes were those with a fold-change of ≥2.0 and a p value of <0.05. Data sets are posted at the Gene Expression Omnibus (GSE#28731). A current gene assignment for the Affymetrix probe sets was done using the Gene ID conversion tool on the DAVID bioinformatic resource (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003; Huang da et al., 2009). To determine over- and under-represented gene ontology (GO) categories, the lists of all genes showing ≥2 fold change and $p<0.05$ for each genotype were uploaded and analyzed with the PANTHER Gene Expression Data Analysis tools on the PANTHER web site (http://www.pantherdb.org) (Thomas et al., 2003, 2006). The expected number of genes is calculated from the percentage of genes from the mouse genome in each category and the number of differentially expressed genes used for the analysis. A p-value cutoff of 0.05 was chosen to identify over-represented GO categories.

Tissue fixation and immunohistochemistry

At 3, 6, 12, and 24 h, 2, 3, 7, 14 and 30 days after stroke or sham-operation, mice were sacrificed for analysis. Animals were deeply anesthetized with isofluorane, and perfused through the left cardiac ventricle, first with 0.9% cold saline and then with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4). Brains were removed and immersed for 2 days at 4 °C in the same fixative and then rinsed with PBS. Coronal sections, 40 μm thick, were made with a Vibratome (VT 1000 S; Leica Microsystems, Wetzlar, Germany).

Immunofluorescence

Immunohistochemistry was carried out on free-floating sections under moderate shaking. Sections were incubated for 1 h in a blocking solution (0.1 M PBS, 0.3% Triton X-100 and 5% equine serum). After three washes in 0.1 M PBS, sections were incubated for 3 days at 4 °C with primary antibody for glial fibrillary acidic protein (GFAP, diluted 1:5, #22522, Immunostar, Hudson, WI, USA), vimentin (diluted 1:100, sc-7557, Santa Cruz Biotechnology, CA), or brain lipid binding protein (BLBP, diluted 1:400, #32423, Abcam, Cambridge, MA). After incubation with primary antibody, sections were rinsed in buffer and incubated for 2 h at room temperature with Alexa Fluor 488- (for GFAP) or 594- (for vimentin and BLBP) conjugated secondary antibodies (diluted 1:300, Invitrogen, Carlsbad, CA), washed three times in PBS, and mounted on glass slides using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

Immunoperoxidase

All washes and incubations were done in a washing buffer containing 0.1 M PBS, 0.3% BSA and 0.3% Triton X-100. After 1 h in a blocking solution endogenous peroxidase activity was quenched for 10 min at room temperature in 3% hydrogen peroxide in 30% methanol. After several washes, sections were incubated for 3 days at 4 °C with a polyclonal anti-rabbit antibody for S100β (diluted 1:200, Z0311, DAKO, Carpinteria, CA), or a monoclonal antibody for glutamine synthethase (GS, diluted 1:100, MAB302, Millipore, Bedford, MA). After incubating sections with primary antibody, sections were