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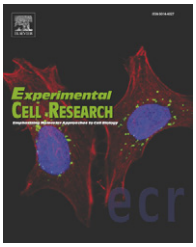

Research Article

Properties of astrocytes cultured from GFAP over-expressing and GFAP mutant mice

*Woosung Cho, Albee Messing**

Waisman Center and Department of Comparative Biosciences, University of Wisconsin-Madison, 1500 Highland Ave, Rm 713, Madison, WI 53705, USA

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ABSTRACT

Alexander disease is a fatal leukoencephalopathy caused by dominantly-acting coding mutations in GFAP. Previous work has also implicated elevations in absolute levels of GFAP as central to the pathogenesis of the disease. However, identification of the critical astrocyte functions that are compromised by mis-expression of GFAP has not yet been possible. To provide new tools for investigating the nature of astrocyte dysfunction in Alexander disease, we have established primary astrocyte cultures from two mouse models of Alexander disease, a transgenic that over-expresses wild type human GFAP, and a knock-in at the endogenous mouse locus that mimics a common Alexander disease mutation. We find that mutant GFAP, as well as excess wild type GFAP, promotes formation of cytoplasmic inclusions, disrupts the cytoskeleton, decreases cell proliferation, increases cell death, reduces proteasomal function, and compromises astrocyte resistance to stress.

Introduction

Glial fibrillary acidic protein (GFAP) is the major intermediate filament protein in astrocytes. Heterozygous missense mutations within the coding region of *GFAP* account for the majority of cases of Alexander disease, a fatal neurodegenerative disorder that typically affects young children [1]. Many patients suffer seizures and/or macrocephaly as their initial clinical sign, and then experience a variety of delays or regression in psychomotor development. MRI of patients with infantile onset reveals a frontal leukodystrophy with characteristic changes in periventricular regions [2]. From its initial description by Alexander [3], attention focused on astrocytes as the instigators of disease because of the hallmark proteinaceous aggregates found within their cytoplasm — Rosenthal fibers. More recent biochemical studies show that Rosenthal fibers are complex mixtures of GFAP, vimentin, αB-crystallin, HSP27, plectin, and p62 (and other unknown components) [4–8], and bear some resemblance to the neurofilament-containing Lewy bodies of neurons and the keratin-containing Mallory bodies of hepatocytes.

Whether Rosenthal fibers per se cause astrocyte dysfunction, and what the precise trigger(s) is for their formation, is not clear. These inclusions have long been known to occur in the context of chronic gliosis or up-regulation of GFAP expression of various causes. The first description of Rosenthal fibers was from a patient with syringomyelia [9], and subsequently they have been observed in a wide variety of conditions including multiple sclerosis [10] and pilocytic astrocytomas [11] (for a more complete review, see [12]). Transgenic studies clearly show that simply elevating levels of wild type GFAP to a sufficient degree will lead to Rosenthal fibers [13], and it is possible that reactive astrocytes (that also up-regulate GFAP) and Alexander disease astrocytes (expressing a mixture of mutant and wild type GFAP) have certain properties in common.

Precisely how mutations in GFAP lead to the pleiotropic manifestations of Alexander disease is not known [14,15]. Nearly half of all patients carry mutations in either of two amino acids,

* *Corresponding author.* Fax: +1 6082634364.
E-mail address: messing@waisman.wisc.edu (A. Messing).

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R79 or R239, although it appears that mutations distributed throughout the protein produce essentially identical Rosenthal fibers and similar disease [16,17]. A number of arguments point to the idea that the GFAP mutations, which are genetically dominant, act in a gain-of-function fashion, and that elevations of total GFAP levels are a major factor in pathogenesis. One way in which this issue has been studied is by transfection of cultured cells, where over-expression of either mutant or wild-type GFAP leads to the formation of cytoplasmic protein aggregates with recruitment of small stress proteins and shifts in GFAP solubility [18,19,20]. Multiple positive feedback loops act to further increase accumulation of GFAP, both by inhibition of proteasomal degradation and by increased expression. Activation of JNK and p38 also occurs, and may further contribute to GFAP accumulation [21]. However, the aggregates formed via transfection either fail to replicate the morphological features of Alexander disease Rosenthal fibers [18], or are studied in non-astrocytic cell lines [20]. In addition, the effects of GFAP alterations on cell lines may not be identical to changes that are induced in bona fide astrocytes.

Mouse models have been created via both transgenic and knock-in approaches that reproduce key aspects of the Alexander phenotype, particularly the formation of Rosenthal fibers identical to those found in the human disease [13], and increased seizure susceptibility [22,23]. To provide new tools for investigating the nature of astrocyte dysfunction in Alexander disease, we have established primary astrocyte cultures from two of these mouse models (a knock-in at the endogenous mouse locus of the R236H mutation [22], and a transgenic over-expressing wild-type GFAP, termed TgGFAP-wt [13]), and studied their properties in culture. We find that mutant GFAP, as well as excess wild type GFAP, promotes formation of cytoplasmic inclusions, disrupts the cytoskeleton, decreases cell proliferation while increasing cell death, reduces proteasomal function, and compromises astrocyte resistance to stress.

Materials and methods

Primary cortical astrocyte cultures

Cortical astrocyte cultures are prepared from 0–2 day old postnatal mice, either heterozygotes (for the R236H mice [22]), hemizygotes (for the TgGFAP-wt mice, 73.7 line [13]), or wild-type controls. To facilitate comparisons between these *in vitro* studies and ongoing *in vivo* experiments utilizing crosses between the R236H knock-in mice and the TgGFAP-wt mice [22], all cultures described here were derived from mice that are F1 hybrids between the two parental background strains (FVB/N and 129S6, both obtained from Taconic Farms). The cortices from individual pups were freed of meninges and placed into DMEM (Gibco) without serum, mechanically dissociated into single cells, suspended in medium containing DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), seeded into T25 flasks (one brain per flask), and maintained in a humidified 5% CO_2 atmosphere at 37 °C. Tail samples were collected simultaneously from the pups for genotyping. At 48 h the serum was reduced to 1% FBS. Medium was changed every 3 days. At 14–16 days *in vitro* (DIV), the flasks were shaken overnight at 200 rpm to remove oligodendrocytes and microglia. The adherent astrocyte population was detached by incubating briefly in 0.25% trypsin-EDTA (Gibco), and then pooled by genotype within the same litter. The cells were then suspended in DMEM with 10% FBS and plated ("passage 2") on either 35 mm dishes, 6-well plates, or 96-well plates (Corning) as needed. Unless stated otherwise, all experiments described here utilized cells grown for varying days *in vitro* (DIV) at passage 2. Tissue culture dishes and plates were pre-coated with 100 μg/ml poly-L-lysine for 1 h in a humidified 5% CO_2 atmosphere at 37 °C, and then allowed to air dry. The purity of these cultures is typically >95% GFAP-positive cells as determined by immunostaining.

Electron microscopy

Primary astrocytes were grown for 3 days at passage 2 on glass coverslips and fixed with 2.5% glutaraldehyde — 2% paraformaldehyde in 0.1 M $NaPO_4$ buffer, pH 7.4. Cells were postfixed before embedding in Polybed 812. Images of thin sections were taken with a Philips CM120 scanning transmission electron microscope at 80 kV at the University of Wisconsin Medical School Electron Microscopy Facility.

GFAP quantitation via sandwich ELISA

Quantitation of total GFAP levels was obtained using a sandwich ELISA on cells plated at a density of 5000 cells/well in white 96-well plates (Becton-Dickinson). After 48 h, cells were lysed with 100 μl buffer containing 1% SDS, 2 mM EDTA, and 50 mM Tris, pH 7.5, supplemented with complete proteinase inhibitor cocktail (Roche Applied Sciences). Separate microtiter plates were coated with the SMI-26 anti-GFAP mouse monoclonal antibody cocktail (1:1000, Covance, diluted in PBS) overnight at 4 °C, rinsed 3 times in PBS, and then blocked with Blotto (5% nonfat milk /PBS) for 2 h at RT. Samples from the astrocyte cell lysates (20 μl per well) were loaded on the plates and brought up to 100 μl with 0.5% Triton X-100/PBS (a buffer used for all subsequent antibody incubations and rinses), and incubated for 2 h at RT. The plates were rinsed three times and then incubated with a rabbit polyclonal anti-GFAP antibody (1:5000, DAKO) overnight at 4 °C. The plates were rinsed and then incubated with HRP-conjugated goat anti-rabbit IgG (1:30,000, Sigma) for 2 h at RT. After three final rinses, peroxidase activity was detected using the SuperSignal Femto Maximum Sensitivity Substrate (PIERCE) with a GloRunner microplate luminometer (Turner Biosystems). The GFAP content in the samples was determined from a standard curve generated using serial dilutions of purified GFAP (Research Diagnostics), and normalized to the total protein content in each sample as determined by the BCA protein assay kit (PIERCE).

To test whether differences between groups might be an artifact of alterations in affinities for the antibodies contained in the SMI-26 cocktail used for the initial capture step, the ELISA was repeated three times using the individual components of this cocktail for capture (with the exception of SMI-21, which recognizes human but not mouse GFAP). All three monoclonals (SMI-23, SMI-24, and SMI-25) gave similar results for both the purified bovine GFAP used in the standard curve, and the comparisons between wild type, R236H, and TgGFAP-wt astrocytes (data not shown).

Astrocyte growth curves

Direct cell counting was conducted on cells grown in 6-well plates or in 35 mm dishes. Cells were plated at a density of 5×10^4 cells/well, and then maintained in DMEM supplemented with 10% FBS.