Published in final edited form as:

Exp Cell Res. 2009 April 15; 315(7): 1260–1272. doi:10.1016/j.yexcr.2008.12.012.

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

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Summary

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.

Keywords

glial fibrillary acidic protein (GFAP); Alexander disease; Rosenthal fiber (RF); astrocytes

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, α B-crystallin, HSP27, plectin, and p62 (and other unknown components) [4,5,6,7,8], and bear some resemblance to the neurofilament-containing Lewy bodies of neurons and the keratin-containing Mallory bodies of hepatocytes.

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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, 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, promote formation of cytoplasmic inclusions, disrupt the cytoskeleton, decrease cell proliferation while increasing cell death, reduce proteasomal function, and compromise 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% CO₂ atmosphere at 37°C. Tail samples were collected simultaneously from the pups for genotyping. At 48 hours 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 hr in a humidified 5% CO₂ 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 NaPO₄ 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 hours, 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 hours 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 hours 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 hours 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. Cells were incubated in a trypan blue solution and the negative cells were counted using a hemocytometer on days 3, 6, 9, and 12 DIV after plating. In separate experiments, cell growth rate was also evaluated using the colorimetric MTS assay (conversion of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) according to the manufacturer's instructions (Promega). For these

experiments, 2000 cells/well were plated in 96-well plates and the cells were assayed at days 1, 6, and 9 DIV after plating. Duplicate wells or dishes were measured for each litter and time point assayed.

BrdU incorporation assay

To measure astrocyte proliferation, ~20,000 cells were plated into each chamber of poly-lysine coated 4-well chamber slides (Nalge Nunc). At various times after plating, cells were incubated in 10 μ M BrdU (Sigma, B5002) in DMEM, overnight at 37°C. The cells were fixed with ice cold methanol for 10 min., rinsed three times in PBS, rehydrated in PBS overnight, incubated in 2N HCl/PBS for 20 min at RT, and then rinsed three times in PBS again. After treatment with blocking buffer (5% donkey serum, 0.2% Triton X-100 in PBS) for 1 hour at RT, the cells were then incubated with polyclonal anti-GFAP (1:1000, DAKO) and monoclonal anti-BrdU (1:1000, Sigma) antibodies in 1% donkey serum / PBS, overnight at 4°C. After rinses in PBS, Alexa 488 and 594 secondary antibodies (all at 1:500 dilutions, Molecular Probes) were applied for 1 hour at RT. Fluorescence was detected with a Nikon Microphot microscope and a 20X PlanApo objective, equipped with a Spot-2 camera (Diagnostic Instruments). Approximately 50 (day 2) to 200 (day 6) cells were counted for each genotype, in triplicate slides, using a 20x objective. Data was obtained from cultures derived from three independent litters.

Cell size measurement

Cells were grown for 14-16 DIV after initial plating, detached by brief incubation in 0.25% trypsin-EDTA, washed twice in DMEM with 10% FBS, and incubated in trypan blue. A 500 μ l cell suspension was loaded into the counting chamber of a Coulter Counter (Vi-Cell XR cell viability analyzer, Beckman). At least 1200 viable cells were measured for each sample. Cell sizes and distribution were analyzed using Vi-CELL software (Beckman).

Cell death

Caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay according to the manufacturer's instructions (Promega). Briefly, cells were plated at 5000 cells/well in white-walled 96-well plates and maintained in 5% CO₂ at 37°C. At various time points (2, 4, and 6 DIV, passage 2) the cells were incubated with the Caspase-Glo reagent at room temperature (RT) for 1 hour in the dark, and luminescence was measured in a plate-reading illuminometer (Glo-Running Microplate Luminometer, Turner Biosystems). The data was normalized to the total protein content in each well as determined by the Bradford assay (BioRad).

Cell death was further evaluated using the LIVE/DEAD Viability/Cytotoxicity assay (Molecular Probes). Astrocytes were cultured for 4 DIV in poly-lysine-coated chamber slides (Nalge Nunc). Cells were rinsed twice with serum-free DMEM and then incubated with 1 μ M EthD-1 and 2 μ M calcein-AM for 30 minutes at RT. Stained cells were rinsed with PBS, mounted in Vectashield hard-set mounting medium (Vector Labs) and visualized on a Nikon Microphot fluorescent microscope using a 40x Plan-Apo objective (NA 0.95). A minimum of 300-400 cells were counted for each slide. Cell death was calculated as the percentage of EthD-1 positive cells compared to the total population.

Fluorescence microscopy

Cells were grown in chamber slides (10,000 cells/well) for 7 days, rinsed in PBS, and then fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 minutes at RT. After permeabilization with 100% EtOH for 2 minutes, the cells were then blocked with 5% normal goat or donkey serum (depending on the species of the secondary antibody) in 0.1% Triton X-100 for 1 hour. Cells were then incubated in the following primary antibodies overnight at 4°C: mouse monoclonal anti-GFAP, 1:1000 (Chemicon); rabbit polyclonal anti-GFAP at 1:1000 (DAKO);

rabbit polyclonal anti- α B-crystallin, 1:250 (Chemicon); rabbit polyclonal anti-vimentin, 1:250 (gift of Dr. Virginia Lee, University of Pennsylvania); mouse monoclonal anti-vinculin, 1:250 (Sigma). Slides were washed three times with PBS and then incubated with secondary anti-mouse or anti-rabbit antibodies conjugated to Alexa 488 or Alexa 594 (Molecular Probes) at a dilution of 1:500 for one hour at RT. To visualize F-actin, cells were incubated in PBS overnight at 4°C in 2.5 μ g/ml Phalloidin-TRITC (Sigma). Slides were mounted in with Vectashield hard-set mounting medium and images obtained from Nikon C1 confocal or Zeiss Axioplan II photomicroscopes at the Waisman Center's Cellular and Molecular Neuroscience Core facility.

Proteasome activity assay

Cells were plated on poly-lysine coated white 96-well plates at a density of 5000 cells/well. After 72 hours, proteasome activity was determined using the Proteasome-Glo Cell-Based Assay (Promega), and the luminescent signal was detected with a GloRunner microplate luminometer (Turner Biosystems). The proteasome activity was normalized to intracellular ATP content of viable cells using the Celltiter-Glo assay (Promega).

Oxidative stress

Cells were plated at a density of 5000 cells/well in 96-well plates. After 48 hours, cells were exposed to various concentrations of H₂O₂ (0-500 μ M) for 24 hours at 37°C. The cells were then washed three times with serum-free DMEM, and cell viability determined using the colorimetric MTS assay using a 2 hr incubation at 37°C (Promega).

Statistical analysis

Statistical analysis was performed using Prism software (version 3.02, GraphPad). Comparing two groups was based on student t-test or two-way ANOVA. Statistical significance was considered for $p < 0.05$.

Results

GFAP levels are increased in R236H and TgGFAP-wt astrocytes

The parental R236H line (a knock-in at the endogenous mouse locus) and TgGFAP-wt transgenic line (transgenics over-expressing wild type human GFAP) from which the primary astrocyte cultures are derived have increased levels of GFAP in brain [13,24,22]. To determine whether such an increase in expression is reflected in cultured astrocytes, we evaluated total GFAP immunoreactivity after 2 DIV (passage 2) in cell extracts using a sandwich ELISA (Figure 1). By this assay the R236H astrocytes contain 2.5-fold more GFAP than controls, and the TgGFAP-wt astrocytes contain 4.9-fold more GFAP than controls.

R236H and TgGFAP-wt astrocytes contain cytoplasmic GFAP inclusions

To evaluate the effects of R236H or TgGFAP-wt expression on the organization of GFAP filaments in cultured astrocytes, we performed immunofluorescent visualization using confocal microscopy (Figure 2A). Inclusions similar to Rosenthal fibers of Alexander disease have previously been seen in primary cultures of astrocytes from a different line of TgGFAP-wt mice [25]. In our experiments, astrocytes from wild type littermate controls formed fine filaments distributed throughout the cytoplasm in a typical cytoskeletal array. In contrast, a proportion of both R236H and TgGFAP-wt astrocytes formed perinuclear inclusions, often with disruption of the normal filament architecture in the immediate vicinity of the inclusion. These inclusions formed in ~3.4% of the GFAP-positive cells in the R236H cultures, and in ~28% of the GFAP-positive cells in the TgGFAP-wt cultures (Figure 2B, C). The proportion of cells displaying inclusions did not change appreciably with additional time in culture (up to

90 DIV), and may even have decreased slightly in the R236H cells. In general, the inclusions in the TgGFAP-wt astrocytes appeared larger than the ones in the R236H cells.

α B-crystallin concentrates in GFAP inclusions in R236H and TgGFAP-wt astrocytes

Expression of α B-crystallin is up-regulated in the brains of human Alexander disease patients and in mouse models [4,13,24], and α B-crystallin co-localizes with GFAP aggregates in cell lines that are transfected with the mutant GFAP's [20]. To evaluate the effects of R236H or TgGFAP-wt expression on the distribution of α B-crystallin in relation to GFAP, we performed immunofluorescent confocal microscopy on cells that were double-labeled with antibodies to both proteins. The basal levels of α B-crystallin in astrocytes derived from control mice were undetectable using our methods (Figure 3, top panel). In R236H astrocytes, bright staining for α B-crystallin was seen co-localized with a proportion of the densely stained GFAP inclusions (Figure 3, middle panel). In TgGFAP-wt astrocytes, the α B-crystallin was also concentrated in the inclusions, sometimes especially within subregions of the inclusions (Figure 3, bottom panel). However, the majority of GFAP inclusions were not immunoreactive for α B-crystallin, perhaps reflecting the limited sensitivity of the technique.

Disruption of cytoskeletal network by R236H or TgGFAP-wt expression

Intermediate filament inclusions induced by expression of mutant forms of keratins, desmin, and GFAP sometimes cause a general collapse of the cytoskeleton, although this effect is highly cell-line dependent [26,27,20]. To characterize the effect of R236H or TgGFAP-wt expression on other elements of the cytoskeleton in primary astrocyte cultures, we examined the organization of vimentin, actin, and vinculin (an actin-associated protein) by confocal microscopy. Vimentin completely co-localized with GFAP in all three types of astrocytes, both in the normal filaments as well as in the inclusions (Figure 4, left panel). Actin stress fibers were apparent in control astrocytes, and in the distal portions of the R236H and TgGFAP-wt astrocytes, but were absent in the immediate vicinity of the inclusions in the latter types of cells (Figure 4, middle panel). Vinculin is an actin-binding protein that in control astrocytes displays both diffuse cytoplasmic and focal cell surface staining, where it reflects participation in the organization of focal adhesion plaques. In TgGFAP-wt astrocytes, however, the cytoplasmic staining for vinculin was greatly diminished, and cell surface foci were absent (Figure 4, right panel). The R236H astrocytes were also affected, but to a lesser extent. Interestingly, the vinculin staining that was present in the R236H astrocytes appeared primarily confined to the edges of the cells (Figure 4F). These experiments indicate that formation of GFAP inclusions results in a cascade of changes throughout the cytoskeletal network, possibly extending to effects at the cell surface.

R236H and TgGFAP-wt astrocyte growth is suppressed in vitro

To assess growth properties of astrocytes of different GFAP genotypes in culture, cells were dissociated from newborn mouse pups, expanded in T-25 flasks, and then passaged into 35 mm dishes (or wells) for analysis. Cell growth was monitored over the following 12 DIV by enzymatically detaching the cells and performing direct cell counts. Astrocytes from both the TgGFAP-wt and R236H mice consistently yielded fewer viable cells than from littermate controls (Figure 5A, C). At 12 DIV the numbers of TgGFAP-wt and R236H cells were only 60% and 75% of controls ($n = 5$, $p < 0.001$), respectively.

To further confirm the apparent growth retardation observed from direct cell counts, we prepared separate cultures and analyzed their growth rates over 9 DIV using the MTS assay (an indirect measure of cell number and viability based on mitochondrial function). Again, the values for TgGFAP-wt and R236H cells were reduced compared to their littermate controls (Figure 5B, D) (74% and 81%, respectively, $n = 5$, $p < 0.05$). These findings suggest that the *in*

vitro growth rate of astrocytes is subtly but measurably impaired by over-expression of wild type GFAP or mutant GFAP protein.

We considered whether differences in cell size might influence growth rate, for instance by allowing earlier contact inhibition in one set than another. By measuring the spherical diameter of cells detached from the culture surface after brief trypsinization, we found no difference between wild type ($13.45 \pm 3.78 \mu\text{m}$), R236H ($14.23 \pm 4.00 \mu\text{m}$) or TgGFAP-wt ($13.98 \pm 3.50 \mu\text{m}$) astrocytes.

Decreased cell proliferation in R236H and TgGFAP-wt astrocytes

To test whether the decrease in cell number observed in R236H and TgGFAP-wt astrocytes reflected a change in cell proliferation, we determined the proportion of GFAP-positive cells incorporating BrdU at various time points in culture (Figure 6A). No differences between groups were noted at 2 DIV (passage 2). However, significantly fewer R236H astrocytes incorporated BrdU than control cells at 6 DIV, and fewer TgGFAP-wt cells incorporated BrdU at both 4 and 6 DIV (Figure 6B). These results suggest that the decreased growth rate for R236H and TgGFAP-wt cells in part reflects a decrease in cell proliferation.

Increased cell death in R236H and TgGFAP-wt astrocytes

We considered whether changes in cell death could account for the alterations in growth rate described above. As one measure of cell death, we examined the activity of caspases 3 and 7 at different times after plating. No differences were noted at 2 DIV. However, by 4 DIV both R236H and TgGFAP-wt astrocytes exhibited increased levels of caspase 3/7 activity compared to cells from littermate controls (Figure 7A, B). To confirm these findings, we then evaluated cell death using morphological techniques by staining cultured astrocytes (at 4 DIV) with calcein AM (viable cells detected by intracellular esterase activity, green) and EthD-1 (dead cells detected by uptake of EthD-1, red) (Figure 7C). The proportion of cells that were positive for EthD-1 uptake was higher in R236H astrocytes compared to cells from littermate controls (7.08% vs. 3.86%, $p < 0.05$) (Figure 7D). Astrocytes from TgGFAP-wt mice displayed a similar difference compared to controls (8.18% vs. 2.1%, $p < 0.01$) (Figure 7E). These results indicate that the reductions in growth rate for both R236H and TgGFAP-wt astrocytes at least partially derive from increased cell death.

R236H and TgGFAP-wt astrocytes have reduced proteasomal activity

We considered whether the R236H and TgGFAP-wt astrocytes exhibited any change in proteasomal function, comparable to what has been observed in cell lines transfected with GFAP constructs [21]. We measured proteasomal activity in R236H and TgGFAP-wt astrocytes at 3 DIV, and found a 20% and 35% decrease, respectively, compared to wild type control cells (Figure 8). This suggests that compromised proteasomal protein degradation may contribute to GFAP accumulation and formation of Rosenthal fibers in primary astrocytes.

R236H and TgGFAP-wt astrocytes are more vulnerable to H₂O₂ toxicity

Expression of both R236H and TgGFAP-wt in mice results in a generalized stress response with activation of the common anti-oxidant response element in astrocytes [24,22]. To test whether this increased baseline of stress renders astrocytes more vulnerable to further injury, we exposed primary cultures derived from controls and both types of disease model mice to various concentrations of H₂O₂. As expected [28], control astrocytes show a clear dose-dependent toxicity following incubation in H₂O₂. However, R236H astrocytes display increased sensitivity compared to controls at 300 μM H₂O₂ (Figure 9A), and TgGFAP-wt astrocytes are more sensitive at both 200 and 300 μM H₂O₂ (Figure 9B). The proportion of cells with inclusions was unchanged after H₂O₂ exposure (data not shown), suggesting that

the increased sensitivity extends to the non-inclusion-bearing cells as well. These findings indicate that expression of mutant GFAP, or overexpression of wild-type GFAP, compromises astrocyte resistance to oxidative stress.

Discussion

An increase in absolute levels of GFAP may be central to the pathogenesis of Alexander disease. Our mouse models employ two distinct genetic means by which such an increase is achieved, either by added copy number of a wild-type sequence, or by expression of a mutant allele. Expression of mutant protein apparently activates multiple downstream pathways, including p38, JNK, and MAPK, that impact GFAP turnover in various and sometimes opposing ways [21,29]. The inhibition of proteasomal function that follows JNK activation is likely to be one mechanism that contributes to GFAP accumulation [21]. The primary astrocyte cultures described here also display increased levels of GFAP. The transgenics (with added copy number) showed higher levels than did the point mutants, which mirrors the relative levels found in brains of adult mice from these two lines and perhaps reflects the dual contribution of copy number and stress pathway activation [22]. However, the levels found in the cultured cells never reached the levels achieved *in vivo*, perhaps reflecting the age at which the cultures were prepared or the absence of normal anatomical context.

Rosenthal fibers form in astrocytes when GFAP levels exceed a certain level, but precisely where that threshold lies is still uncertain. We have found that cultures expressing either mutant or wild type protein, at levels approximately 3-5 fold over endogenous baseline levels, reliably form these inclusions, albeit only in a subset of the cells. One possible explanation for the low number of inclusion-bearing cells is that there is cell-to-cell variation in the expression of GFAP, and that only the highest expressing cells formed inclusions (in which case the threshold is considerably above the 3-5 fold change measured in the whole population). We considered whether increasing time in culture would increase the proportion of astrocytes with inclusions, but the proportion remained relatively stable. Another possible explanation is that the small percentage of inclusion-bearing cells reflects the particular source of tissue used to establish the cultures, cerebral cortex, where relatively few astrocytes ever develop Rosenthal fibers *in vivo*, particularly in the R236H mice [22]. A third possibility is that cells within each culture are relatively uniform with respect to GFAP levels, but differ in some other as yet undefined downstream consequence of GFAP excess. Tanaka et al. [23], using a transgenic model that expresses an R239H transgene (the human equivalent of our R236H knock-in mouse mutant), argue that inclusions formed at an even lower threshold, only 1.3-fold above baseline. Hence the exact stimulus for formation of Rosenthal fibers within each individual astrocyte is still a topic for further investigation.

Whatever the stimulus for Rosenthal fibers, inclusion-bearing astrocytes display a number of abnormalities. There appears to be a generalized disruption of the cytoskeleton, with impacts on both vimentin and actin. It is interesting that Sullivan et al. [30] recently found a role for GFAP in regulating the cell surface distribution of a glutamate transporter, GLAST, which suggests that inclusion-bearing astrocytes may be compromised in this critical astrocyte function. Furthermore, vinculin, an actin-binding molecule that is commonly found in focal adhesion plaques, also appeared to be affected. Using primary cultures of rat astrocytes, Goldman and Chiu [31] found an inverse correlation between GFAP levels and actin. Subsequently, Goldman and Abramson [32] reported that elevating cAMP induced actin depolymerization in astrocytes, with associated loss of stress fibers and vinculin.

The functional significance of vinculin loss in astrocytes is not known, since the global knockout of vinculin has severe developmental defects and dies by mid-gestation [33]. It is conceivable that alterations in vinculin could affect growth control via linkages to integrins at

the cell surface [34], although at present there is no direct evidence for this possibility. Alternatively, alterations in vinculin could influence cell migration and attachment, and such studies are underway. It is interesting that fibroblasts derived from vinculin-null mice display decreased adhesion and increased motility [33], and glioma cells transfected with two of three Alexander disease-associated mutant GFAPs also exhibited increased motility [35]. However, vinculin loss is not simply a consequence of elevations in GFAP, as proteomic analysis of primary rat astrocyte cultures exposed to endothelin-1 to mimic gliosis show a dramatic increase in vinculin instead [36]. Hence there are likely to be fundamental differences in the properties of astrocytes stressed to the point of forming Rosenthal fibers and the milder phenotype of reactive astrocytes.

Immunolocalization of α B-crystallin, a small stress protein that normally associates with GFAP and regulates its assembly into mature filaments, showed intense staining of the inclusions. α B-crystallin is well known as a binding partner for type III intermediate filaments, including vimentin [37], desmin [38], and GFAP [39]. Studies in both C6 cells and in primary astrocytes demonstrate that α B-crystallin can influence GFAP integration into the cytoskeletal network, both by regulating normal assembly and by assisting in recovery from stress [37,40].

In Alexander disease, expression of α B-crystallin is markedly up-regulated and it becomes an abundant component of Rosenthal fibers [4]. Previous studies by Perng et al. [20] have shown that GFAP aggregates in transfected cells can result in a shift in the equilibrium of α B-crystallin from the soluble to insoluble pool, suggesting that sequestration of α B-crystallin in the aggregates may lead to depletion elsewhere in the cell. Complete deficiency of α B-crystallin in astrocytes would likely have a number of effects, including altering the response to cytokines and MAP kinase/NF κ B signaling pathways and increasing cleavage of caspase-3 [41].

Both the GFAP transgenic and GFAP mutant astrocytes demonstrate measurable retardation of growth in culture, which appears to reflect a combination of increased cell death and decreased proliferation. Studies in glioma cell lines have shown an inverse relation between levels of GFAP expression and proliferation [42,43,44], and primary astrocyte cultures derived from GFAP-null animals display increased proliferation compared to wild type controls [45]. One potential mechanism linking proliferation and intermediate filaments is an alteration of phosphorylation [46]. Phosphorylation of intermediate filaments normally increases during mitosis, which perhaps functions to disassemble the filament network [47]. Using keratin 8 as a model, Ku and Omary [48] have proposed that intermediate filaments can act as “phosphate sponges”, placing a brake on proliferation by occupying kinases that would otherwise be available for cell cycle regulation. GFAP is typically phosphorylated at several sites within the head domain, with changes that are linked to stages of the cell cycle [49]. Excessive phosphorylation of GFAP, as might occur in Alexander disease, could thus limit the proliferation capacity of affected cells. Mignot et al. [50], using time-lapse recordings of cells transfected with GFP-labeled GFAP, found that under some circumstances Rosenthal fibers can disappear, which is another potential effect of phosphorylation. An alternative possibility is that altered signaling through either 14-3-3 (the γ isoform of which binds to the phosphorylated form of Ser8 in GFAP [51]) or mTOR (shown to be decreased by expression of mutant GFAPs [29]) could affect proliferation. However, the effects of mTOR and 14-3-3 may vary by cell type, intermediate filament, and isoform of 14-3-3 [52].

The mechanism by which GFAP influences cell death is also not known. Sequestration of α B-crystallin in the inclusions could result in depletion of its soluble cytoplasmic pool and thereby compromise its anti-apoptotic activity [53,54,55,41]. GFAP can be cleaved by caspase-3, and others have argued that caspase activation may be related to alterations of astrocytes that fall short of cell death per se [56,57]. Keratins, vimentin, and nestin all have anti-apoptotic properties, although in some circumstances intermediate filament cleavage products may also

be pro-apoptotic [58,59]. What implications these alterations in astrocyte growth properties have for the behavior of astrocytes *in vivo*, in the brains of Alexander disease patients, is also not clear. No evidence currently exists for an increase in astrocyte death or deficiency of astrocyte numbers in such patients, although the complex neuropathology with secondary tissue destruction and reactive gliosis makes autopsy studies difficult to interpret [60].

Beyond changes in growth, GFAP accumulation and mutant GFAP may impact other areas of astrocyte function. Both Alexander disease brains, and the two types of mouse models studied in this report, exhibit evidence of oxidative stress, with activation of Nrf2 and increased mRNA levels for several genes that are regulated by this transcription factor via the common antioxidant response element (ARE) [24,22]. The expression of at least some keratins also appears to be regulated by Nrf2, the transcription factor that binds to the ARE [61], and Magin [62] has proposed that intermediate filaments may play an important role in protection against oxidative stress. GFAP over-expressing and mutant GFAP astrocytes both display elevated levels of cell death in response to exposure to hydrogen peroxide, which may again reflect depletion of α B-crystallin [63]. A further potential consequence of oxidative stress that is particularly relevant for astrocytes is a compromise in glutamate transport [64]. Studies of transfected cells suggest that mutant GFAPs do exert this effect (J.E. Goldman, personal communication), and studies of primary astrocytes are currently underway. It is important to note that our results of both morphological studies of cell death, and the hydrogen peroxide killing, suggest that GFAP-induced vulnerability extends beyond just the inclusion-bearing cells. Hence it is likely that GFAP toxicity is an early event that manifests before the formation of frank inclusions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Denise Springman, Channi Kaur, and Benjamin August for technical support, and Michael Brenner, James Goldman, Tracy Hagemann, and Roy Quinlan for advice on the manuscript. This work was supported by NIH grants NS42803 and NS060120 (A.M.), and by HD03352 (Waisman Center). W.C. was supported in part by the Wayne and Jean Roper Wisconsin Distinguished Graduate Fellowship.

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FIG 1. GFAP levels are increased in R236H and TgGFAP-wt astrocytes. GFAP content was quantitated by a sandwich ELISA on 1% SDS extracts from cells grown for 2 days (passage 2). GFAP was 2.5-fold elevated in R236H astrocytes, and 4.9-fold elevated in TgGFAP-wt astrocytes, compared to controls. Error bars show ± 1 SD from 6-8 mice. Statistical significance was evaluated by the unpaired t-test (***) $p < 0.001$.

FIG 2. Cytoplasmic GFAP inclusions form in R236H and TgGFAP-wt astrocytes. **A.** Immunofluorescent confocal images using a mouse monoclonal anti-GFAP antibody (Chemicon) demonstrate disruption of the normal filamentous GFAP architecture and formation of perinuclear aggregates in R236H and TgGFAP-wt astrocytes compared to wild type or non-transgenic controls (scale bars = 10 μ m). Insets show ultrastructural images of Rosenthal-fiber like inclusions in each cell type (scale bars = 1 μ m). **B, C.** The percentage of inclusion-bearing cells does not change with time in culture (1 week vs. 3 months, passage 2). Each litter was considered an independent experiment. Error bars show $\pm$ 1 SEM from 3 independent experiments.

FIG 3.

α B-crystallin colocalizes to GFAP inclusions. Astrocytes at 7 DIV (passage 2) were double-labeled with anti-GFAP and anti- α B-crystallin antibodies. **A-C.** The basal expression level of α B-crystallin in control astrocytes is undetectable. **D-I.** The inclusions in both R236H and TgGFAP-wt astrocytes are highly immunoreactive for α B-crystallin. The inclusions in the R236H cells appear smaller and more compact than those in the TgGFAP-wt astrocytes. Confocal immunofluorescent images were obtained as Z-stack projections (60x optical magnification, oil immersion, 0.35 μ m steps). Scale bars = 10 μ m.

FIG 4.

R236H mutation and over-expression of wild type GFAP affects cytoskeleton and cytoskeleton-associated proteins. Confocal images were obtained for astrocytes double-labeled for GFAP (green) along with vimentin, actin, or vinculin (red). **A, D, G.** Vimentin typically co-localized with both normal filamentous GFAP and with the inclusions (indicated by yellow). **B, E, H.** Filamentous actin is disrupted in the immediate vicinity of the GFAP inclusions. **C, F, I.** Vinculin is normally concentrated in adhesion plaques at the cell surface in wild type cells (arrows), but vinculin immunoreactivity becomes low or absent in R236H GFAP mutant or over-expressing astrocytes. Cell counts indicating the proportions of inclusion-bearing cells with or without vinculin staining obtained from one experiment are shown in Supplemental Data, Table 1. Confocal images were obtained at 60x optical magnification. Scale bars = 10 μ m.

FIG 5.

Growth rates of R236H and TgGFAP-wt astrocytes are impaired. Cells were plated on 6-well (50,000 cells/well) or 96-well (2,000 cells/well) plates at passage 2 and evaluated by two measures of growth. Both the R236H mutation and overexpression of wildtype GFAP lead to decreased astrocyte growth as monitored by direct counts (**A**, **C**) or the colorimetric MTS assay (**B**, **D**). Cells from each litter were analyzed in duplicate dishes or wells. Each litter was considered an independent experiment. Statistical significance was evaluated by two-way ANOVA. Error bars show ± 1 SEM, $n = 3$ -5 litters. **E**. Cells were grown for 14-16 DIV at passage 1, and then cell size was measured with an automatic cell counter. At least 1200 cells were measured for each genotype.

FIG 6. DNA replication rates are reduced in R236H and TgGFAP-wt astrocytes. **A.** Cells were incubated with 10 μ M BrdU overnight at 37°C, and then double immunostained using antibodies to both GFAP (red) and BrdU (green). Scale bars = 20 μ m. **B.** Quantitation of BrdU-positive astrocytes at 2, 4, and 6 DIV (passage 2), indicating a significant reduction for R236H cells at 6 DIV, and for TgGFAP-wt cells at both 4 and 6 DIV. Cell counts were performed in triplicate for each genotype, counting 50-200 cells per slide. Bars represent the results of cell counts from 3-4 independent experiments. Error bars show $\pm$ 1 SEM (unpaired t-test, * $p < 0.05$).

FIG 7.

Caspase activity and cell death are increased in R236H and TgGFAP-wt astrocytes assessed at 2-6 DIV (passage 2). **A, B.** Caspase activity of R236H and TgGFAP-wt astrocytes is increased compared to littermate controls. The caspase activity was normalized to total protein in each well. **C.** Cell viability was evaluated at 4 DIV (passage 2). Cells were stained with calcein-AM (2 μ M, green) and EthD-1 (1 μ M, red) to identify live and dead cells, respectively. Images shown are representative of 3 independent experiments. Scale bars = 10 μ m. **D, E.** Stained cells were visualized using a 40x objective, and the percentage that were dead (EthD-1 positive) was determined. Error bars show $\pm$ 1 SEM from 3-4 independent experiments (unpaired t-test, * $p < 0.05$ and ** $p < 0.01$).

FIG 8. Proteasomal function is impaired in **A**) R236H and **B**) TgGFAP-wt astrocytes, compared to astrocytes from littermate controls. Activity was measured using the Proteasome-Glo assay (Promega), and the luminescent signal normalized to cell content using the Celltiter-Glo cell viability assay (Promega). The R236H and TgGFAP-wt astrocytes displayed decreased proteasomal activity of 20% and 35%, respectively. Bars represent the results from 5-7 mice. Error bars show ± 1 SD (unpaired t-test, * $p < 0.05$). RLU: relative light unit.

FIG 9. Susceptibility to H₂O₂-toxicity is increased in **A**) R236H and **B**) TgGFAP-wt astrocytes, compared to astrocytes from littermate controls. Cells were plated at 5,000 cells per well in poly-lysine coated 96-well plates, and maintained in DMEM supplemented with 10% FBS. After 48 hrs, cells were exposed to various concentrations of H₂O₂ for 24 hrs at 37°C. The cells were then washed, and cell viability was assessed with the MTS assay. Cell survival is shown as the percent of the number of non-exposed cells grown for the same period of time in culture. Error bars show ± 1 SEM from 3-4 independent experiments (unpaired t-test, * p < 0.05 and ** p < 0.01).