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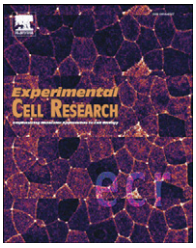

Research Article

In vitro treatments with ceftriaxone promote elimination of mutant glial fibrillary acidic protein and transcription down-regulation

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ABSTRACT

Alexander disease is a rare, untreatable and usually fatal neurodegenerative disorder caused by heterozygous mutations of the glial fibrillary acidic protein (*GFAP*) gene which ultimately lead to formation of aggregates, containing also αB-Crystallin, HSP27, ubiquitin and proteasome components. Recent findings indicate that up-regulation of αB-Crystallin in mice carrying *GFAP* mutations may temper the pathogenesis of the disease. Neuroprotective effects of ceftriaxone have been reported in various animal models and, noteworthy, we have recently shown that the chronic use of ceftriaxone in a patient affected by an adult form of Alexander disease could halt its progression and ameliorate some of the symptoms. Here we show that ceftriaxone is able to reduce the intracytoplasmic aggregates of mutant GFAP in a cellular model of Alexander disease. Underlying mechanisms include mutant GFAP elimination, concurrent with up-regulation of HSP27 and αB-Crystallin, polyubiquitination and autophagy. Ceftriaxone has also been shown to modulate the proteasome system, thus decreasing NF-κB activation and *GFAP* promoter transcriptional regulation, which further accounts for the down-modulation of GFAP protein levels. These mechanisms provide previously unknown neuroprotective targets of ceftriaxone and confirm its potential therapeutic role in patients with Alexander disease and other neurodegenerative disorders with astrocyte involvement.

Introduction

Alexander disease (AD) (MIM 203450) is a rare, progressive and usually fatal devastating disorder of the central nervous system [1] now considered an astrogliopathy [2] with intracytoplasmic Rosenthal fibers (RFs) in dystrophic astrocytes. RFs contain the type III intermediate filament glial fibrillary acidic protein (GFAP), ubiquitin and the two small heat shock proteins (sHSPs), αB-

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Abbreviations: GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HSP27, heat shock protein 27; Cef, ceftriaxone; lact, lactacystin

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Crystallin and heat shock protein 27 (HSP27) [3–5]. Heterozygous mutations of the *GFAP* gene have been identified in association with the vast majority of patients [6–9].

Results from *in vivo* and *in vitro* functional studies are consistent with the view that *GFAP* mutations do prevent, at different extent, formation of a regular cytoskeletal filamentous pattern, reduce the solubility of the protein and hamper correct organization of wild type (wt) GFAP [10–12], thus inducing impairment of the ubiquitin-proteasome system (UPS) and consequently leading to increased cell death [13]. In addition, cytoplasmic inclusions similar to RFs have been shown in murine astrocytes over-expressing wt GFAP, suggesting that formation of aggregates could also be due to a critical cytoplasmic accumulation of the wt protein [14], which would lead to functional consequences similar to those induced by mutant proteins [15].

Several studies have shown that αB-Crystallin over-expression induces disaggregation of cellular inclusions containing wt [16] and mutant [10] GFAP. Consistent with these observations, mice affected by Alexander disease and over-expressing αB-Crystallin have been recently shown to bear a less severe phenotype, likely related to activation of αB-Crystallin downstream pathways leading to protection from cell death [17]. On the other hand, little is known about the role of HSP27 in Alexander disease. HSP27 is a small heat shock protein with chaperone activity which normally regulates intermediate filaments assembly, prevents untimely filament–filament interactions that would lead to protein aggregation [18] and, similarly to αB-Crystallin, is able to protect cells against consequences of protein aggregation [19,20]. In particular, its role in refolding denaturated proteins has been assessed in *in vitro* and *in vivo* models of Huntington's disease (HD) where HSP27-dependent rescue of striatal function in primary neuronal cultures and HD rat models was shown [21]. Moreover, mutations in *HSPB1*, the gene encoding HSP27, have been observed to disrupt neurofilaments assembly and to cause protein aggregation in Charcot–Marie–Tooth 2E and 2F disease [22]. Also, in astrocytes HSP27 likely plays a protective effect against stress induced by proteasomal inhibition [23]. Taken together, these data are consistent with recent experimental findings which indicate that a dynamic and reversible aggregation of mutated GFAP may occur and allow cell survival in an astrocyte model of AD [24]. The likely occurrence of a dynamic proteopathy in AD suggests that therapeutic approaches to prevent or cure GFAP aggregates might be effective [24].

Ceftriaxone is a widely used beta-lactam antibiotic for which neuroprotective effects in various animal disease models have already been demonstrated, including amyotrophic lateral sclerosis, ischemic stroke, HIV-dementia, HD, and multiple sclerosis [25–31]. Ceftriaxone-mediated neuroprotection has been suggested to be related to increased astroglial expression and activity of the glutamate transporter GLT-1 (EAAT2), thus inducing increased glutamate uptake by astrocytes, counteracting the excitotoxicity against neurons [30,32], and leading to activation of the antioxidant defense system including Nrf2 and xCT system [33]. Importantly, we have very recently reported that the chronic use of cycles of ceftriaxone in a patient affected by an adult form of Alexander disease apparently halted the progression of the disease and ameliorated some of the symptoms [34], suggesting that this antibiotic may have a therapeutic role also in Alexander disease.

In this work, we have confirmed the potential beneficial effect of ceftriaxone in an *in vitro* cellular model of Alexander disease, which has already been proven to be a reasonably reliable indicator of disease activity [10]. Treatments with ceftriaxone have resulted in a marked reduction of intracytoplasmic aggregates of mutant GFAP, due to elimination of GFAP mutant proteins and concurrent up-regulation of HSP27 and αB-Crystallin, polyubiquitination and autophagy. A further ceftriaxone-mediated decrease of GFAP protein levels was demonstrated to occur through modulation of the proteasome system which, leading to decreased NF-κB activation, induced transcriptional down-regulation of the *GFAP* promoter.

Overall, these observations confirm the potential therapeutic role of ceftriaxone in patients with Alexander disease and other neurodegenerative disorders with astrocyte involvement.

Methods

Cell culture and ceftriaxone treatments

The U251-MG human astrocytoma cell line was grown in RPMI medium supplemented with 10% FBS (Gibco, New Zealand) 1% penicillin, and streptomycin (Euroclone). Ceftriaxone (Sigma-Aldrich) was diluted in sterile water and added to cells which were previously grown for at least a week in absence of antibiotics.

HSP27 and αB-Crystallin expression analysis

U251 cells were plated at 60% confluence in 60 mm dishes and treated with increasing amount of ceftriaxone (10 μM and 1 mM). After 24 and 48 h, total RNA was extracted (Rneasy mini kit, Qiagen) and reverse transcribed (BioRad). Expression of *HSP27* (*HSPB1*, NM_001540), αB-Crystallin (*CRYAB*, NM_001885.1) and *GFAP* (*GFAP*, NM_002055.3) was analysed by Real Time PCR on a IQ5 thermocycler (BioRad) using specific assays (TaqMan Gene Expression, Applied Biosystem), according to manufacturer's instructions. In particular, each 20 μl reaction contained about 30 ng cDNA, 10 μl Taqman IQ SuperMix (BioRad) and 1 μl of the specific assay. Glyceraldehydes 3-phosphate dehydrogenase (G3PDH), actin and β2-microglogulin were used as endogenous control-genes because their expression was not modulated by ceftriaxone.

Transient transfections

U251-MG cells were transfected with 1 μg of the already described expression vectors encoding for the wild type and the mutant forms, p.[R330G;E332K] or p.R239C, of GFAP fused to the GFP [10] by using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. After transfection, increasing amounts of ceftriaxone (Sigma) were added to cell culture and after 24 or 48 h samples were assayed for protein expression analysis or immunostaining assays.

Immunofluorescence analysis

Forty-eight hours after transfection, GFAP-GFP organization of at least 100 cells in each sample was directly analysed by evaluating the GFP fluorescence with a fluorescence microscope (Zeiss Axiophot; Nikon ACT-2U). For immunostaining assays, cells were prepared by using an already set up protocol [10], then incubated