Congo red, doxycycline, and HSP70 overexpression reduce aggregate formation and cell death in cell models of oculopharyngeal muscular dystrophy

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LETTER TO JMG

The formation of intracellular amyloid-like inclusions by mutant proteins is a feature of two groups of codon reiteration diseases, for which there are currently no treatments. The first group was that was described includes the nine known neurodegenerative conditions caused by polyglutamine (polyQ) repeat expansions resulting from CAG trinucleotide repeat mutations, exemplified by Huntington’s disease (HD). HD is caused by a tract of more than 37 uninterrupted polyglutamines in exon 1 of the HD gene product, huntingtin. Genetic and transgenic studies are consistent with a model where expanded polyglutamines cause disease by conferring a novel toxic function on the disease proteins. The second type of codon reiteration mutation results in autosomal dominant oculopharyngeal muscular dystrophy (OPMD). OPMD is caused by the abnormal expansion of a (GCG)6 trinucleotide repeat in the coding region of the polyadenine binding protein 2 gene (PABP2): a (GCG)6 repeat is expanded to (GCG)13 in most patients. In some rare cases, insertion mutations such as (GCG)6GCA(GCG)2, (GCG)6GACA(GCG)1, and (GCG)6GACA(GCG)2 are seen. In PABP2, (GCG)6 codes for the first six alanines in a homopolymeric stretch of 10 alanines. Thus, disease is associated with expansions of 12 or more uninterrupted alanines in this nuclear protein. OPMD is characterised by aggregates in muscle cell nuclei comprising mutant PABP2 as a major component.

The role of inclusions in these diseases has been vigorously disputed. Nevertheless, strategies that target protein misfolding frequently reduce aggregate formation and cell death in parallel. In mammalian cell based models of both polyglutamine and polyalanine diseases, the mutant proteins are much more prone to aggregate formation than their wild-type counterparts and cause significantly more cell death. In such models, aggregate formation and cell death can be reduced by overexpressing yeast and bacteria derived chaperones that do not appear to protect against some other cell death pathways. A causal role for aggregation in cell death in tissue culture models of OPMD is supported by complementary data from our lab and Rouleau’s group. Rouleau and colleagues found that oligomerisation of PABP2 is mediated by two potential oligomerisation domains (ODs)—deletions in either of these domains inactivated oligomerisation of mutant PABP2 and also reduced the cell death caused by this protein. The similarities between polyglutamine diseases and OPMD have led us to explore whether strategies that protect against polyglutamine aggregation or toxicity are also effective in OPMD models. Our previous studies suggested that mammalian heat shock proteins might be able to play similar roles in both diseases. Members of the HSP70 and HSP40 family members are recruited to polyQ inclusions in vivo and in cell models. We have tested if this is the case in OPMD patients, as we had previously shown that this occurred in a cell model of OPMD. We have previously shown that HDJ1, an HSP40 family member, reduced aggregate formation and cell death in cell models of HD and OPMD. Since HDJ1 is a co-chaperone for HSP70, we have now tested if HSP70 chaperone is effective in cell models of OPMD, as it can be effective in HD.

Since it may be possible to treat these diseases with compounds that reduce aggregate formation, we have been testing a number of anti-amyloid compounds in cell based models of OPMD (where no animal models have been published). We tested Congo red, as data from Ross and colleagues suggested that it blocked the conversion of mutant huntingtin protofibrils into mature fibrils and Sanchez et al showed that Congo red reduced aggregation and cell death in HD models. The latter study also reported that infusion of Congo red into an HD mouse model by the intraperitoneal and intracerebroventricular routes improved survival, weight loss, and motor function, compared with untreated mutant mice. These data provided important insights into the role of inclusions polyglutamine disease pathology and suggested that the beneficial effects of Congo red were due to its

Key points

- Intracellular amyloid-like inclusions formed by mutant proteins result from polyglutamine expansions in Huntington’s disease (HD) and polyalanine expansions in polyadenine binding protein 2 (PABP2) in oculopharyngeal muscular dystrophy (OPMD).
- Here we show further parallels between these diseases and suggest therapeutic strategies for OPMD. Like polyglutamine diseases, HSP70 and HDJ-1 colocalised with PABP2 aggregates in OPMD patient muscle tissue and overexpression of HSP70 reduced mutant PABP2 aggregate formation. Aggregate formation and cytotoxicity in cell models of OPMD were reduced by Congo red or doxycycline.
- Our data highlight the therapeutic potential of these compounds in oculopharyngeal muscular dystrophy.

Abbreviations: DAPI, 4, 6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle’s medium; Dox, doxycycline hydrochloride; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; HD, Huntington’s disease; HSP, heat shock protein; OD, oligomerisation domain; OPMD, oculopharyngeal muscular dystrophy; PABP2, polyadenine binding protein 2; polyQ, polyglutamine
anti-aggregate properties. Unfortunately, the therapeutic potential for Congo red in HD may be minimal, because of its very poor blood brain barrier permeability. On the other hand, brain penetration would not be an issue for a muscle disease like OPMD. We also tested doxycycline as an anti-aggregate compound, since previous studies had suggested that tetracyclines were anti-amyloidogenic.

**MATERIALS AND METHODS**

**Cell culture and transfection**

African green monkey kidney (COS-7) cells were grown in Dulbecco's modified Eagle's medium (DMEM 5471) (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine at 37°C, 5% CO₂. For transfection of plasmid DNA, cells were seeded on coverslips at 0.6-1×10⁵ per well, in 6-well plates, the day before transfection. Cells were transfected with Lipofectamine (Life Technologies) according to the manufacturer's instructions. Constructs are described in refs 11 (PABP2) and 15 (HSP70).

**Compounds and treatment**

Congo red (Sigma) was dissolved in DMEM 5671(Sigma) with a stock concentration of 10 mg/ml. Doxycycline hydrochloride (Dox) was dissolved in water with a stock concentration of 100 mg/ml. All the stock solutions were sterilised through a filter with a 0.2 μm pore diameter. The compounds were diluted to the required concentration in the cell culture medium immediately before use. At 24 h after transfection, the compounds were added to the cells at different concentrations. The cells were incubated with the compounds for another 24 h and then washed with 1x phosphate-buffered saline, fixed in 4% paraformaldehyde, and permeabilised with 0.2% Triton X-100. The cells were then blocked with 5% goat serum for 1h. The colocalisation of endogenous PABP2 with HSP70, or HDJ-1, or anti-HSP70 (StressGene, 1:1000), mouse polyclonal anti-HSP70 (StressGene, 1:1000), mouse polyclonal anti-HSP40 (StressGene, 1:1000), and monoclonal mouse anti-HSP70 (1:200), or by using anti-PABP2 antibody together with the monoclonal mouse anti-HDJ-1 (1:200). The sections were then incubated with the mixture of antirabbit Texas red conjugated goat IgG antibody (Molecular probes, USA) and antimouse FITC conjugated goat IgG antibody (Alexa Fluor G488, Molecular probes, USA).

**Confocal microscopy**

Fluorescently labelled samples were analysed using the laser scanning microscope Zeiss LSM410 equipped with an argon ion laser (wavelength, 488 nm) to excite FITC fluorescence, and a helium neon laser (wavelength, 543 nm) to excite Texas red fluorescence. For double labelling experiments, images from the same focal plane were sequentially recorded in different channels and merged to confirm colocalisation.

**Measurement of aggregate formation and abnormal cell nuclei**

Cells were fixed with 4% paraformaldehyde at 72 h after transfection and counterstained with DAPI. Aggregate formation and nuclear morphology were assessed with a fluorescence microscope. Two hundred enhanced green fluorescent protein (EGFP) expressing cells were counted (with the observer blinded to the slide identity) across the centre region of the slides, to quantify all types of polyalanine aggregates in the cells. We assessed the proportions of PABP2-A17 expressing cells that contained one or more inclusions. We considered cells to have inclusions if the green fluorescent protein (GFP) was abnormally concentrated and sequester heat shock proteins (HSPs) in a manner similar to the right biceps muscle from a patient with dentatorubral pallidoluysian atrophy (non-OPMD disease control, male, 62 years old, with a 15 polyalanine expansion mutation in the PABP2 gene (genotype: (GCG) 11/(GCG)6)), the right gastrocnemius muscle from a patient (female, 61 years old, 15 polyalanines in PABP2; (genotype: (GCG)17/(GCG)6)), the right deltoid muscle from a normal control (20 years old) and the right cnemius muscle from a patient (female, 61 years old, 15 polyalanine expansion mutation in PABP2 with HSP40 was detected by using the rabbit polyclonal anti-PABP2 antibody25 (1:200, kindly provided by Professor Elmar Wahle, Halle, Germany) together with the monoclonal mouse anti-HSP70 (1:200), or by using anti-PABP2 antibody together with the monoclonal mouse anti-HDJ-1 (1:200).

**Statistical analysis**

As we and others have described previously, pooled estimates for the changes in inclusion formation resulting from perturbations assessed in multiple experiments were calculated as odds ratios with 95% confidence intervals:

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\text{% cells expressing construct with inclusions in perturbation conditions} \quad \frac{\% \text{ cells expressing construct without inclusions in perturbation conditions}}{\% \text{ cells expressing construct with inclusions in control conditions}}
\]

Odds ratios and p values were determined by unconditional logistical regression analysis using the general log/linear analysis option of SPSS ver 6.1 software (SPSS, Chicago, USA). p<0.05 was considered to be statistically significant.

**RESULTS**

To test if the intranuclear inclusions in OPMD patients sequester heat shock proteins (HSPs) in a manner similar to what we described in cell models, we used double staining
immunohistochemistry to allow simultaneous analysis of both mutant PABP2 and the relevant HSPs. Fig 1 shows colocalisation of HSP70 and HDJ-1 (HSP40 family) in inclusions in the DAPI stained nuclei of OPMD muscle. Similar findings were observed in samples from two OPMD patients. No colocalisation of these HSPs with PABP2 was seen in two control muscle samples (data not shown). The anti-PABP2 antibody we have used has been previously used to distinguish between cells with and without PABP2 aggregates.9 The strong nuclear staining for the chaperones correlated strongly with the cells with nuclear aggregates (fig 1).

Previously, we showed that GFP tagged mutant PABP2 with 17 alanines (PABP2-A17) resulted in increased intra-nuclear aggregate formation and cell death, compared with otherwise identical constructs with 10 alanines11. HSP70 co-expression with GFP tagged mutant PABP2 with 17 alanines (PABP2-A17) significantly reduced the proportion of GFP positive cells with aggregates, compared with cells cotransfected with an empty vector control (pFlag-empty) (fig 2). HSP70 overexpression reduced the toxicity of mutant PABP2 in parallel with the reduction in aggregates (fig 2). Fig 2B shows that total HSP70 levels increase in cells transfected with HSP70, or in cells treated with sodium arsenite, which induces a heat shock response (as a positive control).

We considered Congo red and doxycycline as chemical chaperones with the potential to reduce mutant PABP2 aggregation and toxicity. COS-7 cells (the only cell model that has been reported to show aggregation and cell death as a readout for mutant OPMD toxicity) were transfected with GFP-tagged mutant PABP2 with 17 alanines (PABP2-A17). Twenty four hours after transfection, we added different concentrations of Congo red and doxycycline for the next 24 h and then assessed the proportions of GFP positive cells with aggregates and cell death (as scored by apoptotic nuclear morphology). We found that both Congo red and doxycycline reduced aggregation and cell death caused by mutant PABP2 (fig 3A). These effects were not caused by these compounds inducing raised levels of stress inducible HSPs, like HSP70 (fig 3B). While Congo red did not protect against cell death caused by incubating cells in staurosporine (2.5 mM) or H2O2 (400 μM)11, protective effects were observed with doxycycline (fig 3C). In these experiments,

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**Figure 1** Colocalisation of HSP70 and HDJ-1 with nuclear PABP2 inclusions in muscle biopsies from OPMD patients. Frozen sections of muscle biopsies from OPMD patients were processed for immunohistochemistry. Texas red conjugated (red) second antibody was used to label PABP2 and FITC conjugated (green) secondary antibody was used to label HSP70 and HDJ-1. Colocalisation was confirmed by analysing images from the same focal plane sequentially recorded in different channels by confocal microscopy.

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**Figure 2** HSP70 overexpression suppresses aggregation of PABP2-A17 and its cytotoxicity. (A), PABP2-A17 (0.5 μg) was cotransfected with an expression vector for HSP70 (pFlag-HSP70, 1.5 μg) or an empty vector (pFlag-empty, 1.5 μg). Cells were fixed and stained with DAPI 72 h after transfection. Aggregate formation and nuclear fragmentation/condensation were counted. The odds ratios were derived from 2–4 independent experiments, each done in triplicate. The error bars represent the 95% confidence intervals for the odds ratios. *p<0.05; **p<0.001; ***p<0.0001; not significant, p>0.05. (B), Western blot showing expression of HSP70 in transfected COS-7 cells. COS-7 cells were either transfected with 2 μg PABP2-A17, treated or non-treated with sodium arsenate (NaAsO2, 50 μM, treated for 1 h) or cotransfected with PABP2-A17 (0.5 μg) and pFlag-HSP70 (1.5 μg), PABP2-A17 and pFlag-empty (1.5 μg). NaAsO2 was used as a positive control to stimulate HSP70 expression.
cells were treated in the same protocol as described previously, and staurosporine and \( \text{H}_2\text{O}_2 \) were added in the final 6 h and 4 h, respectively, of the experiment.

**Discussion**

Both polyglutamine diseases like HD and polyalanine expansion mutations in \( \text{PABP2} \) are associated with intracellular inclusions, whose appearance correlates with cell death in model systems. In this study we have extended the parallels between these different types of codon reiteration mutations by demonstrating HSP70 and HDJ-1 colocalisation in \( \text{PABP2} \) inclusions in vivo, which has been previously observed in HD and related polyglutamine diseases. The failure to detect colocalisation of these HSPs with \( \text{PABP2} \) in wild-type samples or outside the nucleus in OPMD tissues is likely to be because the HSP preferentially associate with the aggregated protein and because \( \text{PABP2} \) is predominantly nuclear in steady state. HSP70 overexpression resulted in a reduction of mutant \( \text{PABP2} \) and HD exon 1 aggregation and cytotoxicity. While this paper was being written, Abu-Baker et al published data also showing colocalisation of HSP70 with \( \text{PABP2} \) aggregates and reduction of these aggregates with overexpression of this chaperone. Since HSP70 can directly protect against certain apoptotic pathways, for instance by inhibiting cytochrome c-mediated caspase activation, it is important to be cautious about inferring a causal relationship between reduced aggregation and cell death in this context. Indeed, this cell death pathway is activated and contributes to the cytotoxicity in HD cell models.

The reduction in aggregation and cell death mediated by Congo red and doxycycline against mutant \( \text{PABP2} \) suggests that these compounds may have therapeutic potential, especially for OPMD. The concentrations that we have used are compatible with previous studies in HD models. We obtained significant reductions in aggregation and cell death caused by mutant \( \text{PABP2} \) with 50 µg/ml (70 µM) Congo red, while Sanchez and colleagues reported reduction in their HD cell models with 100µM. Recently, Smith et al reported that doxycycline reduced aggregation in a slice culture model of HD at 50µM, while we observed protection in our OPMD model with 100µM. Doxycycline appears to be protective by reducing aggregation and also by reducing susceptibility to cell death pathways induced by apoptotic inducers like staurosporine and \( \text{H}_2\text{O}_2 \), the latter effect compatible with the data of Chen et al who showed minocycline reduced caspase activation in a mouse model of HD. However, since tetracyclines are predicted to protect against apoptosis by inhibiting caspasess and caspase 1 is interleukin-1β-converting enzyme, the effects of tetracyclines may also be due to anti-inflammatory activity in certain in vivo disease models. While Congo red may have limited utility in HD due to its poor blood brain barrier permeability, it may be useful for diseases associated with aggregation outside the central nervous system, such as OPMD. Congo red appears to be a general amyloid protein ligand. In the case of HD, it blocks the conversion of protofibril to mature fibre formation in the aggregation process. Modelling studies reveal that Congo red is likely to break the continuity of the ordered structures of the β-sheets characteristic of polyalanine expansions, thus providing a molecular explanation for the effect we observed with the mutant \( \text{PABP2} \) protein. The parallel protection that Congo red affords against the cytotoxicity of expanded polyglutamines and polyalanines is consistent with the idea that abnormal protein aggregation and accumulation may be deleterious in all intracellular amyloidoses, irrespective of the primary mutation.

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Congo red and doxycycline protect against cellular toxicity of mutant PABP2

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