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Journal
Human molecular genetics, 15(18)

ISSN
0964-6906

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Publication Date
2006-09-01

DOI
10.1093/hmg/ddl210

Human Molecular Genetics, 2006, Vol. 15, No. 18 ***2743–2751***
doi:10.1093/hmg/ddl210
Advance Access published on August 7, 2006

Green tea (−)-epigallocatechin-gallate modulates early events in huntingtin misfolding and reduces toxicity in Huntington's disease models

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Received May 16, 2006; Revised and Accepted July 31, 2006

Huntington's disease (HD) is a progressive neurodegenerative disorder for which only symptomatic treatments of limited effectiveness are available. Preventing early misfolding steps and thereby aggregation of the polyglutamine (polyQ)-containing protein huntingtin (htt) in neurons of patients may represent an attractive therapeutic strategy to postpone the onset and progression of HD. Here, we demonstrate that the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) potently inhibits the aggregation of mutant htt exon 1 protein in a dose-dependent manner. Dot-blot assays and atomic force microscopy studies revealed that EGCG modulates misfolding and oligomerization of mutant htt exon 1 protein *in vitro*, indicating that it interferes with very early events in the aggregation process. Also, EGCG significantly reduced polyQ-mediated htt protein aggregation and cytotoxicity in an yeast model of HD. When EGCG was fed to transgenic HD flies overexpressing a pathogenic htt exon 1 protein, photoreceptor degeneration and motor function improved. These results indicate that modulators of htt exon 1 misfolding and oligomerization like EGCG are likely to reduce polyQ-mediated toxicity *in vivo*. Our studies may provide the basis for the development of a novel pharmacotherapy for HD and related polyQ disorders.

INTRODUCTION

Huntington's disease (HD) is caused by an unstable CAG repeat expansion in the first exon of the *IT-15* gene which encodes huntingtin (htt), a ∼350 kDa protein, functionally involved in clathrin-mediated endocytosis, vesicle transport processes and transcriptional regulation (1,2). The trinucleotide expansion translates into an elongated polyglutamine (polyQ) stretch, and the disease appears when the pathological threshold of 37 glutamine residues is exceeded (3). The disorder is characterized by a progressive loss of cortical and striatal neurons and the formation of neuronal inclusions containing aggregated htt protein (4,5). There is evidence that mutant htt aggregate formation is causally linked to the progressive neuropathology of the disease (6), though it is not clear whether large insoluble, fibrillar structures or smaller assemblies of htt are the toxic agents responsible for neuronal damage and loss [reviewed by (7)]. Furthermore, toxicity could arise from the recruitment of other polyQ-containing proteins, i.e. transcription factors or wild-type htt, into the neuronal inclusions, which would result in a loss of their normal cellular functions [see review by (8)].

Several studies have focused on the identification of molecules that are able to interfere with aggregation of mutant htt.

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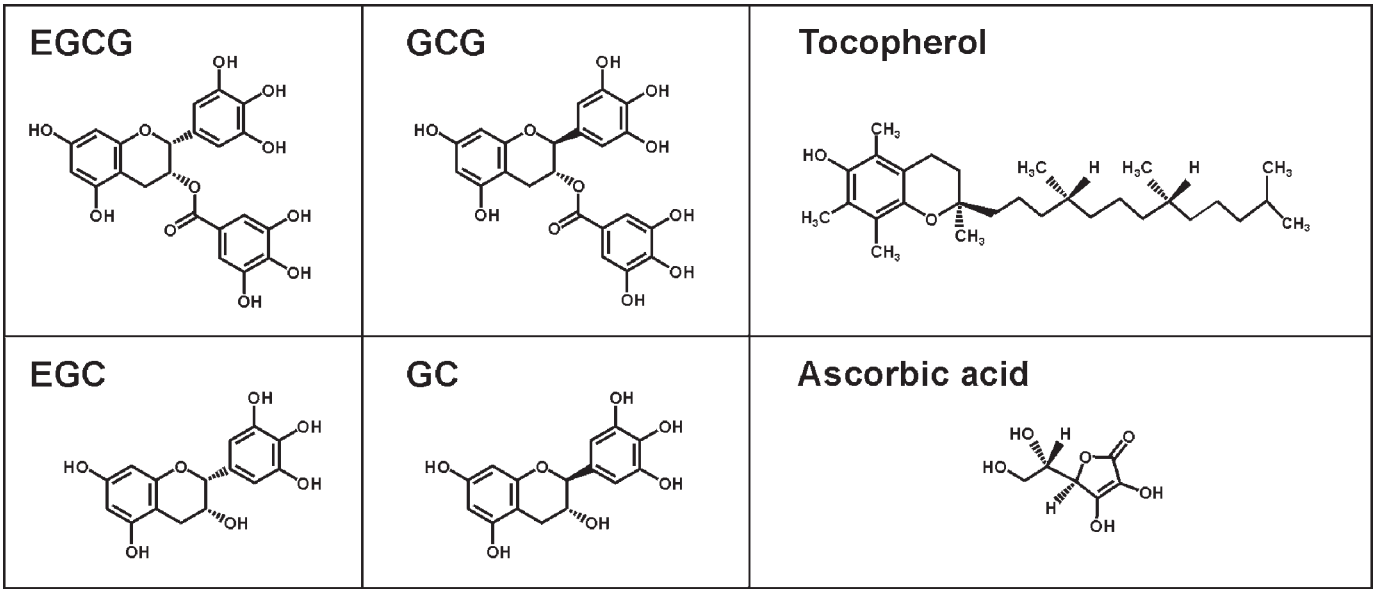

Figure 1. Chemical structures of the aggregation inhibitors tested.

Antibodies directed against the elongated polyQ tract or the N-terminal region of htt efficiently inhibit aggregation *in vitro* or in cell culture models of HD (9,10). Similarly, bivalent polyQ-containing peptides were found to suppress htt aggregation and to inhibit photoreceptor degeneration in fly models of HD (11,12). However, peptides have a high probability of inducing allergic reactions and are barely able to pass the blood–brain barrier.

These problems do not necessarily occur with chemical compounds. In recent years, several research groups have been searching for small molecule inhibitors of polyQ aggregation using cell-free and cell-based assays (13,14). Compounds like Congo red, PGL-34, thioflavine S, gossypol or trehalose have been found to prevent htt aggregation (15). However, it is not known so far how or at which stage of the aggregation process these compounds exert their inhibitory influence. Also, for most of these substances it is unclear whether they have a therapeutic effect in animal models of HD.

A crucial issue in the development of novel therapeutics is tolerance of the drug by the human organism, especially considering that in diseases like HD, drugs are taken chronically for long time periods. Well tolerated are e.g. the flavonoids, a large group of naturally occurring polyphenolic substances that are found in many plants (16). The everyday intake of flavonoids in food is in the range of 50–800 mg, depending on the fruit, vegetables and beverages consumed (16). Previous studies have demonstrated that they are able to modulate various cellular processes; their pharmacological effects have mostly been linked to their antioxidant properties (17). Furthermore, their ability to reduce oxidative stress leads to neuroprotection in models of Alzheimer's and Parkinson's disease (17), suggesting that they could be developed into therapies for neurodegenerative disorders.

In this study, we have screened a library of natural compounds and identified (−)-epigallocatechin-3-gallate (EGCG) and related polyphenols as potent inhibitors of mutant htt exon 1 protein aggregation *in vitro*. We found that EGCG modulates misfolding as well as the assembly of oligomers in cell-free assays and reduces both toxicity and aggregate formation in yeast and fly models of HD. The relevance of our findings with regard to the HD pathomechanism and therapy development is discussed.

RESULTS

Identification of green tea polyphenols as inhibitors of htt exon 1 aggregation

In order to identify inhibitors of htt aggregation, we screened a library of ~5000 natural substances using a membrane filter retardation assay (14). Mutant GST-tagged htt exon 1 fusion protein with 51 glutamines (GST-HDQ51) was incubated for 16 h at 37°C in the presence of both the chemical compounds to be tested and elastase, a protease that very efficiently triggers the aggregation process by completely removing the GST-tag from the fusion protein. The samples were denatured by boiling in SDS/DTT, and aggregates were trapped on a cellulose acetate filter followed by immunodetection with the CAG53b antibody (18). With this approach we detected six plant extracts, which we did not analyze further, and one purified natural compound, EGCG. EGCG is a polyphenol present in green tea (for structure see Fig. 1) that has antioxidant properties and has been shown to cross the blood–brain barrier (19). It suppressed the assembly of HDQ51 aggregates in a concentration-dependent manner with an IC_{50} value of ~1 μM, corresponding to a molar ratio of drug to protein of 2:1 (Fig. 2A and B).

We also tested the stereoisomer (−)-gallocatechin 3-gallate (GCG) and two other related green tea compounds, (−)-gallocatechin (GC) and (−)-epigallocatechin (EGC) that have known antioxidant activities *in vitro* (Fig. 1). GCG

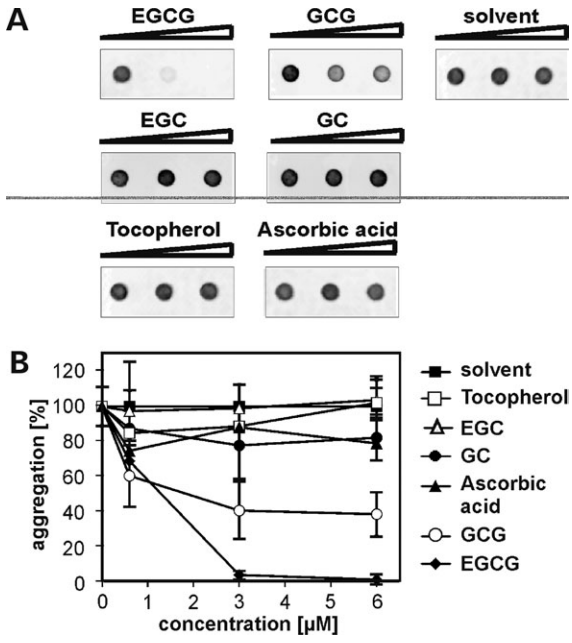

Figure 2. The polyphenols EGCG and GCG inhibit the formation of HDQ51 aggregates *in vitro*. (**A**) Effect of the indicated compounds on HDQ51 aggregation as monitored by the filter retardation assay and immunodetection with the CAG53b antibody. (**B**) Quantification of the filter assay results shown in (A). The amount of aggregates retained on the filter in the solvent control was arbitrarily set at 100%. The data reported are representative for three independent experiments ±SE.

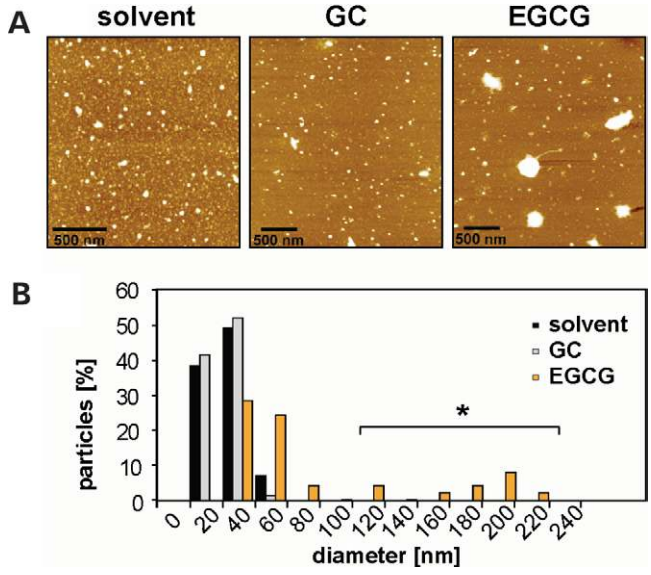

Figure 3. EGCG stimulates the formation of large spherical oligomers. (**A**) GST-HDQ53 was cleaved with PreScission protease and incubated for 5 h with a 2-fold molar excess of EGCG, GC or the solvent alone. Aliquots were analyzed by AFM (image size 3 μm^2 each). (**B**) The particles were grouped with respect to their size (20 nm diameter intervals). Five hundred and forty-one particles were counted for the solvent, 312 for GC and 340 for EGCG. The correlation coefficients between all data sets were 0.9765, 0.9736 and 0.9331 for solvent, GC and EGCG, respectively. The asterisk indicates particles that were only observed after EGCG treatment.

inhibited aggregation to a lesser extent than EGCG (IC_{50} of ~2 μM), whereas GC and EGC, which unlike EGCG and GCG do not possess a gallate moiety, did not show a significant effect on HDQ51 aggregation (Fig. 2A and B). This indicates that the presence of the gallate esters rather than the antioxidative properties of polyphenols in general are crucial for the inhibitory effect *in vitro*. In good agreement with this, also the strong antioxidants ascorbic acid and α-tocopherol did not significantly influence the assembly of SDS-stable htt exon 1 aggregates in the cell-free assays (Figs 1, 2A and B).

EGCG modulates the formation of htt exon 1 oligomers *in vitro*

Previous studies have demonstrated that htt exon 1 fibrillogenesis is a complex process involving the formation of oligomers that can be observed by atomic force microscopy (AFM) (20). Here, we examined whether the addition of EGCG to aggregation reactions modulates the assembly of such structures *in vitro*. GST-tagged fusion protein with 53 glutamines (GST-HDQ53) was incubated with the site-specific PreScission protease to remove the GST tag and to initiate the aggregation of HDQ53. At 5 h after GST cleavage, aliquots were taken from the aggregation reactions and the assembly of oligomers was analyzed by AFM. As shown in Figure 3A and B, the dominant structure observed was a heterologous population of spherical oligomers with a diameter of 20–80 nm, which is in agreement with previous studies (20). Strikingly, in EGCG-treated samples, the density (particles/field) of these structures was significantly reduced, whereas the number of spherical oligomers with a larger diameter (~120–200 nm) was increased (Fig. 3A and B). A similar result was obtained with the stereoisomer GCG (data not shown), whereas the compounds EGC and GC lacking the gallate moiety (Fig. 1) did not significantly modulate oligomerization *in vitro* (data shown exemplarily for GC, Fig. 3A and B). This indicates that EGCG suppresses the formation of small HDQ53 oligomers by stimulating the formation of larger ones.

EGCG interferes with a conformational change in mutant htt exon 1 protein

Schaffar *et al*. (21) demonstrated that proteolytic cleavage of GST-htt exon 1 fusion protein induces a rapid conformational change in the polyQ-containing htt fragment, which can be monitored by FRET assay. We investigated whether EGCG can influence this intramolecular rearrangement upon cleavage of the htt fragment from the GST tag. To monitor the structural change, we used a dot-blot assay and the monoclonal antibody MW1, which specifically registers the expanded polyQ tract (22) and has been used previously to observe the occlusion of the polyQ epitope after cleavage of a GST-htt exon 1 fusion protein with PreScission protease (20). Figure 4A shows that the MW1 antibody very efficiently recognized the uncleaved fusion protein. When the GST tag was removed with elastase, however, 60% of the immunoreactivity was lost immediately after addition and no signal was

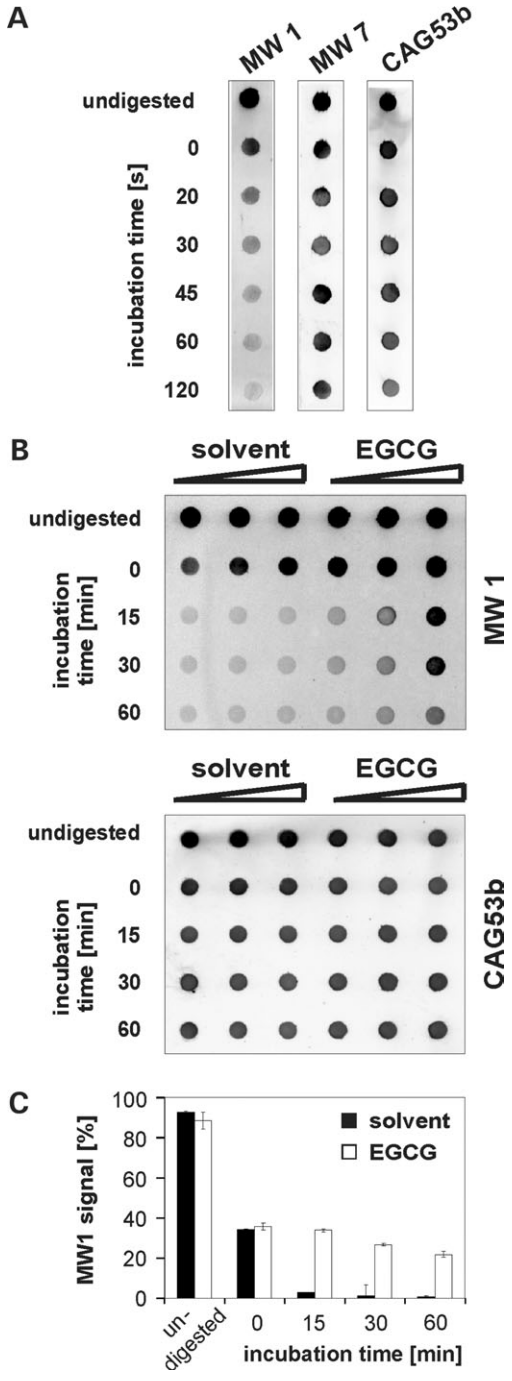

Figure 4. EGCG interferes with a conformational change in HDQ51. (**A**) 0.6 μM GST-HDQ51 was incubated with elastase for the indicated times and samples were spotted onto nitrocellulose for immunodetection with the antibodies MW1, MW7 and CAG53b. MW1 immunoreactivity disappeared rapidly after cleavage, indicating a fast intramolecular conformational change in the PolyQ tract. (**B**) 0.6 μM GST-HDQ51 was incubated with elastase and 0.3, 0.6 or 3 μM EGCG or the solvent at 37°C and spotted onto nitrocellulose at the indicated time points. The disappearance of MW1 immunoreactivity was significantly slowed down by the addition of increasing concentrations of EGCG, indicating that the compound modulates the misfolding of HDQ51 *in vitro*. No such effect was observed with the polyclonal CAG53b antibody, which detects both the GST-tag and the htt exon 1 protein. (**C**) Quantification of the results obtained with the MW1 antibody after the addition of 3 μM EGCG or solvent as shown in (B). The signal obtained for uncleaved reactions was arbitrarily set at 100%. The data reported represent three independent experiments ± SE.

detected after an incubation of 2 min (Fig. 4A). Such a dramatic effect was neither seen with the antibody MW7, which specifically recognizes the poly-proline region in the htt exon1 protein (22), nor with the CAG53b antibody, which detects both the intact GST fusion protein and its fragments after proteolytic cleavage (18). Thus, our findings strongly support previous results (21) indicating that the htt fragment undergoes a very rapid structural rearrangement upon cleavage from GST that can be monitored by the polyQ-specific antibody MW1.

When EGCG was present, the loss of MW1-immunoreactivity was slowed down significantly (but not prevented) in a concentration-dependent manner (Fig. 4B and C). When a 5-fold molar excess of EGCG was added to the aggregation reaction, MW1 immunoreactivity was still clearly detectable after an incubation time of 30 min. This suggests that the compound binds to the unstructured polyQ sequence and interferes with the conformational rearrangement assumed to occur immediately after cleavage (21).

EGCG reduces toxicity and aggregate load in a yeast model of HD

In order to test whether EGCG influences htt exon 1 toxicity *in vivo*, an yeast model of HD was used. The htt exon 1 GFP fusion proteins GFP-HDQ25 and GFP-HDQ72 were overexpressed in yeast and cell growth was measured by monitoring the optical density at 600 nm. As shown in Figure 5A, overexpression of GFP-HDQ25 did not significantly influence yeast cell growth. When GFP-HDQ72 was overproduced, however, cell proliferation was completely blocked, indicating that the GFP fusion protein with the expanded polyQ tract is toxic in yeast. Strikingly, when these cells were treated with 500 μM EGCG yeast growth significantly improved (Fig. 5A), indicating that the compound is able to reduce the toxicity of the GFP-HDQ72 protein *in vivo*.

A recent study demonstrated polyQ aggregation and toxicity in yeast to be dependent on the prion state of the protein Rnq1 (23). To exclude the possibility that yeast growth in EGCG-containing media is due to a spontaneous conversion of Rnq1 from the aggregated to the soluble state, centrifugation assays were performed. Supernatant and pellet fractions were analyzed by western blotting. We found that EGCG-treatment did not influence the aggregation state of Rnq1, indicating that solubilization of Rnq1 cannot be the cause for the EGCG-mediated reduction of polyQ toxicity (Supplementary Material, Fig. S1).

Furthermore, we tested whether EGCG-treated cells expressing the toxic GFP-HDQ72 protein can regrow on SD + Gal medium lacking the compound. We found that the yeast cells, after removal of the compound, do not grow on SD + Gal plates (galactose is required for the induction of GFP-HDQ72 expression), whereas they can grow on SD + Glu medium. This strongly indicates that the EGCG treatment did not induce spontaneous mutations in the yeast genome responsible for the improved growth phenotype (data not shown).

Next, we examined whether EGCG can influence the formation of insoluble protein aggregates in yeast. Fluorescence

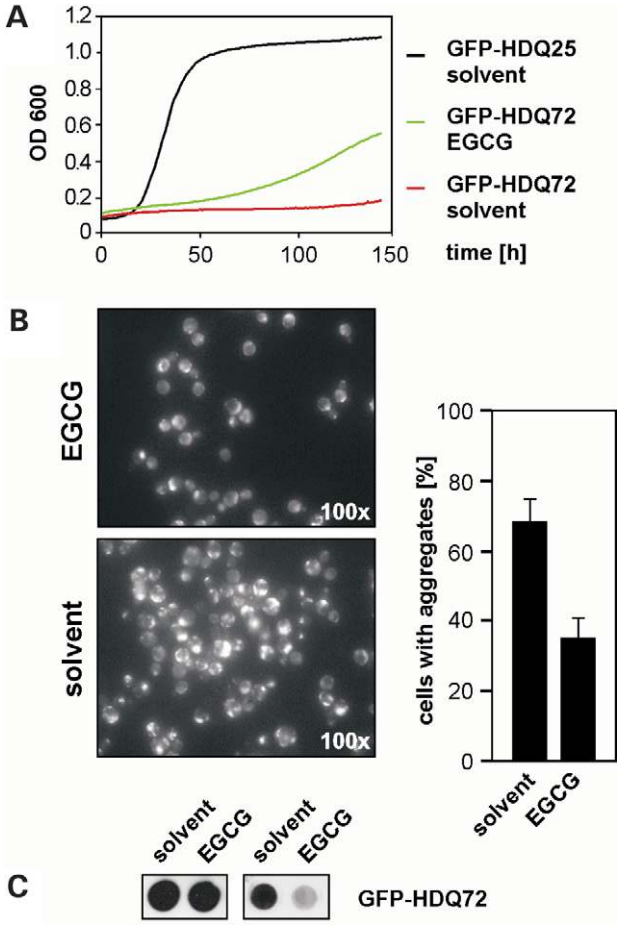

Figure 5. EGCG diminishes toxicity and aggregation of mutant htt fragments in yeast. (**A**) Growth of yeast expressing GFP-HDQ25 or GFP-HDQ72 in the presence or absence of 500 μM EGCG was monitored by measuring the OD_{600} every 30 min over an interval of 144 h. (**B**) Representative fluorescence microscopy images of yeast cells expressing GFP-HDQ72 after cultivation for 4 h in the presence of 500 μM EGCG or the solvent. The percentage of cells containing visible aggregates was determined (a total of 1500 cells was counted), the average results from three independent experiments ± SE are shown. (**C**) Yeast cells expressing GFP-HDQ72 were grown in the presence of 500 μM EGCG or solvent for 6 h. Cell lysates were spotted onto nitrocellulose (left) or subjected to the filter retardation test (right). The total amounts of GFP-htt spotted or the amount of aggregates retained on the filter were detected with a GFP antibody.

microscopy showed that EGCG treatment reduces the number of yeast cells containing GFP-HDQ72 protein aggregates (~40% reduction compared with controls, Fig. 5B). Similarly, when cell extracts were analyzed with the membrane filter retardation assay (24), a lower amount of SDS-insoluble GFP-HDQ72 aggregates was observed in EGCG-treated cells (Fig. 5C), indicating that EGCG suppresses not only toxicity but also protein aggregation in an yeast model of HD.

EGCG reduces photoreceptor degeneration and motor impairment in HD transgenic flies

Previous studies in transgenic flies have demonstrated that overexpression of htt exon 1 protein with an expanded polyQ sequence of 93 glutamines (Htt93Q) causes progressive photoreceptor neuron degeneration that can be monitored by light microscopy (25). The disruption of the regular trapezoidal arrangement of seven visible photoreceptor neurons (rhabdomeres) can be examined as a marker for more widespread neuronal degeneration. To determine whether EGCG improves photoreceptor degeneration, transgenic flies expressing Htt93Q in all neurons from embryogenesis onward were fed with the compound and the number of rhabdomeres per ommatidium was analyzed using the pseudo-pupil technique (25). Freshly eclosed Htt93Q adults were transferred to vials containing increasing doses of compound or solvent alone (DMSO). After 7 days, the number of rhabdomeres was determined. Because of the fact that the transgenic protein is already expressed during development, eyes have begun to degenerate when the flies emerge. Compound efficacy is evaluated by comparing treated flies at day 7 with day 0 control flies. In the absence of EGCG, htt-expressing neurons deteriorate until on average ~3.5 photoreceptor neurons/ommatidium remain after 7 days. When increasing amounts of compound were present, a dose-dependent slowing down of the degenerative process could be observed. With the highest EGCG concentration tested (100 μM), an average of ~4.2 neurons/ommatidium was found after 7 days, corresponding to a rescue of ~29% (Fig. 6B). A visual representation of rescue of the number of rhabdomeres/ommatidium is shown in Figure 6A.

Finally, we tested whether EGCG treatment can improve motor function in the HD transgenic flies using a climbing assay (25). Flies overexpressing HttQ93 show a progressively abnormal movement and climbing behavior with age because of impaired motor and neuronal function (Fig. 6C). This phenotype, however, was dramatically improved when the HD flies were mated and raised on sugar supplemented with 500 μM EGCG. While untreated transgenic flies, at an age of 18 days, could climb on an average 4–5 cm/60 s in a vertical glass cylinder, the EGCG-treated flies climbed about 18 cm in the same time, indicating that the compound not only reduces photoreceptor degeneration but also significantly improves the abnormal motor ability in the transgenic flies. Together, these studies indicate that EGCG is not only a potent inhibitor of polyQ-mediated htt exon 1 protein aggregation *in vitro* but is also able to reduce toxicity in different *in vivo* models of HD.

DISCUSSION

Growing evidence suggests that the misfolding and aggregation of htt is central to HD pathogenesis. Using a well-established filter assay, we screened a library of natural substances and identified EGCG and related compounds from green tea as potent inhibitors of htt exon 1 aggregation. Green tea derivatives are very attractive drug candidates because they are naturally occurring phytopharmaca with known neuroprotective effects (16). EGCG e.g. has been shown to reduce oxidative stress and neurotoxicity in different model systems of Alzheimer's and Parkinson's disease and to modulate the expression of cell survival and cell death genes (17). Furthermore, EGCG was reported to pass the blood–brain barrier in mammals (19) and to be safe for humans when tested in clinical studies (26).

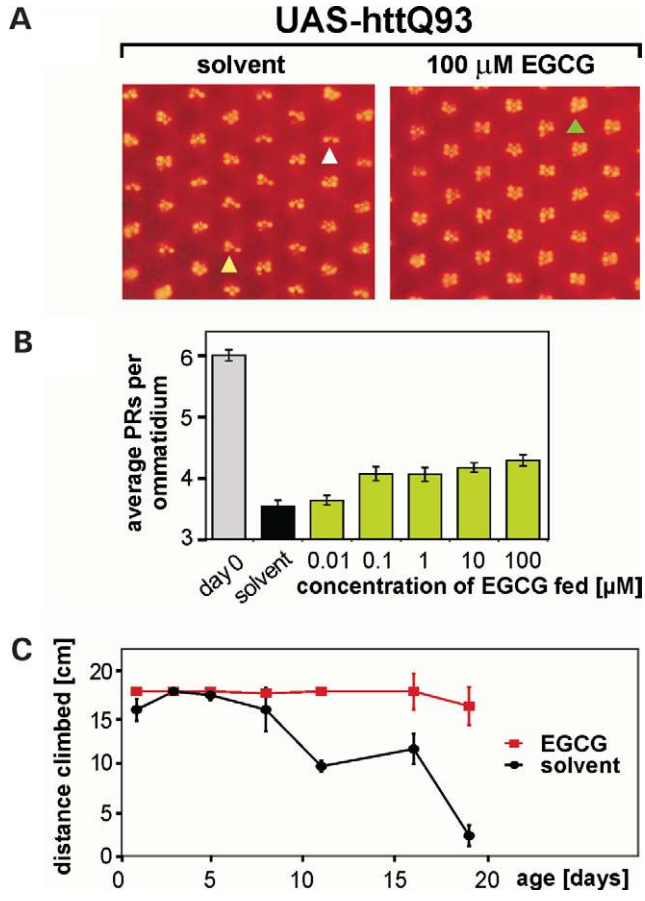

Figure 6. EGCG diminishes the toxicity of mutant htt fragments in fly models of HD. (**A**) Rescue of degeneration of photoreceptor neurons (PR) in *Drosophila* expressing mutant htt fragments with 93 glutamines. Representative deep pseudopupil images of eyes from 7-day-old EGCG-treated and solvent-treated flies. Left panel: Solvent-treated flies show extensive degeneration with many ommatidia containing either three (white arrowhead) or four (yellow arrowhead) rhabdomeres. Right panel: Flies fed 100 μM EGCG show less degeneration, most ommatidia contain four to five rhabdomeres with some retaining seven (green arrowhead). (**B**) Newly eclosed flies were kept on food supplemented with different amounts of EGCG (0.01, 0.1, 1, 10 and 100 μM) or solvent only. The average number of photoreceptor cells per ommatidium was scored 7 days post-eclosion. Error bars represent ±SE. Day 0 shows the neuronal loss that has occurred during pupal development prior to drug treatment. Values ≥0.1 μM are all significantly different ($P < 0.002$) from the no drug control, 0.01 μM EGCG had no significant effect. Maximal rescue in the representative experiment shown was 29%. (**C**) Age-dependent deterioration of climbing abilities in flies expressing mutant htt fragments with 93 glutamines (UAS-httQ93) in neurons was monitored by placing the flies on the bottom of a vertical glass tube and measuring the distance covered in 60 s. Flies mated and raised on food with 500 μM EGCG performed significantly better than solvent-treated controls.

Our data obtained from *in vitro* models of HD demonstrate for the first time that green tea polyphenols are able to modulate early steps in the aggregation process of an amyloidogenic polyQ-containing protein. The inhibitory effect on the assembly of mutant htt exon 1 fragments in the cell-free assays is concentration-dependent and does not require the antioxidant properties of the polyphenols. Nevertheless, the known beneficial activities of EGCG and its derivatives such as radical scavenging, reduction of reactive oxidative species or chelating of metal ions may contribute to the decrease of htt aggregation and toxicity in the *in vivo* models of HD (17).

The aggregation of htt exon 1 fragments *in vitro* is a multistep and, potentially, multipathway process that involves an initial conformational change in the polyQ tract (21), the formation of annular or spherical oligomers and protofibrils and the self-assembly of mature fibrils (27). Two different models for the conversion of polyQ proteins into amyloid-like fibrils have been proposed. On the one hand, it has been suggested that multiple conformations of misfolded htt monomers coexist and give rise to several distinct oligomeric or amorphous assemblies as well as fibrillar structures (20). On the other hand, misfolded htt monomers are believed to proceed via transient oligomers or protofibrils into amyloid-like structures (28). In this model, oligomers are metastable aggregation intermediates that form during the nucleation phase and act as seeds for the assembly of large fibrillar structures. However, the precise assembly pathways for the different types of htt aggregates observed *in vitro* are currently unclear. Moreover, it is unknown which type of protein assembly causes dysfunction and toxicity in mammalian cells.

Using an *in vitro* aggregation assay, we found that the epitopes of expanded polyQ tracts very rapidly become inaccessible for the MW1 antibody after proteolytic cleavage. This observation is in good agreement with the data presented previously (21) and suggests that fragments of mutant htt undergo a conformational change very early in the aggregation process. In our cell-free assay, EGCG significantly prolonged the accessibility of the polyQ epitope for the MW1 antibody in a concentration-dependent manner. This indicates that the compound modulates the intramolecular rearrangement of mutant htt exon1 prior to aggregate formation, influencing the initial misfolding step in the aggregation cascade.

We propose that in untreated reactions, protease cleavage of the GST fusion protein triggers a very rapid intramolecular change in the polyQ-containing htt exon 1 fragment that leads to a compact β-sheet-rich structure. This compaction is most likely caused by the formation of hydrogen bonds between the main chain and side chain amides in the polyQ tract. In EGCG-treated samples, however, either the formation of stable hydrogen bonds is slowed down, or the binding of EGCG molecules to htt might induce the formation of a stable protein-drug adduct that has a novel structure and cannot be recognized by the polyQ-specific MW1 antibody.

Previous studies have shown that gallate esters are important for the association of polyphenols with proteins, probably because they mediate weak hydrophobic interactions with proline-rich regions in target proteins, which is a prerequisite for subsequent hydrogen bond formation (29). Our findings that only EGCG and GCG, but not the related compounds GC and EGC are able to modulate htt exon 1 aggregation *in vitro* are in agreement with the observations that the gallate moiety is critical for the binding of polyphenols to proteins. We suggest that the proline-rich region in the htt exon 1 protein might function as an anchoring point for EGCG binding and the adjacent polyQ sequence causes the formation of stable hydrogen bonds between the compound and the polypeptide chain. However, more detailed studies will be necessary to characterize the structure of the polyphenol-htt complex formed *in vitro*.

AFM analysis also revealed that the addition of EGCG to aggregation reactions changed the formation of htt exon 1 oligomers *in vitro*. EGCG significantly reduced the density of small oligomers with a diameter of 20–80 nm while larger, spherical oligomers with a diameter of 120–200 nm had appeared. This is a clear indication that the compound not only alters the rapid conformational change of the htt exon 1 fragment after proteolytic cleavage of the fusion protein, but also has a modulating impact on later steps in the aggregation process. We propose that the larger spherical oligomers, which are formed after EGCG treatment are off-pathway for fibril formation and are more biologically inert than the small oligomeric structures that form in the absence of the chemical compound. Thus, EGCG may reduce polyQ toxicity in a similar way as the molecular chaperones Hsp70 and Hsp40 do because it attenuates the assembly of small spherical oligomers that appear very early in the aggregation process (20).

The effect of EGCG on polyQ-mediated htt exon 1 aggregation and toxicity was examined in an yeast model of HD. We found that the compound significantly reduced both the formation of SDS-stable htt aggregates as well as toxicity in this *in vivo* model system, supporting the hypothesis that compounds that function as chemical chaperones and modulate early steps in the aggregation pathway (misfolding and oligomerization) have a high potential for therapy development (30). This assumption is further strengthened by our finding that EGCG ameliorates the pathological effects of mutant htt in a fly model of HD. Currently, the molecular mechanisms by which htt fragments with pathogenic polyQ sequences exert their toxic effects in a cellular environment are unclear. Misfolded β-sheet-rich htt monomers or small soluble oligomers form abnormal protein–protein interactions with other polyQ proteins such as the transcription factors TBP and CBP *in vitro*, suggesting that such interactions might also occur *in vivo* in neuronal cells and contribute to HD pathogenesis (21). EGCG might reduce the polyQ-mediated htt toxicity in mammalian cells by changing the conformation of the disease protein and by preventing the formation of destructive protein–protein interactions.

We conclude that EGCG is a potent inhibitor of polyQ aggregation that has beneficial effects *in vivo*. It may protect neuronal cells expressing a mutant htt protein from its noxious properties. Finally, our study reveals EGCG to have considerable potential as drug candidate for the development of treatments of HD and of protein misfolding and amyloid diseases in general.

MATERIALS AND METHODS

Proteins, antibodies and chemical compounds

GST-HDQ51 and GST-HDQ53 were overexpressed in bacteria and purified as described (14,31). The recombinant proteins were stored at −80°C after shock-freezing in liquid nitrogen. Before each experiment, the proteins were either centrifuged at 230.000*g* or 18.000*g* for 20–30 min at 4°C.

The polyclonal anti-htt antibody CAG53b has been described (18). The monoclonal anti-polyQ and anti-polyproline antibodies MW1 and MW7 (22), respectively, were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). The anti-GFP antibody was purchased from Roche (Indianapolis, USA).

The compounds EGCG, GCG, (−)-epigallocatechin (EGC) and (−)-gallocatechin (GC), ascorbic acid and α-tocopherol were purchased from Sigma–Aldrich (Hamburg, Germany). Stock solutions of the compounds of 10 mM were prepared in DMSO and stored at 4°C.

Compound screening and filter retardation assay

A compound library containing ∼5000 natural substances was screened for inhibitors of htt aggregation as described (14). Total removal of the GST-tag from the fusion protein was achieved by elastase treatment (3 min at 37°C in 150 mM NaCl, 20 mM Tris–HCl pH 8.0 and 2 mM $CaCl_2$) before the addition of chemical compounds. HDQ51 of 0.6 μM was then incubated with compound (0.6, 3, 6 μM) or the solvent DMSO alone for 16 h at 37°C to allow aggregate formation.

For the filter retardation assay, aliquots were mixed with 2% SDS and 50 mM DTT (final concentrations) and denatured at 98°C for 7 min. Aliquots corresponding to 125 ng of GST-HDQ51 protein were filtered through a cellulose acetate membrane (0.2 μm pore size, Schleicher & Schuell, Dassel, Germany). Aggregates captured on the membrane were detected with the CAG53b antibody, an alkaline phosphatase-conjugated secondary antibody and the fluorescent substrate AttoPhos. Signals were quantified using the AIDA image analysis software (Raytest, Straubenhardt, Germany).

Analysis of epitope accessibility

Aggregation reactions with GST-HDQ51 were set up as for the filter retardation assay, however, elastase and the chemical compounds were added to the protein at the same time to monitor early compound effects. At different time points, aliquots corresponding to 2 μg fusion protein were spotted onto Protran™ nitrocellulose membranes (Schleicher & Schuell). Membranes were then immunodetected with primary antibody (MW1, MW7 or CAG53b), an alkaline phosphatase coupled secondary antibody and the fluorescent substrate AttoPhos. Signals were quantified using the AIDA image analysis software.

AFM analysis

GST-HD53Q protein was prepared at a concentration of 6 μM in buffer A (50 mM Tris–HCl pH 7, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, 0.5 μM leupeptin, 0.5 μM pepstatin A). At time zero, 12 μM compound and PreScission protease (4 units/100 μg fusion protein) (Amersham Biosciences, Piscataway, USA) were added to initiate removal of GST and HDQ53 aggregation. Aliquots of HDQ53 protein were removed from the reactions and diluted into 50 mM Tris–HCl pH 7. The protein was spotted on freshly cleaved mica, allowed to adhere for 2 min and then washed with 200 μl distilled water. The samples were partially dried with compressed air, dried to completion at room temperature and imaged in air with a digital multimode NanoscopeIII scanning probe

microscope operating in tapping mode. Representative 3 μm^2 images obtained in three separate experiments were used for size/density analysis with custom written software (32).

Yeast methods

Yeast strains employed in this study were in the W303 (*MATα can1-100 ade2-1 his3-11, 15 trp1-1 ura3-1 leu23,112*) genetic background and contained integrated plasmids (pRS303 backbone) for the expression of green fluorescent protein- (GFP) HDQ25 or HDQ72 fusion proteins under the control of the inducible *GAL1* promotor.

Growth of yeast cultures in selective media containing raffinose (2%) as the sole carbon source to mid-log phase followed by growth in galactose (2%) containing selective media induced GFP-HDQ25/72 expression.

For the growth assays, yeasts were grown overnight in selective media with glucose (2%). Cells were washed and diluted to an OD_{600} of 0.02 in media containing 2% galactose. Growth curves were determined with the Bioscreen instrument by taking OD_{600} measurements at 30 min intervals over a period of 72 h.

Filter retardation assays of aggregated material were performed as described (24) with total lysates prepared from yeast cells expressing GFP-HDQ72. Dot-blots were performed like filter retardation assays except that PVDF membranes were used. Aggregates and total htt protein on the membranes were immunodetected with the GFP-antibody.

The percentage of cells containing GFP-HDQ72 aggregates was determined with a Zeiss Axioplan II microscope and the Openlab (ImproVision, Lexington, USA) software. In total, 1500 cells of three independent cultures were counted.

Drosophila melanogaster methods

Expression of the htt protein in transgenic flies is driven by the bipartite expression system upstream activator sequence (UAS)-GAL4 (yeast transcriptional activator). Stocks *w*; $P(w^{+mC}$; $w+$; *elav-GAL4/CyO*) and *w*; $P(w+mC=$ *UAS-Q93httexon1*$)^{P463}$ were mated in order to obtain flies expressing mutant htt fragments in all neurons from embryogenesis on. Flies were mated at 25°C on standard food supplemented with the compound to be tested, transferred to fresh food daily and assayed for neurodegeneration at day 7 post-eclosion (25). Concentrations used in the feedings were: 0.01, 0.1, 1, 10 and 100 μM, using a 20 mM stock in DMSO. Vials were normalized to the same concentration of DMSO, always $\leq$0.5% to control for DMSO effects (25). The plotted values are an average of 10 eyes from 10 flies assayed at each concentration with a minimum of 100 ommatidia (omm) counted and averaged per eye. The significance of the resulting differences in average number of PRs was analyzed using the Wilcoxon rank-sum test. Error bars in Figure 5A represent $\pm$SE. Percent rescue was calculated as [(ave no. PR/omm in drug-fed flies) − (ave no. PR/omm in no-drug control flies)] ÷ [(ave no. PR/omm at day zero) − (ave no. PR/omm in no-drug control flies)] × 100. The average number of PR/omm present at day 0 in a representative sample of flies is measured prior to drug feeding.

For the climbing assay, flies were mated and raised on media containing 500 μM EGCG or the DMSO solvent. At different timepoints after eclosion, 20 flies were placed in a vial and the height half of them were able to climb from the bottom within 60 s was determined. The values shown are the average of five determinations $\pm$SE.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank S. Schnoegl for critical reading of the manuscript and editorial support. The project was funded by the NGFN grants 01GR471 and 01GR474, the APOPIS grant LSHM-CT-2003-503330, Deutsche Forschungsgemeinschaft grants Wa-1151/5-1 and He-1928/8-2, the Huntington's Disease Society of America (HDSA), the HDF, NINDS (NS54753) and the High Q foundation.

Conflict of Interest statement. The authors do not declare any conflict of interest.

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