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Binding of epigallocatechin-3-gallate to transthyretin modulates its amyloidogenicity

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ABSTRACT

More than 100 transthyretin (TTR) variants are associated with hereditary amyloidosis. Approaches for TTR amyloidosis that interfere with any step of the cascade of events leading to fibril formation have therapeutic potential. In this study we tested (−)-epigallocatechin-3-gallate (EGCG), the most abundant catechin of green tea, as an inhibitor of TTR amyloid formation. We demonstrate that EGCG binds to TTR "in vitro" and "ex vivo" and that EGCG inhibits TTR aggregation "in vitro" and in a cell culture system. These findings together with the low toxicity of the compound raise the possibility of using EGCG in a therapeutic approach for familial amyloidotic polyneuropathy, the most frequent form of hereditary TTR amyloidosis.

Structured summary:
MINT-7294529: *TTR* (uniprotkb:P02766) and *TTR* (uniprotkb:P02766) *bind* (MI:0407) by *comigration in non-denaturing gel electrophoresis* (MI:0404)

1. Introduction

Recent studies revealed that the polyphenol (−)-epigallocatechin gallate (EGCG), the most abundant catechin in green tea, may be used for the prevention and treatment of diseases involving amyloid fibril formation [1,2]. In the particular case of neurodegenerative diseases, it was shown that EGCG inhibits both α-synuclein and amyloid-β fibrillogenesis [1]. In both instances, protein aggregation is a key factor for the development of the disease. That is also the case of transthyretin (TTR) amyloidoses that are protein aggregation diseases associated with peripheral neuropathy. More than 100 TTR variants with a single amino acid substitution have been related to hereditary amyloidosis [3]. The most frequent variant is TTR V30M, in which a residue of valine (V) is substituted by a methionine (M) originating a particular form of the disease designated familial amyloid polyneuropathy (FAP) [4]. Other TTR variants are associated with more or less aggressive pathologies. For instance, TTR L55P is associated with the most aggressive form of the disease and is the most amyloidogenic TTR variant known. Isolated TTR L55P easily forms fibrils in physiological conditions (pH 7.4, 37 °C) [5]. TTR Y78F is also a variant very prone to amyloid formation. In the soluble form, this variant presents epitopes common to TTR amyloid fibrils thus resembling an early intermediate of TTR fibrillogenesis [6]. Therefore, this variant can be used to screen inhibitors at very early stages of amyloid fibril formation.

The amyloidogenic potential of TTR variants has been related to the stability of the TTR tetramer [7]. The TTR molecule is a tetramer of identical subunits of 127 amino acid residues each [8]. The occurrence of amino acid substitutions in the monomer induces subtle structural alterations that decrease the stability of the tetramer that dissociates into conformational altered monomers that aggregate to form amyloid fibrils [9,10]. Several small compounds have been suggested for TTR amyloidosis therapy. Those compounds might act as inhibitors of TTR tetramer dissociation impairing amyloid fibril formation or as disruptors of TTR fibrils [11]. Among those, there are non-steroidal anti-inflammatory drugs (NSAIDs) or derivatives [12] and natural substances such as flavonoids [13] and xanthones of plant origin [14]. Most of the compounds previously tested

Abbreviations: TTR, transthyretin; T_4, thyroxine; EGCG, (−)-Epigallocatechin-3-gallate; FAP, familial amyloidotic polyneuropathy

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compete with thyroxine (T_4), a physiologic TTR ligand, for the binding to TTR but present low binding affinity or low selectivity of binding to TTR and in some cases, high toxicity. Therefore it is of most interest to find better compounds to inhibit amyloid fibril formation. EGCG is a natural compound with very low toxicity that has been tried in different amyloidoses [15]. In the present work we evaluate the ability of EGCG to bind to TTR, its capacity to stabilize the molecule and to inhibit amyloid fibril formation.

2. Materials and methods

2.1. Plasma samples

Whole blood from seven TTR V30M heterozygote carriers and from seven control individuals was collected with EDTA and centrifuged. The plasma was separated and frozen at −20 °C.

2.2. Recombinant proteins

Recombinant wild-type TTR (TTR WT), TTR V30M, TTR L55P and TTR Y78F variants were produced in a bacterial expression system and purified as previously described [16].

2.3. EGCG competition with T_4 for the binding to TTR

Five microliters of plasma or 10 μg of the recombinant protein were incubated with [^{125}I]T_4 (specific radioactivity 1250 μCi/μg; Perkin–Elmer, MA, USA) in the presence or absence of EGCG (20× molar excess). EGCG (Sigma–Aldrich, USA) was solubilised in water or phosphate buffer saline (PBS). The plasma proteins were then separated by native PAGE. The gel was dried, subjected to phosphor imaging (Typhoon 8600; Molecular Diagnostics, Amersham Biosciences), and analyzed using the ImageQuant program version 5.1.

Competition of EGCG with T_4 for the binding to TTR WT was quantitatively assayed by incubating TTR (30 nM) with a trace amount of [^{125}I]T_4 and with increasing concentrations (0–10 μM) of competitor, either T_4 or EGCG. Bound T_4 was separated from free T_4 by gel filtration [16].

2.4. Nitroblue tetrazolium (NBT) staining assay

EGCG binding to TTR was assayed by nitroblue tetrazolium (NBT) staining [17]. Recombinant TTR WT and TTR V30M were incubated in the presence of 10× molar excess of EGCG for 2 h at 37 °C, or PBS (as control) and separated by SDS–PAGE (15% polyacrylamide). The gels were either silver stained or electroblotted onto nitrocellulose membrane (GE Healthcare). The membranes were washed with water and stained with glycinate/NBT solution (0.24 mM NBT in 2 M potassium glycinate, pH 10) for 20 min. The membranes were again washed and stained with Ponceau S (0.1% in 5% acetic acid) resulting in red stained bands, while the NBT stained bands remained purple.

2.5. Mass spectrometry analysis

Recombinant WT TTR (2 mg/mL in PBS) was incubated at 37 °C for 1 h in the presence or absence (control) of EGCG at 0.36 mM (10× molar excess).

The samples were diluted to 0.1 μg/μL in water:methanol:formic acid (50:50:1). Electrospray mass spectrometry (ESI-MS) was performed on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). Samples were applied at 10 μL/min flow rate. The needle voltage was 3000 V, source block at 80 °C and desolvation temperature was 150 °C. Data were acquired with a MassLynx 4 data system (Micromass, Manchester, UK). ESI-MS spectra were deconvoluted using the MaxEnt 1 algorithm.

2.6. Study of EGCG–TTR interaction by Isothermal Titration Calorimetry (ITC)

The details of the microcalorimeter unit (ThermoMetric AB, Järfalla, Sweden) used have been described previously [18,19]. The calorimetric vessel was filled with 0.9 mL of a 120 μM TTR solution in 20 mM sodium phosphate, pH 8.0, and the titration consisted of serial additions of a 1500 μM EGCG solution in the same buffer. The experiments were performed at 308.15 ± 0.01 K. The heat of dilution to correct the binding curves was the constant heat per injection in the last five injections. Experiments were performed in "fast mode", and the curves were dynamically corrected [19], integrated using the Origin 7 software and the heat evolved per injection calculated, using the previously determined calibration constant.

2.7. Assessment of TTR tetrameric stability by Isoelectric Focusing (IEF)

Briefly, 30 μL of human plasma were incubated with 5 μl of 10 mM EGCG solution for 1 h at 37 °C and subjected to native PAGE. The TTR gel band was excised and applied to a partial denaturing (4 M urea) IEF gel containing 5% (v/v) ampholytes pH 4–6.5 (GE Healthcare) run at 1200 V for 6 h [16,20]. Proteins were stained with Coomassie Blue, the gels were scanned (HP Scanjet 4470c; Hewlett–Packard) and subjected to densitometry using the ImageQuant program.

2.8. Studies of TTR aggregation and fibril formation by Transmission Electron Microscopy (TEM)

To study the effect of EGCG on TTR aggregation, soluble TTR Y78F (2 mg/mL) in PBS was incubated at 37 °C for 0, 2, 4 and 6 days alone or with EGCG 0.36 mM (10× molar excess) or 3.6 mM (100×). Ultrastructural analysis of the samples was performed by TEM as detailed previously [21].

To study the effect of EGCG on TTR fibril formation, we used pre-formed aggregates. TTR Y78F aggregates were prepared by incubation of the soluble protein (2 mg/mL) for 2 days at 37 °C, pH 7.4, while TTR WT and TTR V30M aggregates were prepared by acidification of the protein solutions (2 mg/ml) with 0.05 M sodium acetate (pH 3.6) and incubation for 72 h at room temperature. The samples were centrifuged at 15 000×*g*, 4 °C for 30 min. The pellets were re-suspended in water and the aggregated TTRs were incubated at 37 °C for 0, 4 and 6 days alone or with EGCG 3.6 mM. Conditions for TEM analysis were as described previously [21].

2.9. Cell culture and dot-blot filter assay for aggregate detection

To test EGCG as inhibitor of TTR aggregation we used a rat Schwannoma (RN22) (American Type Cell Collection) cell line stably co-transfected with TTR L55P cDNA and grown in conditions previously described [21]. The cells were incubated for 48 h with 1 μM EGCG in the medium and then for further 3 days (80% cell confluence) with EGCG simultaneously with 100 μM $ZnSO_4$. The cells were then incubated with serum-free medium with $ZnSO_4$ and EGCG for additional 24 h. TTR in the medium was quantified by ELISA, and the volume corresponding to 500 ng was applied onto a 0.2 μm pore cellulose acetate membrane filter (Schleicher and Schuell) under vacuum. TTR aggregates, retained in the membrane, were immunodetected using rabbit anti-human TTR antibody (DAKO) (1:500 dilution) followed by a secondary antibody,

anti-rabbit HRP (horseradish peroxidase) conjugate (1:1500). Detection was performed with ECL® (GE Healthcare). Dot blots were quantified using the ImageQuant program. Experiments were repeated at least three times in duplicate. All values are expressed as means ± S.E.M. Comparison between groups was made using the Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. EGCG binding to TTR

To determine if EGCG competed with T_4 for the binding to TTR in plasma from heterozygote TTR V30M carriers and from control individuals (Fig. 1A) and to recombinant TTR WT and TTR V30M (Fig. 1B) the samples were incubated with [^{125}I]T_4 in the presence or absence of EGCG. Subsequently the proteins were separated by native PAGE [16]. In whole plasma, three main proteins bind T_4 as indicated in Fig. 1A: thyroxine binding globulin (TBG), albumin and TTR. As expected, TTR V30M presented significant lower band intensity than TTR WT, both in plasma and recombinant, due to lower binding affinity to [^{125}I]T_4. However no significant alteration of the TTR band intensity was found when comparing the same sample in the presence and absence of EGCG. This indicates that EGCG did not compete with T_4 for the binding to plasma TTR, neither in TTR V30M heterozygote carriers nor in control individuals. Noteworthy, when incubated with EGCG, TTR presented a different electrophoretic mobility suggesting that the catechin alters TTR migration through binding in a region of the molecule other than the T_4 binding site. The results obtained with isolated WT and V30M TTR (Fig. 1B) were consistent with the plasma ex vivo data (Fig. 1A).

We also tested EGCG competition with [^{125}I]T_4 for the binding to recombinant TTR WT by a quantitative assay [16]. EGCG did not displace [^{125}I]T_4 from TTR meaning that it does not compete with T_4 for the binding to TTR (data not shown), confirming the above described data.

3.2. Characterization of the EGCG–TTR interaction

It has been reported that EGCG reacts with proteins forming complexes that are specifically stained with NBT [22]. To investigate EGCG binding to WT and V30M TTR, each protein was incubated in the presence or absence (control) of EGCG and subjected to SDS–PAGE. We included a bovine serum albumin (BSA) sample as positive control for EGCG binding. After electrophoresis, the proteins in the gel were either silver stained (Fig. 2IA) or electroblotted onto a nitrocellulose membrane. The membranes were subsequently stained with Ponceau S (Fig. 2IB), to confirm blotting efficiency, followed by NBT/glycinate staining (Fig. 2IC). Samples incubated with EGCG (+), TTR WT and V30M, showed purple protein bands after NBT staining, while no colour reaction occurred in the controls (−). Thus, EGCG binds to TTR and the interaction is stable under the denaturing conditions of SDS–PAGE.

The stability of the EGCG–TTR interaction suggested that the complex could be detected by MS analysis. Therefore, TTR WT was incubated with EGCG and the MS analysis (Fig. 2II) showed a peak with a mass of 14 523 Da while the TTR alone presented a peak of 14 065 Da. The mass difference between these two peaks is 458 Da, the mass of an EGCG molecule, indicating that one molecule of EGCG binds to the TTR monomer under the tested conditions.

The EGCG–TTR interaction was further characterized by ITC. The results obtained (*Q* values) were analyzed so as to derive the thermodynamic parameters characterizing the binding event. The best fitting of the data was obtained for a model that considers one set of binding sites. The values obtained were for the binding constant $K = 2.5 \times 10^6 \pm 9 \times 10^5$ and the enthalpy of the binding process, $\Delta H = -30 \pm 1$ kJ mol^{-1} with an occupancy of 0.145 ± 0.004 per mole of protein. From these values we calculate a ΔG value of -37 kJ mol^{-1}, as well as an entropy change upon binding of -23 J K^{-1} mol^{-1}.

3.3. Effect of EGCG on TTR tetramer stability

The effect of EGCG on TTR stability was investigated, ex vivo, by IEF (Fig. 3A and B). Several plasma samples from heterozygote carriers of TTR V30M and from control individuals were incubated with EGCG or iododiflunisal (IDIF), a potent TTR stabilizer, (kindly provided by Dr. Gregorio Valencia, CSIC, Barcelona, Spain), previously tested by us and used as reference [16]. Under the conditions used, and in the absence of stabilizers, TTR presented a characteristic band pattern, which includes the monomer, an oxidized form of the monomer and several bands of lower p*I*, representing different forms of tetramers. The samples incubated with EGCG display 30% increase of the tetramer/total protein (tetramer + monomer + oxidized monomer) ratio, in both normal individuals and heterozygote carriers of TTR V30M (Fig. 3C) as compared to controls, indicating that EGCG stabilizes the TTR tetramer and it also alters TTR tetramer migration in IEF.

3.4. Effect of EGCG on TTR aggregation in vitro

The effect of EGCG on TTR aggregation was evaluated by ultrastructural analyses of TTR Y78F treated with two different

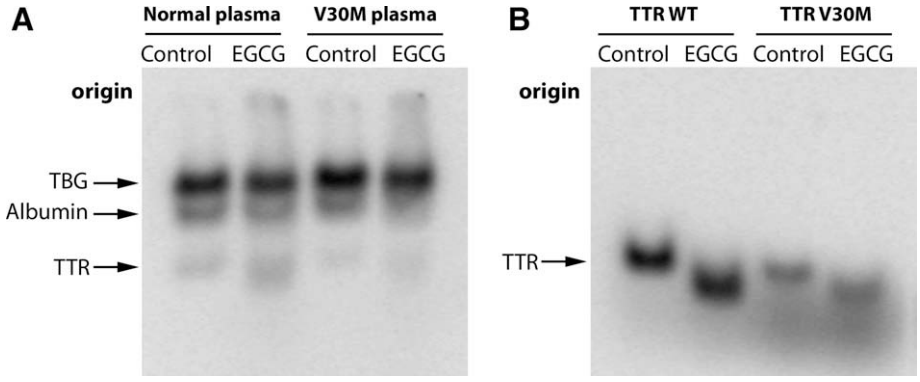

Fig. 1. Competition of EGCG with T_4 for the binding to TTR in human plasma (A) and recombinant TTR (B). Samples were incubated with [^{125}I] T_4, with or without EGCG, and analyzed by PAGE. TTR migration is altered for the samples incubated with EGCG.

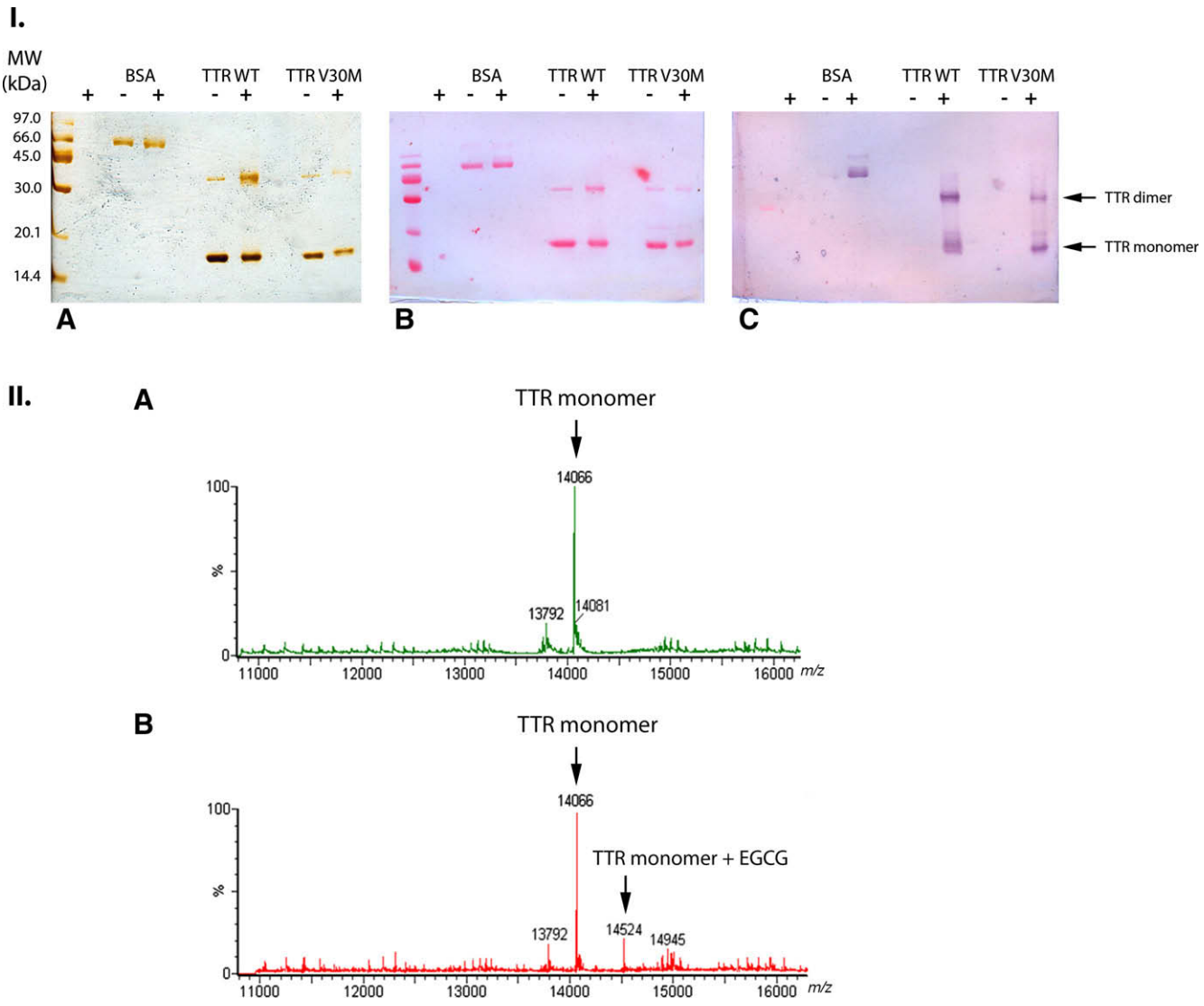

Fig. 2. I. (A) Silver staining of proteins treated (+) or non-treated (−) with EGCG after SDS–PAGE. (B) Ponceau S staining of nitrocellulose membrane after electroblotting of a gel equivalent to (A). (C) NBT/glycinate staining of the membrane of (B). II. Mass spectra of recombinant TTR WT (A) and recombinant TTR WT incubated with EGCG (B).

EGCG concentrations. TTR Y78F is very amyloidogenic and forms fibrils under physiological conditions (PBS, pH 7.4, 37 °C) [16]. The results obtained are depicted in Fig. 4I, left panels: after 2 days at 37 °C, the protein alone presented as a heterogeneous preparation formed mainly by oligomers; occasionally, aggregates (arrowhead) and short twisted fibrils (arrow); at 4 days incubation there was further aggregation of the protein (arrowheads) and formation of larger aggregates. After 6 days, the preparation was largely constituted by short fibrils of different diameter (arrows) and aggregates were still present (arrowheads). The TTR Y78F preparations treated with EGCG were evaluated at the same time points: after 2 days at 37 °C, small aggregates (arrowhead, middle panel, 2 days) and oligomers were detected in the 10× EGCG concentration but no short fibrils were visible while incubation with EGCG 100× resulted in round particles resembling the soluble protein after 2 days incubation (Fig. 4 right panel, arrowheads). This effect was maintained throughout the period studied (right panels, 4 and 6 days). Similar results were achieved using EGCG 10x, at later stages (4 and 6 days, Fig. 4 middle panels). Our results indicate that EGCG is able to prevent TTR Y78F aggregation in vitro.

3.5. Effect of EGCG on TTR aggregation in a cell culture system

The effect of EGCG on TTR aggregation was also investigated in a rat Schwannoma cell line that secretes TTR L55P to the medium where it forms small aggregates [16]. The aggregated protein is retained in a membrane filter and is immunodetected by an anti-TTR antibody. In the present work, we added EGCG to the cells growing medium and compared with the effect of iododiflunisal [16]. The blot and relative effect of EGCG on TTR aggregation are presented in Fig. 4II. The inhibition of TTR aggregation is higher in the medium of EGCG treated cells than in the medium of iododiflunisal (reference compound) treated cells relative to control medium, non-treated cells. The results confirm that EGCG inhibits TTR aggregation in this cell system.

3.6. Effect of EGCG on TTR fibril formation

The action of EGCG was also tested in pre-formed aggregates prepared from TTR Y78F, TTR WT and TTR V30M. These preparations were incubated with or without EGCG (100× molar excess) for 18 days at 37 °C.

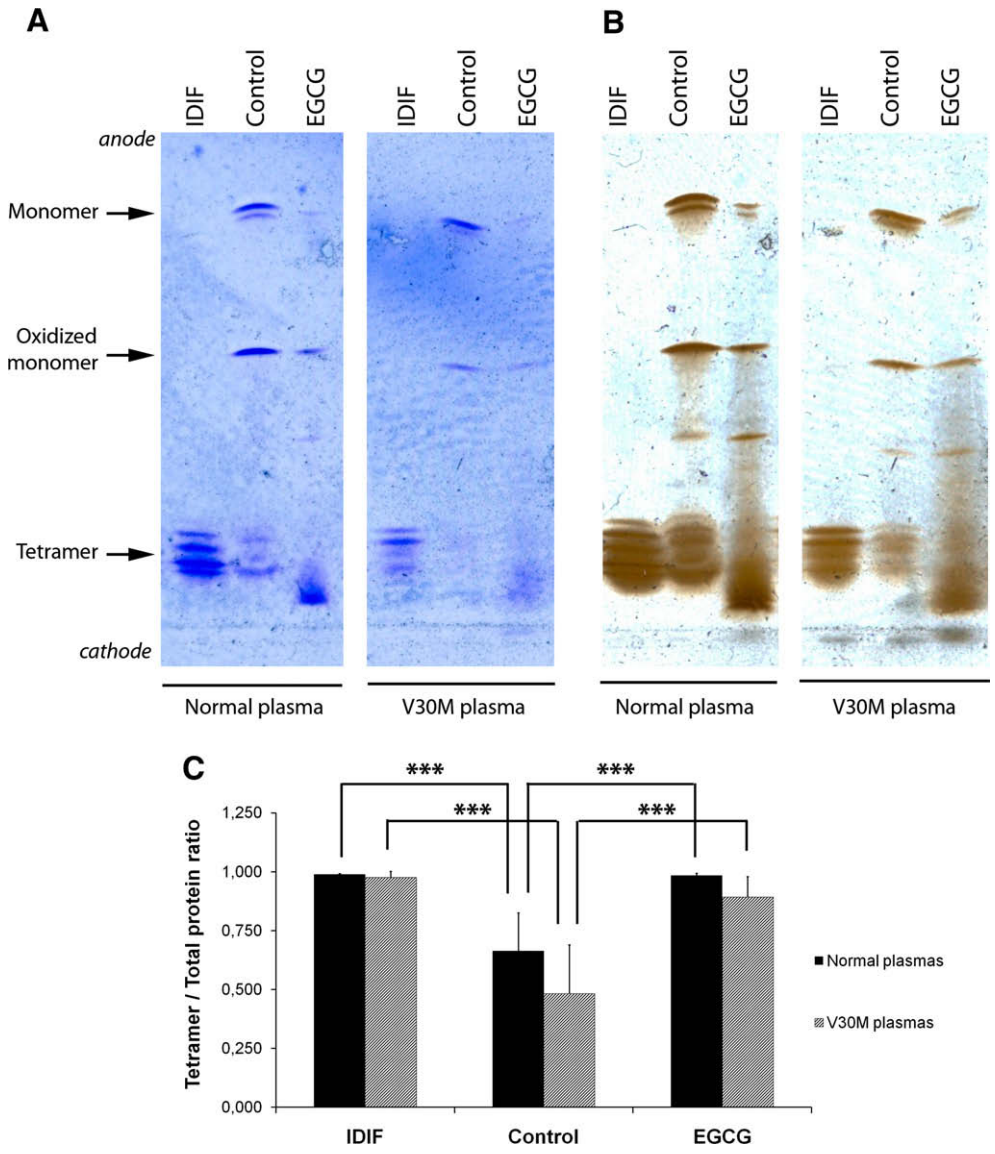

Fig. 3. Analysis of plasma TTR by IEF after Coomassie Blue staining (A) and silver staining (B). Samples treated with EGCG present stronger TTR tetramer bands and less intense reduced and oxidized monomer bands as compared to controls. These gels are representative of others run in parallel. (C) The histogram shows densitometry of IEF gel: TTR tetramer/total TTR bands ratio for normal and V30M TTR protein, $^{***}P \leqslant 0.001$.

The results obtained for TTR Y78F are illustrated in Fig. 5. Before EGCG addition, the preparation was composed mainly by oligomers, small aggregates and short twisted fibrils (day 0). After 2 days incubation with EGCG only a small number of aggregates and short fibrils were detected, as compared to the control (data not shown) indicating that EGCG disrupted TTR Y78F fibrils. This effect was even more evident at 4 days incubation, when the preparation was mainly constituted by small spherical (off-pathway) aggregates. Extended incubation for 18 days showed increase of the number of small round species throughout the preparation (data not shown).

Concerning TTR WT and V30M fibrils, formed by acidification, a similar fibril disruption effect was achieved in the presence of EGCG for the same time points (4 and 18 days) (data not shown).

4. Discussion

In this work we investigated the effect of EGCG on TTR amyloid fibril formation. We demonstrate that EGCG binds to TTR in vitro and ex vivo it inhibits TTR amyloid fibril formation in vitro and in a cellular system.

TTR interaction with EGCG is very stable since it is resistant to highly denaturing conditions and was detectable by MS analysis. We concluded that one EGCG molecule binds to each TTR monomer since only one extra peak corresponding to the mass of the TTR monomer plus the mass of a molecule of EGCG was detected. The TTR–EGCG interaction was further characterized by ITC. The value obtained for the binding constant shows that the ligand does bind strongly to the protein under the studied conditions even though the occupancy obtained was very low.

Curiously, the binding of EGCG to TTR induces a significant alteration of TTR migration in electrophoresis. The same does not happen with TTR ligands that bind at the T_4 binding channel as is the case of most of the TTR fibril inhibitors known [23]. This reflects the different mode of binding of EGCG as compared to other TTR inhibitors. Indeed we were expecting that EGCG competed with T_4 for the binding to TTR because there are reports on the competition of flavonoids [13]. EGCG does not bind at the T_4 bind-

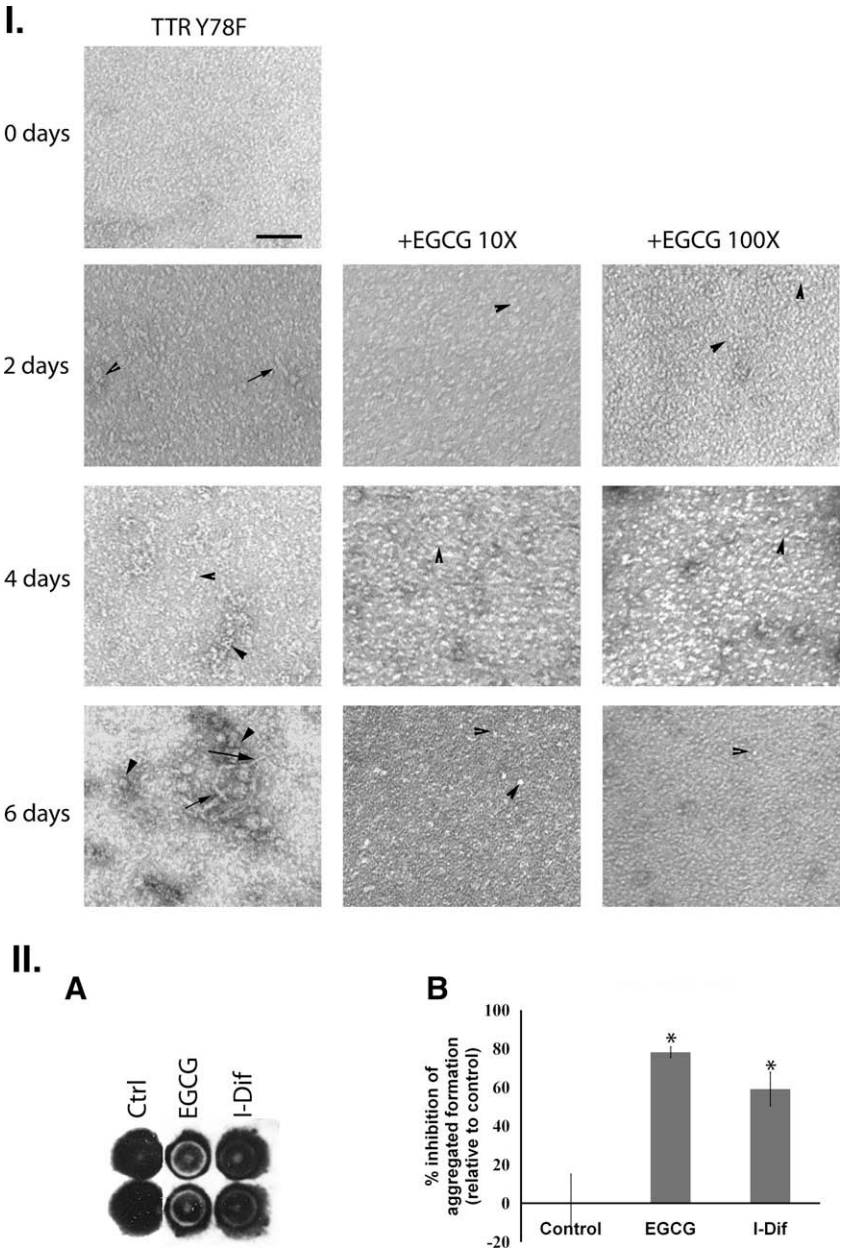

Fig. 4. I. TEM analysis of TTR Y78F aggregation in the presence or absence of EGCG (10× or 100× EGCG molar excess), at different time points. II. (A) Filter assay of the medium from cells grown in the absence (control) or in the presence of EGCG or iododiflunisal (IDIF). (B) Representation of the percentage of aggregation inhibition based on quantification of the dots obtained in A ($^{*}P \leqslant 0.05$).

ing site in TTR, possibly it binds at a region located at the surface of the molecule, altering its conformation and consequently its electrophoretic mobility. The results obtained by IEF in partial denaturing conditions suggest that, ex vivo, EGCG induces TTR stabilization since there is a decrease in the amount of TTR monomer in the treated samples as compared to the non-treated ones. In addition, the amount of TTR tetramer increases and there is also an alteration of the TTR tetramer migration in this electrophoresis system. Stabilization of the TTR tetramer is one of the mechanisms by which TTR amyloid fibril formation inhibitors might act. Thus, TTR stabilization by EGCG can explain the inhibition of TTR aggregation as studied by TEM and cell culture studies. A recent report on the effects of EGCG on α-synuclein and Aβ-42 [1] suggests that EGCG binds to unfolded proteins and redirect them to an alternative non-amyloidogenic aggregation pathway. However the possibility of EGCG binding to structured proteins is not excluded, as well as the possibility of dissociation and remodeling of preformed toxic amyloid aggregates [1]. More recently, binding of EGCG to native folded prion protein has been reported. This interaction promotes the formation of random coil structures and leads to aggregation in an amorphous non-fibrillar form of the protein [24]. In either case, binding of EGCG to the unfolded or folded amyloidogenic protein opens new hypotheses to interfere with the amyloid fibril formation process. We further verified that EGCG also has the capability to function as an amyloid fibril disruptor in vitro through its action over pre-formed aggregates.

Most of the previously proposed inhibitors of TTR amyloid fibril formation raise problems of toxicity and selectivity of binding to TTR [11]. Concerning EGCG, the highest dosages tested in humans refer to safe administration of 800 mg/day for at least 4 weeks [25,26] however this dosage results in a peak plasma concentration of EGCG of approximately 1 μM [26]. This value corresponds only

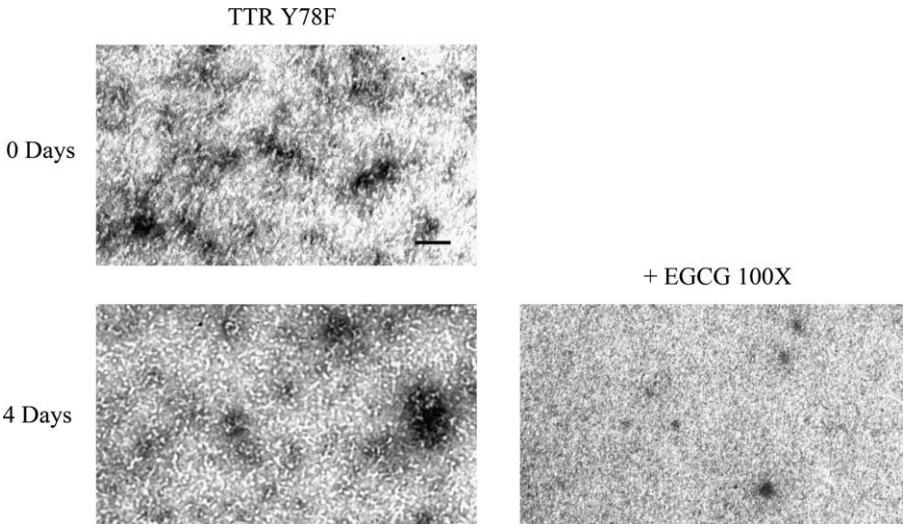

Fig. 5. Effect of EGCG on TTR Y78F pre-formed fibrils (day 0). Aliquots of the preparation were incubated in the absence (control) or presence of EGCG (100× molar excess) and observed 4 days after incubation by TEM.

to about one third of plasma TTR concentration while in the present work we used an EGCG excess of 10–100 times TTR concentration. We also must consider that the interaction of EGCG with TTR is not specific, EGCG binds to other amyloidogenic proteins and most importantly it binds to serum albumin [27]. However we should stress that we only tested EGCG in vitro and that the repeated administration of the compound in a therapeutic approach should result in an increase of the bioavailability of the compound. Thus, only in vivo studies using animal models for TTR amyloidosis [28] will allow evaluation of the usefulness of this compound on therapy for TTR amyloidoses.

It seems also relevant that EGCG crosses the blood–brain barrier [29] and therefore it can bind TTR in the cerebrospinal fluid. TTR is a plasma protein mainly synthesized by the liver but it is also produced by the choroid plexus of the brain and is secreted to the cerebrospinal fluid where it is a major protein component [30]. In most cases of TTR amyloidosis there is no amyloid deposition in the central nervous system but there are a few TTR variants (about 10 variants) associated with TTR amyloid deposition in the leptomeninges [31]. Thus, EGCG therapy might be particularly important in those cases.

In conclusion, studies of EGCG binding to TTR should proceed in order to characterize, at the molecular level, the interaction of TTR with EGCG, in particular the amino acid residues involved. It would be interesting to investigate the influence of TTR ligands, other than T_4, in particular RBP (retinol binding protein) on EGCG–TTR interaction. Taken together the results obtained prompt us to proceed with in vivo studies, in particular with the study of the effects of EGCG in a transgenic mice model for FAP [28].

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