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**Anti-apoptotic treatment reduces transthyretin deposition in a transgenic mouse
model of Familial Amyloidotic Polyneuropathy**

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Abstract

Tauroursodeoxycholic acid (TUDCA) is a unique natural compound that acts as a potent anti-apoptotic and anti-oxidant agent, reducing cytotoxicity in several neurodegenerative diseases. Since oxidative stress, apoptosis and inflammation are associated with transthyretin (TTR) deposition in Familial Amyloidotic Polyneuropathy (FAP), we investigated the possible TUDCA therapeutical application in this disease. We show by semi-quantitative immunohistochemistry and Western blotting that administration of TUDCA to a transgenic mouse model of FAP decreased apoptotic and oxidative biomarkers usually associated with TTR deposition, namely the ER stress markers BiP and eIF2 α , the Fas death receptor and oxidation products such as 3-nitrotyrosine. Most important, TUDCA treatment significantly reduced TTR toxic aggregates in as much as 75%. Since TUDCA has no effect on TTR aggregation “in vitro”, this finding points for the “in vivo” modulation of TTR aggregation by cellular responses, such as by oxidative stress, ER stress and apoptosis and prompts for the use of this safe drug in prophylactic and therapeutic measures in FAP.

Key words: Tauroursodeoxycholic acid (TUDCA), amyloid, protein aggregation, cytotoxicity., apoptosis, ER stress

Introduction

Tauroursodeoxycholic acid (TUDCA) is a hydrophilic bile acid normally produced endogenously in humans at very low concentrations. TUDCA is formed in the conjugation pathways of ursodeoxycholic acid (UDCA), which is commonly used in bile acid replacement therapy for the treatment of certain cholestatic syndromes. Recent reports have shown that both hydrophilic bile acids, UDCA and TUDCA, can prevent hepatic cytotoxicity through several mechanisms. For example, TUDCA prevents the production of reactive oxygen species (ROS) and thus acts as an anti-oxidant (1).

Additionally, TUDCA mitigates mitochondrial insufficiency and toxicity preventing apoptosis, namely by inhibiting Bax translocation from the cytosol to mitochondria. In hepatocytes, this inhibition results in reduced mitochondrial membrane perturbation, release of cytochrome c and activation of downstream caspases (1-4). Moreover TUDCA proved to be neuroprotective in a genetic mouse model of Huntington Disease (HD). Drug treatment lead to marked reduction in striatal cell apoptosis and degeneration, and in intracellular inclusions typical of HD (5). The neuroprotective effect of this drug was also confirmed in rat models of Parkinson Disease (PD) (6), ischemic and hemorrhagic stroke (7-8).

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant hereditary disease characterized by the extracellular deposition of amyloid fibrils in the connective tissue, affecting particularly the peripheral nervous system (9). Amyloid fibrils are composed of mutant transthyretin (TTR), a homotetrameric protein that transports thyroxine (T₄) in the

plasma (10). Research on the dynamics of TTR aggregation into amyloid fibrils, characterized different molecular species suggesting a model involving several steps, namely dissociation of the tetramer and polymerization of the resulting monomers into non-fibrillar oligomers, leading to protofibrils that elongate further into mature fibrils (11). According to this proposed mechanism, stabilization of the tetramer would be a fundamental step for the inhibition of amyloid fibril formation.

Cytotoxicity caused by the aggregation and tissue deposition of TTR Val30Met has been described in FAP. Oxidative stress, inflammation and activation of caspase -3 were detected in a pre-symptomatic stage of the disease when aggregation of non-fibrillar TTR is detected (12).

Increased levels of oxidative metabolites, including hydroxynonenal (HNE) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) are found in tissues presenting TTR aggregation not only in transgenic animals but also in human FAP biopsies (13); furthermore, Fas and caspase-8 activation are observed in tissues with TTR deposition, suggestive of activation of death receptor pathways not involving the mitochondria (13). Recent data from our group showed activation of the classical Unfolded-Protein Response (UPR) pathways in tissues not specialized in TTR synthesis but presenting extracellular TTR aggregates (14).

Transgenic mice for human TTR Val30Met mutation, in a null background serves as an animal model for TTR deposition and constitute an important tool to confirm drug activities and to optimize therapeutic strategies. This animal model for TTR amyloidosis shows deposition of non-fibrillar mutant TTR starting at 3 months of age; TTR amyloid fibrils are detected at 9 months of age, with particular involvement of the gastrointestinal tract (15, 16).

Thus, we reasoned that sub-chronic administration of TUDCA in this animal model could exert effect on apoptotic pathways and oxidative environment normally associated with FAP. We herein report the “in vivo” effect of TUDCA, a well characterized and widely used drug in the FAP animal model.

ACCEPTED MANUSCRIPT

Materials and Methods

Transgenic mice

3 months old transgenic mice bearing the human TTR Val30Met mutation, in a TTR null background - hTTR Met30 (n=15) were used for the experiment (15). Animals were kept and used strictly in accordance with National and European Union Guidelines for the care and handling of laboratory animals. Animals were treated with TUDCA (Calbiochem, San Diego, CA, USA), at a concentration of 500 mg/ kg/ day in the drinking water, during a 3 month period. An age-matched control group of animals was maintained in the same conditions, drinking regular tap water. After 3 months treatment, animals were sacrificed after anesthesia with ketamine/xylazine. All mice tissues, including esophagus, stomach, small and large intestine were immediately excised and frozen at -70°C or fixed in 4% neutral buffered formalin and embedded in paraffin for light microscopy techniques.

Immunohistochemistry

5µm-thick sections were deparaffinated in histoclear (National Diagnostics, Atlanta, Georgia, USA) and dehydrated in a descent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/100% methanol and sections were blocked with 4% fetal bovine serum and 1% bovine serum albumin in PBS. Primary antibodies used were: rabbit polyclonal anti-Fas (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100), rabbit polyclonal anti-3-Nitrotyrosine (Chemicon International, Temecula, CA, USA, 1:500), goat polyclonal anti-BiP (Santa Cruz Biotechnology, Santa

Cruz, CA, USA, 1:50) and finally, rabbit polyclonal anti-TTR (Dako, Carpinteria, CA, USA, 1:1000), which were diluted in blocking solution and incubated overnight at 4°C. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma-Aldrich, St. Louis, MO, USA), using 3-amino-9-ethyl carbazole (Sigma-Aldrich, St. Louis, MO, USA), or diaminobenzidine as substrates. On parallel control sections, the primary antibody was replaced by blocking buffer and by non-immune immunoglobulins corresponding to the species used for primary antibodies, namely rabbit and goat; staining was absent under these conditions.

Semi-quantitative immunohistochemistry (SQ-IHC) analysis was performed using Scion Image software. This application enables the measurement of the area occupied by pixels corresponding to the immunohistochemical substrate's color that is normalized relatively to the total area. Each slide was analyzed in 5 different representative areas. Results shown represent % occupied area (\pm SD).

Total protein extracts

Mice stomach (approximately 5 mg) was homogenized on ice in a small glass rod homogenizer in 1 ml of lysis buffer that contained EDTA, EGTA, MOPS, Triton, PMSF and proteases inhibitors (Amersham Biosciences, NJ, USA). Total homogenates were centrifuged at 14,000 rpm for 30 minutes. Supernatants were separated and proteins quantified by the Bradford protein assay (BioRad, CA, USA).

Western Blot

Equal amounts of protein from mice stomach lysates were separated in a 15% SDS – PAGE and transferred onto a nitrocellulose Hybond- C membrane (Amersham) in a semi-dry system. The primary antibodies used were: goat polyclonal anti-BiP (Abcam, Cambridge, UK, 1:1000), rabbit polyclonal anti- eIF2 α [P] (Biosource, CA,USA, 1:500) and mouse monoclonal anti- β actin (Sigma, MO, US, 1:5,000). The enhanced chemiluminescence method ECL-plus (Amersham Biosciences, NJ, USA) was used to develop the blots. Quantitative analyses of western blots images were performed using the Scion software (Scion Corporation) and results are represented as the average of the mice representative of a group (treated versus non treated).

Study of TTR aggregation by TEM (transmission electron microscopy)

TEM analysis was used to follow TTR aggregation, as previously described (17). Soluble TTR Tyr78Phe (substitution of a tyrosine for a phenylalanine at position 78) at 2 mg/mL was dialysed against PBS and incubated at 37°C in the absence or presence of TUDCA at a molar ratio of 1:100. The protein concentration was determined by the Lowry method (18). After 48 h of incubation, samples were analysed by TEM. For visualization by TEM, sample aliquots were diluted (1:50) in PBS and negatively stained with 1% uranyl acetate. The grids were visualized with a Zeiss microscope (model EM10C), operated at 60 kV.

TTR tetrameric stability assays by isoelectric focusing (IEF) under semi-denaturing conditions

30 μ l of mice plasma were subjected to native PAGE and the gel band containing TTR was excised and applied to an IEF gel. IEF was carried out in a polyacrylamide gel containing 5% ampholytes pH 4–6.5 and in the presence of 4 M urea, at 1200 V for 6 h, essentially as previously described (17). These semi-denaturing conditions allow the visualization of bands corresponding to TTR monomers and tetramers (which include hybrid species composed of reduced and oxidized TTR monomers), and also of an oxidized form of the monomer. Proteins were fixed with 20% (v/v) of trichloroacetic acid and stained with Coomassie Blue, and the dried gel was scanned (hp Scanjet G3010; Hewlett Packard) and subjected to densitometry (program Image Quant, version 5.1, Molecular Dynamics). Four samples of each treated and untreated (control) serum samples were analyzed and the tetramer/monomer ratios evaluated. The results were expressed as the mean and standard deviation of the ratios.

TTR quantification

TTR was quantified by radial immunodiffusion (RID) using the Human Prealbumin Bindarid – Radial Immunodiffusion Kit (The Binding Site, Birmingham, UK).

Statistical analyses

All data examined were expressed as mean \pm S.E. Comparison between groups was made using the Student's t test. A p value of less than 0.05 was considered statistically significant.

Results

TUDCA has no effect on TTR aggregation and stabilization

We first investigated the effect of TUDCA on TTR fibril formation, namely on the aggregation of Tyr78Phe TTR. This variant is very prone to fibril formation (17). Incubation of TUDCA with soluble TTR Tyr78Phe at 37 °C for 48h led to aggregate formation, not distinguishable from the control without drug (figure 1A). When the period of incubation was increased to 15 days, fibril formation could be visualized, indicating that TUDCA neither inhibits TTR aggregation nor has fibril disruption activity (data not shown).

The effect of TUDCA on TTR tetramer stabilization was investigated by IEF in the presence of 4M urea. Plasma samples from treated hTTR Met30 and control untreated mice were compared concerning band patterns and tetramer/monomer ratios. In parallel, plasma from control mice was incubated with iododiflunisal (dissolved in DMSO) as a reference for the stabilization effect (17). Under the used conditions, TTR presented a characteristic pattern of two main bands, representing monomers (normal and oxidized form) and several bands of lower pI, representing different forms of the tetramer (19). Mice receiving TUDCA at a concentration of 500 mg/ kg/ day did not show TTR tetrameric stabilization when compared to control animals (figure 1B); thus, the proportion of tetramer to monomer was found to be 0.51 in TUDCA treated mice, as compared to 0.54 in control samples, as determined by densitometric analyses.

TTR was quantified using radial immunodiffusion with anti-TTR antibody. Random plasma samples from mice treated with TUDCA (n=5) and controls (n=5) were compared and the results were 275.5 ± 151.1 μg TTR/mL and 386.6 ± 216.1 μg TTR/mL respectively, which do not differ significantly.

TUDCA decreases TTR deposition in the gastrointestinal tract of hTTR Met30 transgenic animals

The anti-apoptotic drug TUDCA was given at a concentration of 500 mg/ kg/ day to hTTR Met30 mice. This dose was previously used (20) in rats and produced no adverse effect. The treatment was performed during three months and started when mice were 3 months old, before they present TTR deposition in the gastrointestinal tract, the major target organ in this animal model. Mice seems to tolerate well the 500 mg/kg/day dose, since they do not exhibit any sign of disease and the weight gain was similar to control animals (data not shown).

We analysed the effect on TTR deposition by performing TTR immunohistochemistry (IHC) in different organs. Non-treated animals presented widespread TTR staining, as expected, in the gastrointestinal tract. In the group of TUDCA treated mice, we detected reduction of approximately 75% on TTR deposits in the stomach as can be seen in the representative IHC images and respective quantification of figure 2A. A similar decrease in TTR staining was detected in the esophagus and large bowel (colon) (figure 2A).

These results sustained that TUDCA acts as a potent blocker of TTR deposition.

Oxidation is significantly decreased by TUDCA “in vivo”

We assessed 3-nitrotyrosine (3-NT) levels in TUDCA treated mice as a protein marker of oxidative stress. 3-NT is described as being increased not only in axons of FAP patients but also in tissues from transgenic mice (12, 16). 3-NT immunohistochemistry was performed in the gastrointestinal tract of TUDCA treated animals and age matched controls; approximately two fold decrease in the NT levels in the esophagus and stomach of drug treated mice was observed; to a lesser extent, 3-NT was decreased in the colon as well (Figure 2C).

TUDCA decreased ER stress and apoptosis “in vivo”

The engagement of death receptors on the cell surface was addressed as a possible apoptotic pathway involved in FAP. Ligand-induced aggregation of members of the tumor necrosis factor (TNF) receptor family, such as Fas (CD95) attracts FADD, which recruits procaspase-8 molecules. The autocatalytic activation of caspase 8 by Fas receptor can directly trigger the effector caspase 3 or, through Bid, interfere with Bcl-2 family members (21,22). Thus, we proposed to analyse by IHC the Fas receptor in TUDCA treated mice and controls. We observed that treated mice presented decreased levels of Fas along the gastrointestinal tract, as can be seen by representative pictures of the esophagus, stomach and larger bowel (colon) of hTTR Met30 mice that were given TUDCA (figure 2B); control animals presented the expected TTR deposition for six months old hTTR Met30 mice.

Since the ER is receiving increased attention as an important compartment participating in stress induced apoptosis, activation of the ER stress response was investigated by

quantification of the ER chaperone BiP. The involvement of ER stress as a target for TUDCA action was confirmed by a decrease of approximately 70% of the ER chaperone BiP, in the esophagus and colon of TUDCA treated mice (figure 3A), and a less pronounced decrease of 35% in the stomach. The staining was observed particularly at the mucosa level. To confirm the IHC results for ER stress in the gastrointestinal tract, we quantified BiP and eIF2 α [P] in stomach lysates of control (n=5) and TUDCA treated mice (n=6). The levels of ER chaperones were lower in mice that received the drug, suggesting TUDCA action at ER stress level (figure 3B).

Discussion

In this study we showed that administration of TUDCA, a non-toxic hydrophilic bile acid to a transgenic mouse model of FAP, significantly reduced TTR toxic aggregates and decreased apoptotic and oxidative biomarkers usually associated with TTR deposition. The interest of this application arose from the knowledge that apoptotic and oxidant conditions can cause aggregation of amyloidogenic A β peptide (23) and tau (24) and that in AD a complex crosstalk is observed between calcium desregulation, reactive oxygen species (ROS) and protein aggregation (25-27); since these events are also associated with TTR aggregation in FAP (13,14), we proposed to investigate the effect of sub-chronic administration of TUDCA to hTTR Met30 mice starting at 3 months of age, in the beginning of TTR deposition. After 3 months treatment we observed that while non-treated animals presented widespread TTR staining, particularly in the gastrointestinal

tract, TUDCA treated mice group had approximately 75% reduction in TTR deposition in the same organs.

Next we investigated if some of the markers selected by us as representative of FAP apoptosis were affected by TUDCA administration (13). Contrary to work published in the literature on primary human and mouse hepatocytes reporting Fas receptor expression as not significantly affected by TUDCA (4, 28), we observed “in vivo” decreased levels of Fas in the gastrointestinal tract in hTTR Met30 treated mice. Ramalho and colleagues (29) described that TUDCA reduced caspase 2 and 6 activation, but not caspase 8, modulating Bcl-2 and Bax levels. Based on our data, we consider that under the used conditions and in this particularly mouse model, TUDCA effect was not dependent on mitochondrial pathways. The anti-apoptotic protein Bcl-2 was unaffected after 3 months treatment (data not shown). In the same way, Bax did not present any difference between treated and control animals (data not shown). The differences found between the other models described in the literature and FAP could be due to the fact that TTR deposition is extracellular, in opposition to other neurodegenerative diseases with intracellular aggregation.

Recent studies challenge the central role of mitochondria in apoptosis and suggest that some apoptotic signals, namely those occurring in the ER, may bypass mitochondria to directly activate caspases (30,31). Xie and colleagues described that TUDCA abolishes ER stress by reducing calcium efflux, BiP induction and Caspase 12 activation (32).

The significative decrease of the ER stress markers BiP and iE2 in the GI tract of the animals in the present report is highly likely to be related to the well known properties of TUDCA on ER stress; Recent data demonstrated clearly activation of the classical UPR

pathways in FAP tissues not specialized in TTR synthesis but presenting extracellular TTR aggregate and fibril deposition; cytotoxicity by Ca^{2+} efflux from the ER in cell cultures incubated with TTR oligomers evidenced ER stress associated with extracellular aggregates (14).

As a final point, we investigated whether TUDCA could affect oxidative stress on the tissues damaged by TTR deposition. In the group of TUDCA treated mice, we observed a decrease in the well established biomarker 3-NT for protein oxidative nitration in the gastrointestinal tissues concordant with the decrease on TTR aggregation. This result was in accordance with previous report of TUDCA protection properties of rat liver proteins and lipids against oxidative damage (33).

Although the mechanism by which TUDCA decreases TTR deposition in transgenic mice remains to be elucidated, TUDCA effect as a TTR stabilizer and inhibitor of fibril formation was discarded; we believe that TUDCA can act at multiple levels of apoptotic and oxidative mechanisms early in the FAP pathology, and that oxidative stress and apoptosis impact on extracellular TTR deposition. A possible mechanism is the influence of secreted metabolites generated by oxidative stress and apoptosis on TTR aggregation; for instance, in Alzheimer's disease it was reported that abnormal oxidative metabolites, including hydroxynonenal (HNE) and cholesterol-derived aldehydes, modify $\text{A}\beta$ and accelerate the early stages of amyloidogenesis (34). As ER stress and oxidative stress are biomarkers associated with TTR deposition in FAP patients and in asymptomatic carriers of Val30Met TTR, TUDCA treatment in FAP might reduce apoptotic stress and consequently lower extracellular TTR deposition and the toxic effects induced by TTR aggregates, as our "in vivo" experiments suggest.

The only efficient therapy available till now for FAP is liver transplant. Liver transplant abolishes the synthesis of the variant TTR (at least by the liver) avoiding further amyloid deposition and delaying the progression of the disease. However, this is a very invasive therapy with a high risk for the patients and limited availability of livers for transplant (35). Moreover, liver transplant does not prevent TTR synthesis by the choroid plexus and the eye, the other major sites of TTR synthesis. Furthermore, not all patients respond to this therapy. For those reasons, therapies based on the development and administration of compounds inhibiting amyloid fibril formation and the toxic affects induced by TTR aggregates are ideal alternative strategies.

Our group previously published the effect of tetracyclines in disrupting TTR amyloid fibrils “in vivo” (36); these descriptions lead to ongoing clinical trials in FAP with doxycycline. To have a drug that avoids polymerization “in vivo” such as TUDCA will certainly have prophylactic value since cytotoxicity begins early before amyloid deposition; a therapeutic value can also be considered for TUDCA administration in FAP patients, after adaptation of TUDCA dosage and regiments, namely if administered in conjunction with an amyloid disrupter such as doxycycline .

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Legends to figures

Figure 1- (A) Aggregation of TTR Tyr78Phe observed by TEM. The initial preparation (0 h) contains round particles resembling TTR Tyr78Phe. After 48 h at 37 °C, the protein aggregates and forms oligomers of different sizes. Incubation with TUDCA for 48 h does not prevent aggregation, and large aggregates are clearly visible. 20x magnification; (B) Analysis of mice plasma TTR by IEF. The different visualized molecular species are indicated. Note the stronger bands corresponding to tetramers and the absence of the monomer band in samples treated with iododiflunisal (reference for the stabilization effect); TUDCA treated mice parallels the result of control mice. IDIF, iododiflunisal. (* $p < 0.05$)

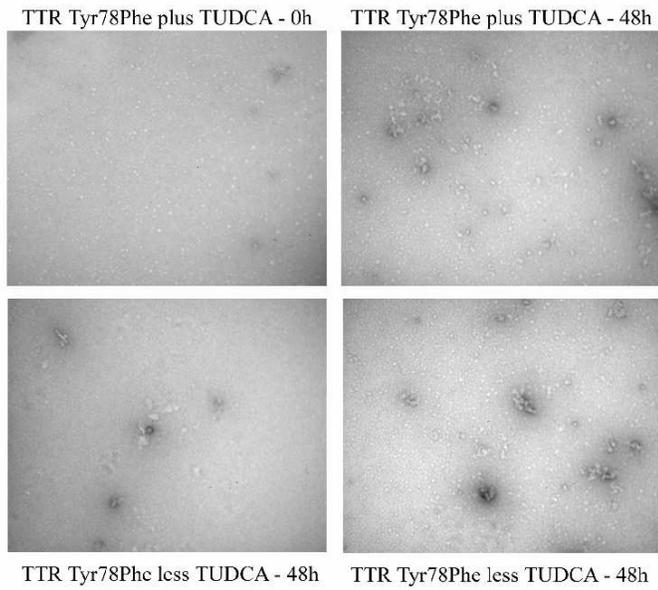
Figure 2 - Representative immunohistochemistry of TTR (A), Fas (B) and 3-NT (C) in esophagus, stomach and colon of TUDCA treated mice (lower panel; $n=15$) and age matched controls (upper panel; $n=10$). 20x magnification; Quantification of immunohistochemical images of TTR, Fas, and 3-NT staining are represented as percentage of occupied area \pm SD (* $p < 0.05$, ** $p < 0.01$, # $p < 0.005$).

Figure 3 – (A) Representative immunohistochemistry of BiP in esophagus, stomach and colon of TUDCA treated mice (lower panel; $n=15$) and age matched controls (upper panel; $n=10$). 20x magnification; Quantification of immunohistochemical images are represented as percentage of occupied area \pm SD (* $p < 0.05$, # $p < 0.005$). (B) Representative anti-BiP and anti- eIF2 α [P] western blots of stomachs from TUDCA

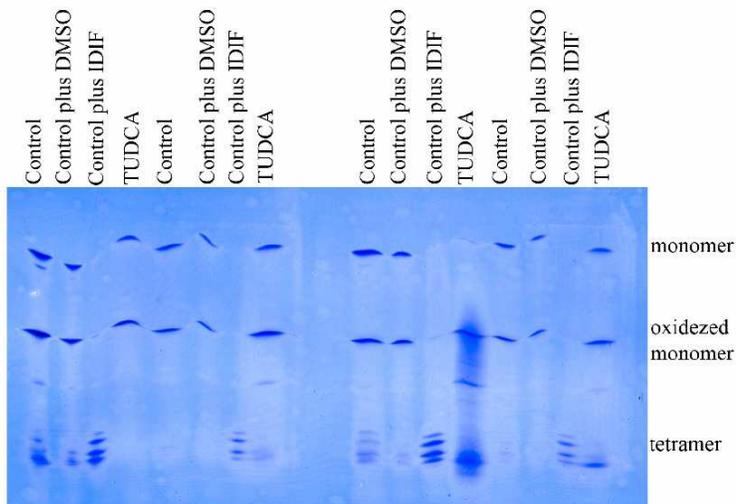
treated and non-treated mice. Histogram: normalized BiP/ β -actin and eIF2 α [P] / β -actin density quantifications \pm SD (* $p < 0.05$). TUDCA treated mice (n=6) parallels the result of control mice (n=5).

ACCEPTED MANUSCRIPT

A



B



Coomassie Blue Stain

