Loss of functional albumin triggers acceleration of transthyretin amyloid fibril formation in familial amyloidotic polyneuropathy

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Transthyretin (TTR)-related familial amyloidotic polyneuropathy (FAP) is characterized by systemic accumulation of amyloid fibrils caused by a point mutation in the *TTR* gene. Despite the urgent need for alternative therapeutic strategies, the pathogenesis of FAP still remains elusive. In our study reported here, we focused on albumin, the most abundant protein in plasma, and described the role of albumin in the TTR amyloid-formation process. Patients with FAP evidenced significantly decreased serum albumin levels as the disease progressed. Biacore analysis showed that albumin had a binding affinity for TTR and exhibited higher affinity for TTR amyloid than native TTR. Albumin functioning as an antioxidant effectively suppressed TTR amyloid formation. In patients with FAP, albumin was significantly oxidized as the disease progressed. Moreover, loss of functional albumin accelerated TTR deposition in analbuminemic rats possessing a human variant *TTR* gene. Taken together, these results indicate that albumin may have an inhibitory role in the TTR amyloid-formation process.

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Transthyretin (TTR)-related familial amyloidotic polyneuropathy (FAP), which is induced by amyloidogenic transthyretin (ATTR), is an autosomal dominant form of fatal hereditary amyloidosis characterized by systemic accumulation of amyloid fibrils in peripheral nerves and other organs.[1,2] To date, more than 100 different point mutations in the *TTR* gene have been reported, most of which are amyloidogenic.[2–5] Of the different types of ATTR-related amyloidosis, ATTR Val30Met (V30M), found worldwide, is the most common.[1] Because the liver predominantly synthesizes TTR, liver transplantation has been thought to be a promising therapy for halting the progression of clinical FAP symptoms.[6–8] However, because no other effective therapy is available as of this moment, development of alternative therapeutic strategies based on the mechanism of TTR amyloid fibril formation is urgently needed.

Although much work has been carried out to identify the various types of ATTR-related FAP,[4] the precise mechanism of TTR amyloid formation remains to be elucidated. TTR normally behaves as a soluble tetramer and binds to retinol binding protein and thyroxine in plasma.[9] It has been proposed that tetrameric TTR is not itself amyloidogenic, but that dissociation of the tetramer into a compact non-native monomers with low conformational stability can lead to amyloid fibril formation.[10] The rate of TTR tetramer dissociation, which is believed to be the rate-limiting step in amyloid fibril formation, is strongly influenced by point mutations in the *TTR* gene.[11] In addition, post-translational modification of TTR is also thought to have a key role in amyloid fibril formation.[12–14] Our recent *in vitro* studies showed that nitric oxide-mediated modification of TTR may serve an important function in amyloid formation, which

indicates that oxidative stress facilitates amyloid formation.[15] Clinical phenotypes of patients with FAP, including the age at onset of disease and patterns of amyloid deposition, differ even in patients with the same *TTR* gene mutation. Some *ATTR* gene carriers never show any clinical manifestations throughout life. A factor or factors other than TTR mutation may therefore have an important role in the amyloid formation mechanism.

Human serum albumin, the most abundant protein in plasma, serves as a transporter of various ligands and an antioxidant in blood circulation.[16,17] Human serum albumin is a mixture of a reduced form (human mercaptalbumin: HMA) and an oxidized form (human nonmercaptalbumin: HNA). Albumin is the major antioxidant in plasma, and a large proportion of all the serum antioxidant properties can be attributed to albumin.[18] Previous work has shown the total reactive antioxidant potential in plasma, considered as an index of the level of antioxidants, decreased in patients with FAP.[19] In addition, more recent work demonstrated that albumin suppressed amyloid formation of amyloid-β(Aβ), a component of amyloid fibrils in Alzheimer's disease, by reducing oxidative stress.[20,21] These data suggest that albumin functing as an antioxidant may perform a crucial role in amyloid formation in FAP.

In this study, we therefore investigated the role of albumin in TTR amyloid formation. We analyzed, quantitatively and qualitatively, the function of albumin that we isolated from serum samples obtained from patients with FAP ATTR V30M. To evaluate the effect of albumin on amyloid formation, we performed sandwich ELISA with monoclonal anti-TTR$_{115-124}$ antibody, which reacts specifically with amyloid fibrils and preamyloid deposits. In addition, we generated analbuminemic rats with human ATTR V30M transgenic (Tg) rats and evaluated the effect of albumin on TTR deposition *in vivo*.

MATERIALS AND METHODS
Materials
Both wild-type (WT) TTR and ATTR V30M were purified from serum samples obtained from healthy volunteers and homozygotic FAP ATTR V30M patients, respectively, as described previously.[15,22] Albumin was isolated from serum samples from healthy volunteers by means of ion-exchange chromatography. Albumin-containing bound fatty acids (FA-albumin) was donated by the Chemo-Sero-Therapeutic Research Institute (Kaketsuken, Kumamoto, Japan) and was isolated by using a Sephadex G-25 column (Nihon Waters K.K., Tokyo, Japan). Defatted albumin and *N*-ethyl-maleimide (NEM)-albumin were prepared by treating albumin with charcoal and NEM, respectively, as described previously.[23] α-1 Acid glycoprotein (AGP) and transferrin (Tf) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used in the studies were analytical grade.

Serum Samples from FAP Patients and Healthy Volunteers
For evaluation of serum protein levels, we used serum samples from 20 patients with FAP ATTR V30M (stage I: 9, stage II: 4, stage III: 3, and stage IV: 4) and 12 healthy volunteers. The clinical stage of FAP was classified as described previously.[24] All serum samples were collected by means of venipuncture. Venous blood was allowed to clot for 30 min at room temperature, after which it was centrifuged for 10 min at 3000 r.p.m. to obtain plasma for analysis of total protein, albumin, and TTR. Informed consent was obtained from each subject. All studies with human samples were conducted according to the current version of the Helsinki Declaration. The Ethical Committee of Kumamoto University approved this study.

Biacore Assays
The binding affinity of albumin for TTR was analyzed with the Biacore 2000 system (Biacore, Uppsala, Sweden). The surface of a C5 sensor chip was activated by injection of a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysuccinimide, with the flow rate at 5 μl/min for 7 min. Albumin, which was diluted in 10 mM sodium acetate (pH 4.5) to a concentration of 5 μg/ml, was injected (5 μl/min) into flow cell 2 to achieve an immobilization level of 50 resonance units (RU), which produced an Rmax of 100 RU. The sensor chip surface was then deactivated by injection of 1M ethanolamine-HCl at pH 8.5, at 5 μl/min for 7 min. The final immobilization level was 60 RU. Flow cell 1 was activated and deactivated without coupling of protein to serve as a reference cell. Interaction between albumin and TTRs was assessed by injecting TTRs, diluted in HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4), at fourfold increasing concentrations (starting concentration: 0.1 μM; top concentration: 25.6 μM) into the flow cells at a flow rate of 30 μl/ min for 5 min. Association- (ka) and dissociation-rate constants (kd) of the interaction between albumin and TTR were calculated with BIA evaluation 2.0 software, and the affinity constant (K_D) was calculated from kd/ka. WT TTR purified from serum samples obtained from healthy volunteers was used for Biacore assays. Denatured TTR was prepared by means of 8 M urea treatment. TTR amyloid was prepared in 20 mM sodium acetate and 100 mM NaCl at pH 3.0 in an Eppendorf tube by incubation at 37°C for 5 days.[15]

Amyloid Fibril Formation Induced by WT TTR And ATTR V30m
To evaluate the effect of various serum proteins on amyloid fibril formation, TTRs were diluted in 20 mM sodium acetate and 100 mM NaCl at pH 3.0 in an Eppendorf tube to a final TTR concentration of 20 μM with or without various serum proteins. The resultant stationary solutions were incubated at 37°C for 5 days in the dark.[15]

ELISA

To quantify TTR amyloid fibrils *in vitro*, the peroxidase-antiperoxidase method for sandwich ELISA was used. PBS containing 0.05% Tween 20 was used as a buffer for washing and dilution. Briefly, the monoclonal anti-TTR$_{115-124}$ antibody, which reacts specifically with the surface epitope (TTR115-124) that is exposed only on amyloid fibrils and preamyloid deposits of TTR,[25] was used to coat a 96-well microtiter plate, followed by overnight incubation at 4°C. Wells were washed three times with PBS containing Tween 20. All additional washing steps were carried out in the same way after each procedure. Nonspecific binding sites were blocked with 0.5% gelatin, followed by incubation at room temperature for 2 h. Thereafter, 50-μl of samples were added and incubated at room temperature for 2 h. Horseradish peroxidase-labeled polyclonal rabbit anti-human TTR antibody was used as the detecting antibody. Absorbance was detected at 450 nm was detectable after incubation with with 2, 2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (KPL, Gaithersburg, MD).[26]

Non-boiled SDS-PAGE

Non-boiled SDS-PAGE was performed under non-denaturing conditions. A measure of 1 μg of the TTR samples incubated for 37°C for 5 days at pH 3.0 as described above was neutralized with PBS to obtain a final pH greater than 6.5. After neutralization, samples were mixed with 5% SDS sample buffer and loaded on 15% polyacrylamide gels, which were stained with Coomassie Brilliant Blue. Intensities of the bands were evaluated by densitometric analysis using ATTO densito (ATTO, Tokyo, Japan).

Determination of Thiol Content

Thiol content was measured as an index of oxidative stress in plasma for the following reasons: (1) ∼80% of the total free thiol content in plasma is derived from the cysteine residue at position 34 (Cys34) of albumin; (2) the Cys-34 of albumin is highly accessible to reactive oxygen species and carbon-centered free radicals and is highly oxidized during the pathological conditions; (3) because of the half-life of albumin (∼20 days), the state of Cys34, which indicates the redox state of albumin, is a more sensitive index of the degree of systemic oxidative stress than is plasma carbonyl content.[27] Thiol concentrations in plasma were measured by means of the Ellman's method.[28] Reaction mixtures were prepared by adding 20 μl of plasma to 100 μl of 5 mM 5, 5′-dithiobis (2-nitrobenzoic acid) in 100 mM potassium phosphate (pH 7.0). After a 60-min incubation of the mixture, thiol concentrations were evaluated at an absorbance of 405 nm.

Chromatography of Oxidized Albumin

High-performance liquid chromatography (HPLC) was performed to determine the oxidation of plasma albumin as described previously.[27] Frozen plasma samples obtained from healthy volunteers and heterozygotic FAP ATTR V30M patients were thawed, and then 5 μl aliquots were analyzed with a Shodex Asahipak ES-502N column (Showa Denko, Tokyo, Japan). The values of the reduced form (HMA) and the oxidized form (HNA) of plasma albumin were estimated from HPLC chromatograms by dividing the area of each fraction by the total area corresponding to albumin. To obtain these respective areas, a symmetrical resolution graphing method was used. All solvents used for HPLC experiments were filtered through a Millipore Sterivex-GS filter unit (0.22 mm). Samples were stored under the sterile conditions at the designated temperatures.

Animals

Rats with analbuminemic (Nagase analbuminemic rats (NARs)) were isolated from Sprague-Dawley rats of CLEA Japan (Japan CLEA, Kanagawa, Japan).[29] Tg rats possessing a human *ATTR V30M* gene (ATTR V30M Tg rats) were generated as previously described.[30] Analbuminemic ATTR V30M Tg rats (V30M Tg NAR) were developed by mating NAR and ATTR V30M Tg rats, and were genotyped by PCR analysis of rats ear DNA. In this study, 9-month-old rats were analyzed by immunohistochemistry for human ATTR V30M. Animals were maintained in a specific pathogen-free environment at the Center for Animal Resources and Development, Kumamoto University.

Immunohistochemical Staining

Paraffin-embedded 4-μm thick sections were prepared and deparaffinated in xylene and rehydrated in graded alcohols. Deparaffinized sections were heated for 20 min in an autoclave apparatus. Slides were then treated with periodic acid for 10 min at room temperature, after which they were incubated in 5% normal serum for 1 h at room temperature in a moist chamber. The primary antibody was rabbit polyclonal anti-TTR (Dako, Glostrup, Denmark; Cat #: A0002) used at a 1:50 dilution. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (Dako) diluted 1:100 in buffer. Reactivity was visualized with the DAB Liquid System (Dako). Sections were counterstained with hematoxylin.

Digital Quantitation of TTR Deposition

The sections were examined under light microscopy, and the entire field of the rat colon was digitized using an OLYMPUS DP71 camera and DP-BSW-V3.1 software. Briefly, individual images of the section were captured, and then merged to produce an image of the whole colon section. Semi-quantitative analysis of immunohistochemical images was carried out with Adobe Photoshop Element 7.0 software (Adobe Systems, San Jose, CA), which performs automated particle analysis in a measured area: that is, the area occupied by pixels corresponding to the immunohistochemical substrate color is counted and normalized relative to the total area.[31] TTR staining in the colon area was assessed by using the color selection tool of the program, and the volume of

Table 1 Serum protein levels of FAP patients

Protein	Healthy volunteers	Stage I	Stage II	Stage III	Stage IV
TP (g/dl)	7.74 ± 0.50	7.37 ± 0.85	7.12 ± 0.97	5.89 ± 0.66[a]	5.27 ± 0.56[a]
Alb (g/dl)	4.77 ± 0.29	4.46 ± 0.47	3.99 ± 0.51[a]	3.31 ± 0.30[a]	3.05 ± 0.33[a]
TTR (mg/dl)	26.68 ± 4.20	19.53 ± 6.52[a]	19.48 ± 1.82	25.66 ± 8.94[a]	17.38 ± 3.47[a]

Abbreviations: TP, total protein; Alb, albumin; TTR, transthyretin.
[a]$P < 0.01$ *vs* healthy volunteers.

TTR deposition per total area was determined. In all, 10 different selected areas of each slide used for semiquantitative immunohistochemistry were analyzed independently by two investigators.

Western Blot Analysis

Equal amounts of serum protein from rats were fractionated via 12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 2.5% non-fat milk and were incubated overnight at 4°C with the rabbit polyclonal anti-TTR primary antibody (dilution 1:1000, Dako; Cat #: A0002), followed by incubation with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (dilution 1:1000, Dako) as a secondary reaction for 1 h at room temperature. The immunocomplex was visualized with the ECL western blot detection system (GE Healthcare Bio-Science, Piscataway, NJ).

Statistical Analysis

All data are expressed as means ± s.d. Statistical evaluation was carried out by means of the paired *t* test. A *P* value of < 0.05 was taken as statistically significant.

RESULTS

Serum Protein Levels of FAP Patients

We first assessed serum protein concentrations in FAP patients at each clinical stage and in healthy volunteers. As shown in Table 1, the total protein levels in serum samples from FAP patients were significantly lower than those in samples from healthy volunteers. It was notable that serum albumin levels also significantly decreased in FAP patients as the disease progressed.

Binding Affinity of Albumin for TTR

To determine the involvement of albumin in the pathogenesis of TTR-related FAP, we evaluated the binding affinity of TTR for various plasma proteins by using Biacore analysis. As shown in Table 2, albumin had a higher binding affinity for TTR than for the other serum proteins, such as AGP and Tf. In addition, albumin had a higher affinity for amyloid and denatured TTR than native TTR (Table 3).

Table 2 Binding affinity of TTR for albumin

Serum protein	K_D (M)
Albumin	1.49×10^{-3}
AGP	ND
Tf	ND

Abbreviations: AGP, α1-acid glycoprotein; Tf, transferrin; K_D, dissociation constant; ND, not detected.

Table 3 Binding affinity of albumin for TTR

TTR	K_D (M)
Native TTR	1.49×10^{-3}
Denatured TTR	9.71×10^{-4}
Amyloid TTR	6.17×10^{-5}

K_D: dissociation constant.

Effect of Albumin on TTR Amyloid Formation

To obtain further evidences about the involvement of albumin in the pathogenesis of FAP, we used sandwich ELISA with monoclonal anti-TTR$_{115-124}$ antibody, which reacts specifically with amyloid fibrils and preamyloid deposits, to determine the effect of albumin on TTR amyloid formation. Of various serum proteins, only albumin showed potent inhibition of TTR amyloid formation compared with AGP and Tf at concentrations reflecting their levels in serum (Figure 1a). Albumin significantly suppressed amyloid formation of both WT TTR and ATTR V30M in a dose-dependent manner (Figure 1b).

Effect of Albumin on the Stability of Tetrameric Forms of TTR

Because stabilizing the tetrameric forms of TTR has been reported to prevent amyloid formation,[10] we used non-boiled SDS-PAGE to determine the effect of albumin on the stability of TTR tetramers. Figure 2 shows that albumin stabilized the
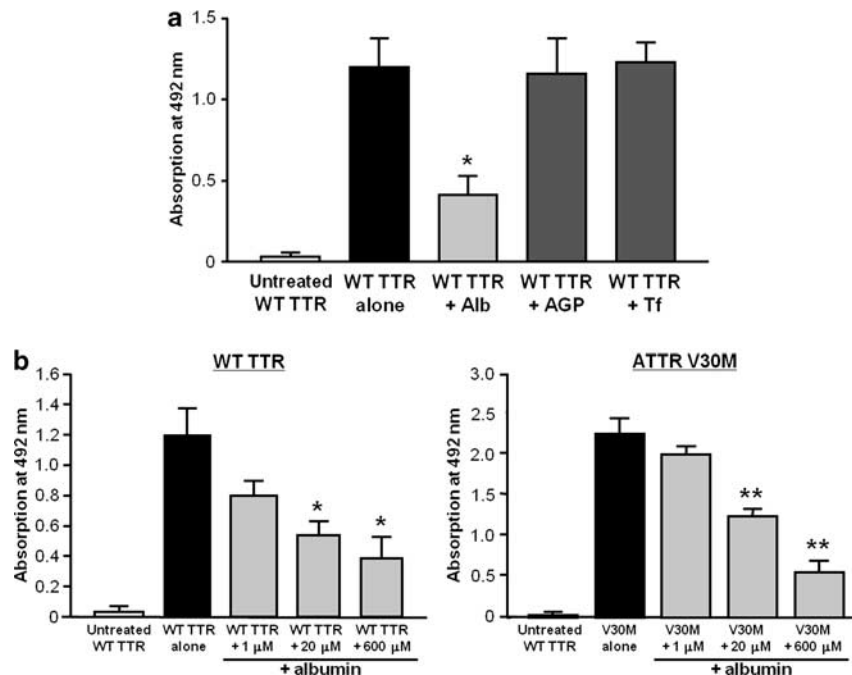
Figure 1 Effect of albumin on transthyretins (TTRs) amyloid formation. (**a**) Samples of 20 μM wild-type (WT) TTR were incubated with or without serum proteins (600 μM albumin (Alb), 20 μM α1-acid glycoprotein (AGP), or 33.3 μM transferrin (Tf)) at 37°C for 5 days in acetate buffer (pH 3.0). The concentrations of the proteins reflected their relative concentrations in serum. WT TTR amyloid formation was detected by means of ELISA with anti-TTR$_{115-124}$ antibody. Each bar represents the mean ± s.d. ($n = 5$). *$P < 0.05$ vs WT TTR alone. (**b**) WT TTR (left panel) and amyloidogenic transthyretin (ATTR) V30M (right panel) amyloid formation in the presence or absence of albumin as detected by ELISA. Each bar represents the mean ± s.d. ($n = 5$). *$P < 0.01$ vs WT alone. **$P < 0.01$ vs ATTR V30M alone.
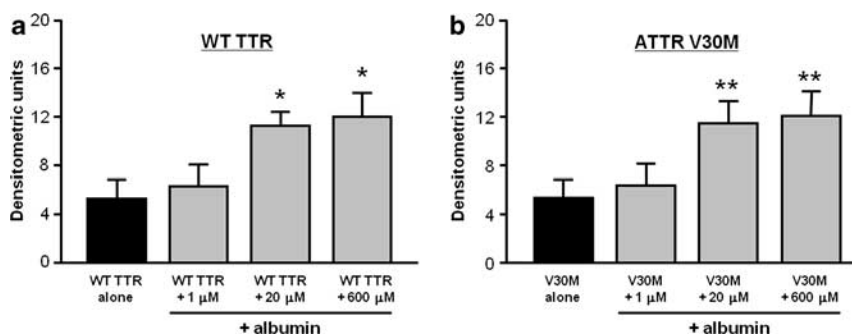

Figure 2 Effect of albumin on the stability of tetrameric forms of transthyretins (TTRs). Samples of 20 μM wild-type (WT) TTR (**a**) and 20 μM amyloidogenic transthyretin (ATTR) V30M (**b**) were incubated with or without albumin (1–600 μM) at 37°C for 5 days in acetate buffer (pH 3.0). Samples were analyzed by non-boiled (non-reducing) SDS-PAGE as described in the text. The stability of TTR tetramers was evaluated by densitometric analysis of the intensity of non-boiled SDS-PAGE bands. Each bar represents the mean ± s.d. ($n = 5$). *$P < 0.01$ vs WT TTR alone; **$P < 0.01$ vs ATTR V30M alone.

tetrameric forms of both WT TTR (a) and ATTR V30M (b) in a dose-dependent manner (Supplementary Figure 1).

Antioxidant Effect of Albumin on TTR Amyloid Formation

To elucidate the antioxidant effect of albumin on TTR amyloid formation, we investigated the effect of albumins that were modified to have different antioxidant effects. As Figure 3a demonstrates, fatty acids (FA)-albumin, which had a strong antioxidant effect,[23] showed a greater inhibitory effect on amyloid formation. The inhibitory effect of defatted

albumin (non-FA-Alb) was consistently weaker than that of native albumin (Figure 3a). Moreover, NEM-albumin, with a weaker antioxidant effect,[23] had less of an inhibitory effect than did albumin alone (Figure 3b).

Oxidative Stress in Circulating Blood of FAP Patients

To further understand the role of antioxidant properties of albumin in FAP disease progression, we evaluated oxidative stress in circulating blood of FAP patients. Compared with healthy volunteers, patients with FAP had significantly reduced plasma thiol content, an index of oxidative stress,
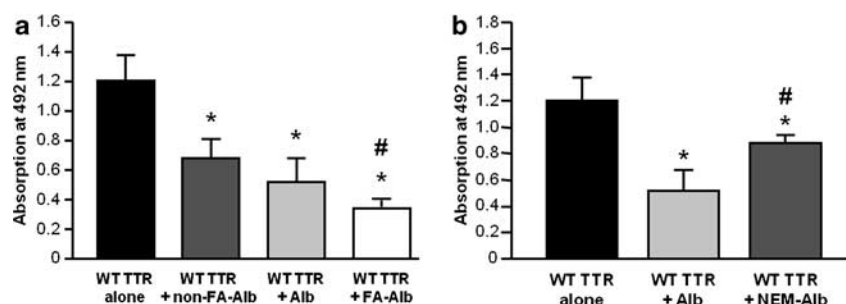
Figure 3 Antioxidant effect of albumin on transthyretin (TTR) amyloid formation. (**a**) The effect of fatty acids (FA) bound to albumin on wild-type (WT) TTR amyloid formation was determined by means of ELISA with anti-TTR$_{115-124}$ antibody. WT TTR (20 μM) was incubated at pH 3.0 with or without different types of albumins: unmodified albumin (Alb), albumin containing bound fatty acids (FA-Alb), and defatted albumin (non-FA-Alb). Each bar represents the mean ± s.d. ($n = 5$). *$P < 0.01$ vs WT TTR alone; #$P < 0.01$ vs WT TTR incubated with non-FA-Alb. (**b**) The effect of N-ethylmaleimide (NEM)-albumin on WT TTR amyloid formation was determined by ELISA. Each bar represents the mean ± s.d. ($n = 5$). *$P < 0.01$ vs WT TTR alone; #$P < 0.01$ vs WT TTR incubated with albumin.
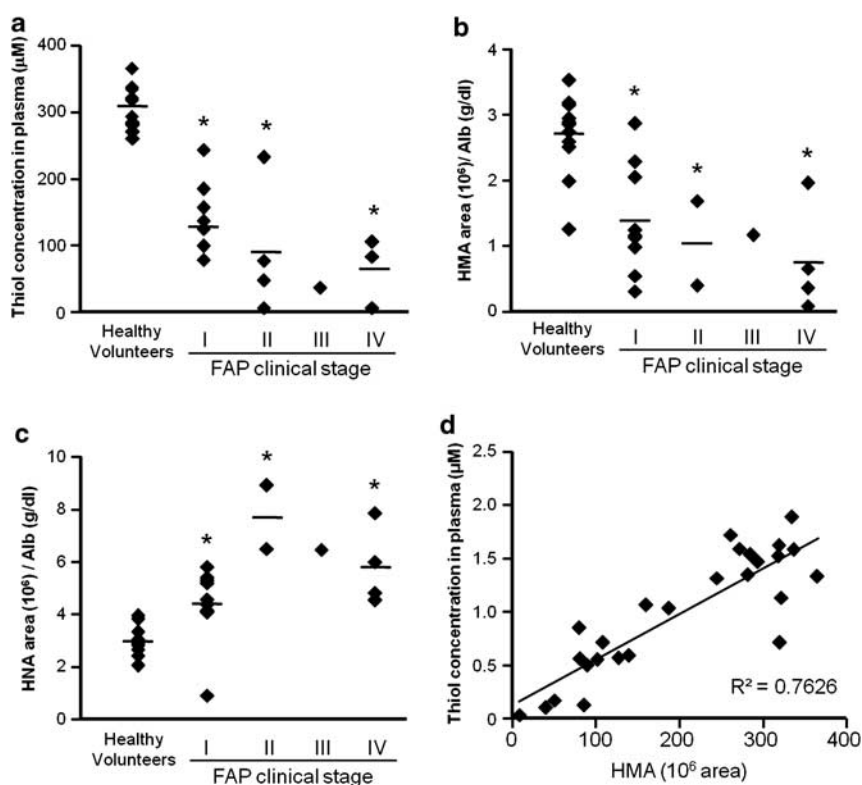

Figure 4 Oxidative stress in circulating blood of familial amyloidotic polyneuropathy (FAP) patients. (**a**) Thiol concentrations in plasma were measured by Ellman's method as described in the text. *$P < 0.01$ vs healthy volunteers. (**b** and **c**) High-performance liquid chromatography (HPLC) was performed for analysis of plasma albumin. Value of either the reduced form (human mercaptalbumin (HMA)) (**b**) and the oxidized form (human non-mercaptalbumin (HNA)) (**c**) of plasma albumin were estimated from HPLC chromatograms by dividing the area of each fraction by the total area corresponding to albumin. *$P < 0.01$ vs healthy volunteers. (**d**) The relationship between thiol concentration and HMA. The line shows a linear regression of thiol concentration in plasma and the levels of reduced albumin.

which indicated increased oxidative stress with disease progression (Figure 4a). In addition, by monitoring the redox state of Cys34 in purified albumin, HMA, the reduced form of albumin, decreased in FAP patients (Figure 4b), whereas HNA, the oxidized form of albumin, significantly increased in FAP patients as the disease progressed (Figure 4c). A good correlation was found between the index of oxidative stress

and the reduced form of albumin, which indicated that oxidative stress in plasma in FAP patients depended largely on the existence of functional albumin (Figure 4d).

TTR Deposition in Analbuminemic Attr V30M Tg Rats
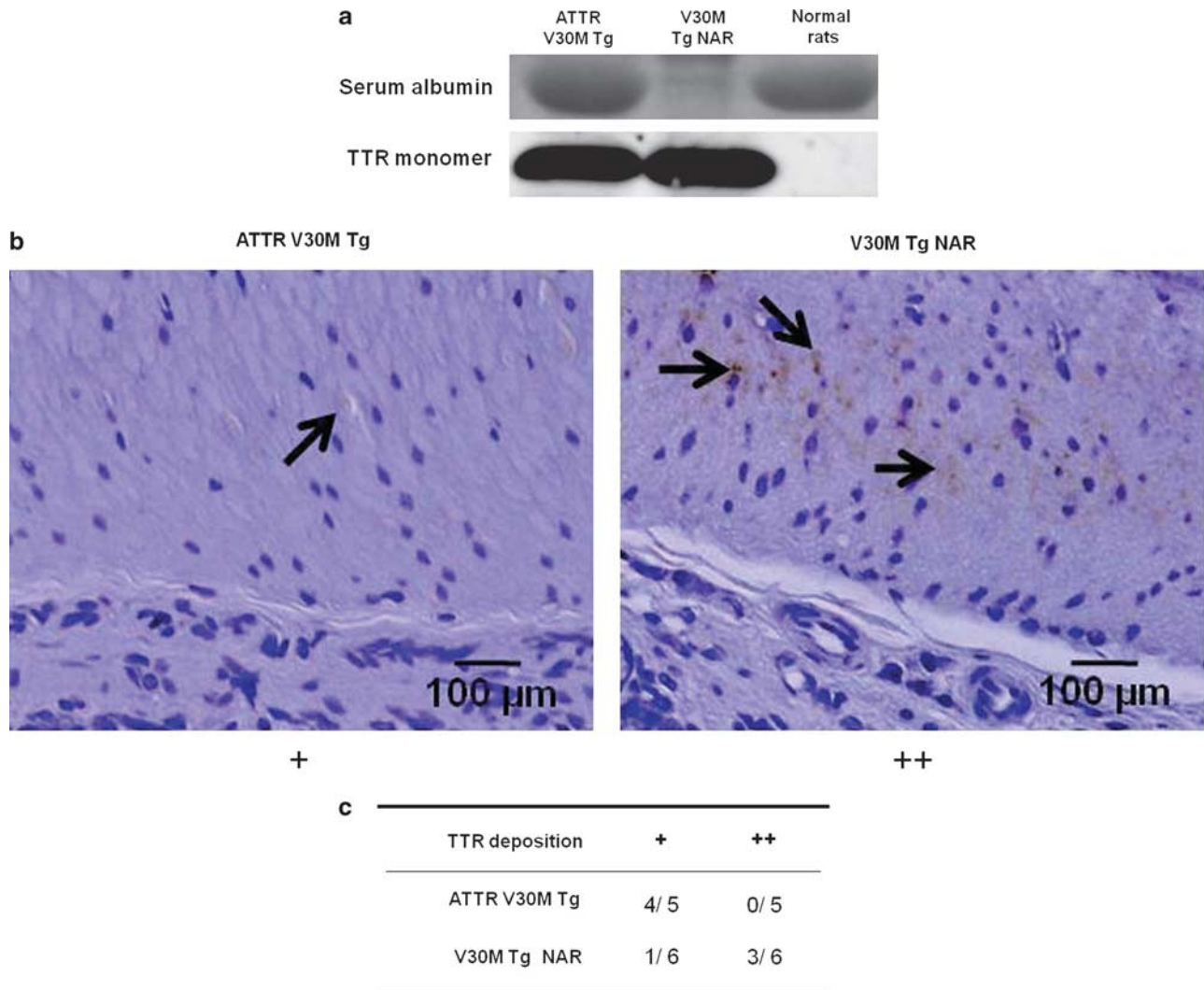To confirm the crucial role of albumin in the pathogenesis of FAP, we generated analbuminemic Tg rats possessing a

Figure 5 Transthyretin (TTR) deposition in analbuminemic amyloidogenic transthyretin (ATTR) V30M Tg rats. (**a**) Equal amounts of serum protein from rats were analyzed by SDS-PAGE for albumin (upper panel) and by western blotting for human ATTR V30M (lower panel). (**b**) Immunoreactivity with polyclonal anti-human TTR antibody in the colon of transgenic (Tg) rats possessing a human *ATTR V30M* gene (ATTR V30M Tg rats) ($n = 5$) and analbuminemic ATTR V30M TG rats (V30M Tg Nagase analbuminemic rats (NARs)) ($n = 6$) at 9 months after birth. (**c**) Comparison of the numbers of rats per degree of TTR deposition. The degree of TTR deposition was divided into two grades: + slight (40 000 pixels < the volume of TTR deposition per total area < 80 000 pixels) and + + moderate (80 000 pixels < the volume of TTR deposition per total area).

human *ATTR V30M* gene (V30M Tg NAR) and evaluated the effect of albumin on TTR deposition *in vivo*. As Figure 5a reveals, analbuminemic V30M Tg rats indeed had no albumin but expressed human ATTR V30M. Because our previous report showed that non-fibrillar deposits of human ATTR V30M in the gastrointestinal tract of ATTR V30M Tg rats started 10–12 months after birth, which was an index of TTR deposition, we next determined whether analbuminemic V30M Tg rats showed TTR deposition in the gastrointestinal tract. In agreement with our previous data, these analbuminemic V30M Tg rats showed more TTR deposition in the colon at earlier age (9 months old) than did V30M Tg rats (which possess albumin) (Figures 5b and c, Supplementary Figure 2).

DISCUSSION

In this study, we provided evidence that albumin has important roles in the pathogenesis of FAP. Serum albumin value significantly decreased and the oxidized form of albumin significantly increased in FAP patients during disease progression. Albumin as an antioxidant effectively suppressed amyloid formation of both WT TTR and ATTR V30M *in vitro*. Furthermore, loss of functional albumin accelerated TTR deposition *in vivo*.

Although liver transplantation has become a well-established therapy for FAP, this therapy has given rise to several problems, and no practical alternatives have been developed.[7,8,32,33] Despite the need for such alternative therapeutic strategies, the pathogenesis of FAP, especially the precise

mechanism of TTR amyloid formation, remains to be elucidated. Of particular interest in our study here is the first evidence of the involvement of albumin in the pathogenesis of FAP. Albumin is a multifunctional protein that is synthesized and secreted by liver cells.[34,35] Albumin is one of the main proteins in blood, and, because of its high plasma concentration, allows it to regulate colloid osmotic pressure of plasma and to serve as a transport and depot protein for numerous compounds.[34] It is well documented that serum albumin levels are affected and decreased in various disease conditions, such as hepatic disorders, renal diseases, and burns.[36] The data presented in Table 1 clearly demonstrate significantly decreased serum albumin levels as the disease progressed, which occurs because FAP patients suffer from malnutrition and/or renal disorders as a result of amyloid deposition in the gastrointestinal tract and kidney.[36–38]

In addition, Biacore analysis showed that albumin had a binding affinity for TTR and exhibited a higher affinity for TTR amyloid (Tables 2 and 3). Under physiological conditions, albumin binds to fatty acids, which are poorly soluble in an aqueous environment, because serum albumin has a number of binding sites for hydrophobic ligands.[35,39] During the process of amyloid formation, soluble TTR self-assembles into insoluble amyloid fibrils through a preamyloid state.[8] Multiple hydrophobic regions of TTR are exposed in amyloid forms of TTR,[25] so albumin may bind to TTR via hydrophobic interactions. These data therefore suggest that albumin may be closely associated with the process of TTR amyloid formation. In fact, it should be noted that the binding affinity of albumin for ATTR V30M isolated from both heterozygotic and homozygotic FAP ATTR V30M patients was also confirmed by Biacore analysis (Supplementary Table 1). Further investigation is needed to analyze the kinetic changes related to albumin during the progression of FAP, and to elucidate detail of the mechanism of binding of albumin and TTR in serum of FAP patients.

TTR usually behaves a tetramer in blood circulation. After an amino-acid substitution occurs in the TTR molecule, the tetrameric form of the molecule becomes more unstable, which leads to amyloid formation.[8] To clarify the role of albumin in TTR amyloid formation, we developed a novel strategy that evaluates the amount of TTRs amyloid fibrils via ELISA with a monoclonal anti-TTR$_{115-124}$ antibody. During TTR amyloid formation, the surface epitope (TTR115-124) in amyloid forms of TTR has been documented to be exposed.[25] Inasmuch as anti-TTR$_{115-124}$ antibody reacts only with TTR amyloid fibrils and preamyloid deposits but not with native TTR, we used this antibody in the ELISA assay to quantitate TTR amyloid formation. With this method, we demonstrated that albumin effectively suppressed TTR amyloid formation. As shown in Figure 1, of major serum proteins, only albumin showed potent inhibition of amyloid formation of both WT TTR and ATTR V30M. No inhibitory effect of AGP and Tf on TTR amyloid formation

was observed, even at higher concentrations (Supplementary Figure 3).

Certain studies have demonstrated that albumin inhibits the amyloid fibril formation of Aβ in Alzheimer's disease.[20,21] Albumin prevents the formation of fibrillar Aβ aggregates by physical interaction.[40,41] Albumin not only interacts preferentially with the prefibrillar oligomeric species of Aβ, but also targets and masks exposed hydrophobic sites in the oligomers, which prevents addition of more monomers and growth of prefibrillar assemblies.[21] Because our data showed that albumin had a binding affinity for TTR, albumin probably physically interacts with TTR via hydrophobic interactions and prevents the growth of TTR prefibrillar assemblies and polymerization. In addition, as we mentioned above, because stability of tetrameric TTR is widely accepted as an important factor in TTR amyloid formation,[11] we also examined the effect of albumin on stability of the TTR tetramer. Non-boiled SDS-PAGE showed that albumin significantly stabilized the tetrameric forms of both WT TTR and ATTR V30M. No effect of AGP and Tf was observed at the concentration (20 μM) at which albumin significantly stabilized the tetrameric forms of TTRs (Figure 2), indicating that this effect is not caused by the effect of protein concentration (Supplementary Figure 4). Taken together, these findings suggest that stabilization of TTRs may be one mechanism by which albumin inhibits TTR amyloid formation.

Oxidative stress has been implicated in the amyloid formation process in several types of amyloidosis.[15,42–45] We previously reported evidence of oxidative stress in deposits of amyloid in tissues of patients with FAP.[45] Our more recent *in vitro* studies also showed that nitric oxide-mediated modification of TTR may have an important role in amyloid formation.[15] Amyloid is commonly deposited around vessels that are the primary site of action of nitric oxide generated from endothelial cells and smooth muscle cells. *S*-Nitrosylation of ATTR V30M via the cysteine at position 10 was two times greater than that of WT TTR, and *S*-nitrosylated ATTR V30M had higher amyloidogenicity than unmodified ATTR V30M.[15] Moreover, structural studies revealed that *S*-nitrosylation of ATTR V30M induced a change in its conformation, as well as instability of tetrameric TTR.[15] In our this study, results with modified albumins demonstrated that albumin as an antioxidant inhibited TTR amyloid formation. Albumin is well documented as a very abundant and important circulating antioxidant, and its antioxidant property is mainly regulated by Cys34.[17,46] Modification of the antioxidant effect of albumin by fatty acids significantly affected its inhibition of TTR amyloid formation (Figure 3a). NEM-albumin, with a diminished antioxidant potential because of modification of Cys34, had a reduced inhibitory effect (Figure 3b). Modification of Cys34 by chloramine-T also abolished the inhibitory effect (data not shown). These data suggest that the antioxidant properties of albumin may have a key role in amyloid formation and that Cys34 is responsible for the inhibitory effect on amyloid formation.

Because our recent studies showed that nitric oxide-mediated modification of TTR induced a change in TTR structure, leading to reduced tetrameric stability and enhancing the amyloidogenicity of TTR,[15] the antioxidative properties of albumin may be the main factor that can stabilize the tetrameric form of TTR. To obtain more data on the antioxidant properties of albumin, we evaluated evidence of oxidative stress in circulating blood of FAP patients. Compared with healthy volunteers, FAP patients had significantly reduced plasma thiol content, which indicated greater oxidative stress (Figure 4a). In addition, with regard to the redox state of Cys34 in albumin, FAP patients demonstrated significantly increased levels of the oxidized form of albumin, in contrast to decreased levels of the reduced form of albumin, as the disease progressed (Figures 4b and c). Oxidation of Cys34 in albumin reportedly leads albumin to lose its capability to function as an antioxidant.[17,46] Inasmuch as a good correlation existed between the index of oxidative stress and the reduced form of albumin (Figure 4d), oxidative stress in plasma of FAP patients may be caused mainly by the loss of albumin's antioxidant function, which in turn would lead to more TTR amyloid formation.

Finally, our *in vivo* studies revealed that analbuminemic rats with an *ATTR V30M* gene showed a tendency to exhibit more severe TTR deposition at much earlier age (9 months old) compared with the V30M Tg rats (which posses albumin). This finding indicated that the loss of functional albumin accelerated TTR deposition *in vivo*. In addition to providing information on the effect of albumin under physiological conditions, these *in vivo* data suggest that the analbuminemic V30M Tg rat may become an animal model of FAP. Although attempts have been made to establish animal models of FAP,[47–49] a suitable model is not yet available. Because FAP is an adult-onset disease and prolonged time may be required for developing TTR deposition,[50] the analbuminemic V30M Tg rat may be a useful tool for examining the effect of different treatments on amyloid fibril formation within a shorter time frame (9 months old). More interesting is the finding that analbuminemic V30M Tg rats apparently showed more TTR deposition in the heart than did the V30M Tg rats with albumin (Supplementary Figure 5). Because it is well-known fact that a cardiovascular system is continuously exposed to oxidative stress, the heart tissues may be susceptible to the effect of lacking albumin. Further investigation is needed to evaluate the phenotype of analbuminemic V30M Tg rats in greater detail and prove the value of these rats as a novel animal model of FAP.

In conclusion, our data provide the first evidence that albumin has key roles in the pathogenesis of FAP. Albumin is widely used in clinical settings as a biomaterial for medical and pharmaceutical applications because of its availability, biodegradability, lack of toxicity, and non-immunogenicity.[51,52] Thus, our studies may not only bring new insights into the pathogenesis of FAP, but also suggest novel therapeutic strategies for FAP.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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