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Cholesterol and anionic phospholipids increase the binding of amyloidogenic transthyretin to lipid membranes

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Abstract

Deposition of transthyretin (TTR) amyloid is a pathological hallmark of familial amyloidotic polyneuropathy (FAP). Recently we showed that TTR binds to membrane lipids via electrostatic interactions and that membrane binding is correlated with the cytotoxicity induced by amyloidogenic TTR. In the present study, we examined the role of lipid composition in membrane binding of TTR by a surface plasmon resonance (SPR) approach. TTR bound to lipid bilayers through both high- and low-affinity interactions. Increasing the mole fraction of cholesterol in the bilayer led to an increase in the amount of high-affinity binding of an amyloidogenic mutant (L55P) TTR. In addition, a greater amount of L55P TTR bound with high affinity to membranes made from anionic phospholipids, phosphatidylglycerol (PG) and phosphatidylserine (PS), than to membranes made from zwitterionic phospholipid phosphatidylcholine (PC). The anionic phospholipids (PS and PG) promoted the aggregation of L55P TTR by accelerating the nucleation phase of aggregation, whereas the zwitterionic phospholipid PC had little effect. These results suggest that cholesterol and anionic phospholipids may be important for TTR aggregation and TTR-induced cytotoxicity.

Keywords: Transthyretin (TTR); Cholesterol; Phospholipid; Aggregation; Amyloid; Binding

1. Introduction

Neurodegenerative diseases associated with amyloidosis involve the build-up of proteinaceous deposits in the extracellular compartment of the nervous system [1,2]. Although the amyloidogenic proteins in their native state have little similarity in structure and perform different biological functions, the common structure of amyloid fibrils has led to the suggestion that the mechanisms of protein aggregation and neurotoxicity may be similar for different amyloidoses [3–5].

Familial amyloidotic polyneuropathy (FAP) is a fatal hereditary neuropathy characterized by systemic deposition of amyloid formed from misfolded transthyretin (TTR) [6,7], a transporter protein for thyroxine (T_4) and retinol in the plasma and the brain [8]. Native TTR consists of four identical subunits, each containing 127 amino-acid residues which form a predominantly β-sheet structure [9]. A large number of mutations have been identified in TTR, most of which cause amyloidosis [7,10]. It has been suggested that amyloidogenic mutations destabilize the tetrameric structure of TTR, leading to the formation of an amyloidogenic intermediate (probably a TTR monomer) which can self-assemble to form amyloid [11,12]. Although the mechanisms of TTR-induced cytotoxicity are still poorly understood, previous studies have reported that amyloidogenic TTR may cause its cytotoxic effects by disrupting intracellular Ca^{2+} homeostasis [13], inducing

Abbreviations: Aβ, β-amyloid protein; AFM, atomic force microscopy; DLS, dynamic light scattering; ER, endoplasmic reticulum; FAP, familial amyloidotic polyneuropathy; HOPG, highly oriented pyrolytic graphite; K_D, dissociation constant; PC, dimyristoyl-L-α-phosphatidylcholine; PE, dimyristoyl-L-α-phosphatidylethanolamine; PG, dimyristoyl-L-α-phosphatidylglycerol; PrP, prion protein; PS, dimyristoyl-L-α-phosphatidylserine; RU, response unit; SM, sphingomyelin; SPR, surface plasmon resonance; SUVs, small unilamellar vesicles; T_4, thyroxine; TTR, transthyretin; VGCCs, voltage-gated Ca^{2+}-channels; WT, wild-type

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endoplasmic reticulum (ER) stress [14] and up-regulating pro-inflammatory cytokines [15]. Several studies have identified TTR oligomers as the major form of TTR that cause cytotoxicity [13,16].

Recently, we reported that TTR binds predominantly to lipids in the plasma membrane via electrostatic interactions, and that this binding is correlated with the cytotoxic effect of amyloidogenic TTR [17]. Previous studies of other amyloidogenic proteins have also shown that binding to lipids in the plasma membrane is an important step in the aggregation and cytotoxicity [18,19]. It has been suggested that binding to membrane lipids may induce protein misfolding and aggregation by providing a favorable local environment [20–22]. Variations in lipid composition of the membrane can modify the protein–lipid interactions. For example, the concentration of cholesterol is correlated with the membrane binding of amyloidogenic proteins, such as β-amyloid protein (Aβ) [19,23] and prion protein (PrP) [18]. Cholesterol has also been identified as a major component of the lipids that are associated with various amyloid deposits [24]. It has been suggested that membrane-bound Aβ [25] or PrP [26] may act as a nucleus for aggregation. By modulating the organization of the plasma membrane, cholesterol may contribute to the aggregation [27–29] and cytotoxicity [18,19,27] of amyloidogenic proteins. In addition, anionic phospholipids have also been reported to be involved in the binding of some amyloidogenic proteins [22,30,31]. It is not known which lipid species in the membrane preferentially bind TTR, and how lipid composition influences this binding.

In the present study, the effects of cholesterol and different phospholipids on the binding and aggregation of TTR were studied. We show that the binding of an amyloidogenic TTR mutant, L55P TTR, to phospholipid bilayers is influenced by both the concentration of cholesterol and type of phospholipids in the membrane. We also show that anionic phospholipids promote the aggregation of L55P TTR. These results suggest that alterations in membrane lipid composition may play a role in the aggregation and cytotoxicity of TTR.

2. Materials and methods

2.1. Materials

The wild-type (WT) TTR and a highly amyloidogenic mutant, L55P TTR, were expressed in *Escherichia coli* and the native tetramers purified using ion-exchange and size-exclusion chromatography to a purity of >95% [17]. Dimyristoyl-L-α-phosphatidylcholine (PC), dimyristoyl-L-α-phosphatidylserine (PS), dimyristoyl-L-α-phosphatidylglycerol (PG), and dimyristoyl-L-α-phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Highly oriented pyrolytic graphite (HOPG) of ZYB quality was the product of Advanced Ceramics (Cleveland, OH). Sphingomyelin (SM) and cholesterol were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2. Preparation of liposomes from phospholipids

Artificial liposomes with different compositions were prepared as described in a previous study [17]. Briefly, the lipids were dissolved in chloroform/methanol (3:1, v/v) at a concentration of 2 mM. Lipid mixtures were prepared with a composition based on that of the grey matter of human brain [32], except that the cholesterol concentration was 0, 25 or 50% on a molar basis of the total lipids, while the remaining lipids were in a constant molar ratio of 6:5:2:2 (PC:PE:PS:SM). The lipids were dried under a constant flow of N_2 gas, and then *in vacuo* overnight, to create a uniform lipid film which can form liposomes by hydration. The dried lipids were resuspended in 20 μM phosphate buffer (pH 7.4) containing 150 mM NaCl to a final concentration of 1 mM. After sonication for 30–60 min, the lipid suspension was extruded seventeen times through a polycarbonate filter membrane (50 nm pore size) using a LiposoFast extruder (Avestin, Ottawa, Canada) to produce unilamellar liposomes which were 50±4 nm in diameter as determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). These small unilamellar vesicles (SUVs) were later used to prepare a lipid bilayer on the surface of biosensor chips for membrane binding studies [17,33].

2.3. Surface plasmon resonance analysis of membrane binding

The binding of TTR to lipid bilayers was studied in real time by surface plasmon resonance (SPR) using a Biacore 3000 biosensor (Biacore AB, Uppsala, Sweden) and was performed essentially as described previously [17]. Artificial liposomes were immobilized onto the surface of an L1 biosensor chip to form a lipid bilayer. TTR preparations (100 μl, in 20 mM phosphate buffer containing 150 mM NaCl, pH at 7.4) were centrifuged at 15,000×*g* for 1 min to remove insoluble materials, and then applied to the lipid bilayer at a flow rate of 30 μl/min for 200 s. The running buffer used was 20 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. A 10-min wash by the running buffer was performed after each injection to allow for dissociation of TTR. The lipid bilayer was then regenerated by an injection of NaOH (10 μL of a 10-mM solution) at 50 μl/min. The amount of binding was determined by changes in the instrumental response unit (RU). As described previously [17], the amount of binding at equilibrium (R_{eq}), which represents the maximum binding of TTR at a given concentration, was calculated. The binding data were then analyzed using Scatchard plots in which R_{eq} was plotted against R_{eq}/C (where C=TTR concentration). Although TTR preparations at different aging times contained various forms of the protein with disparate molecular masses, TTR concentrations in all preparations were calculated by assuming that all TTR molecules were present as tetramers.

2.4. Analysis of TTR aggregation by solution turbidity assay and dynamic light scattering

TTR aggregation was monitored by changes in solution turbidity and particle size as TTR was incubated (aged) at 37 °C in various solutions. As described previously [13], solution turbidity was monitored by measuring the absorbance of the solution at a wavelength of 330 nm using a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA). Changes in particle size were measured by means of DLS using a Zetasizer Nano ZS [13].

2.5. Atomic force microscopy

Atomic force microscopy (AFM) was used to examine changes in TTR morphology during the time course of aggregation and was performed as described in a previous study [13]. TTR solutions (20 μl, 0.1–1 μM) were deposited on a freshly cleaved HOPG surface and incubated at 37 °C for 30 min. After five washes with deionized distilled water, the HOPG surface was then dried under a constant flow of N_2 gas. TTR samples were examined by a Digital Instruments Nanoscope IV Multimode scanning probe microscope (Veeco Instruments, Woodbury, NY). NSC15 125 μm silicon cantilevers with a tip length of 15 μm (Mikromasch, Tallinn, Estonia) were used for data acquisition. Images were obtained in the tapping mode at oscillation frequencies of 200–300 kHz with constant adjustment of the force exerted on the cantilever. AFM images were analyzed by the WSxM 4.0 software (Nanotec Electronica S.L., Madrid, Spain).

3. Results

3.1. Effect of cholesterol on membrane binding of TTR

As previous studies have shown that cholesterol can increase the binding of Aβ to lipid membranes [19,28], we first examined

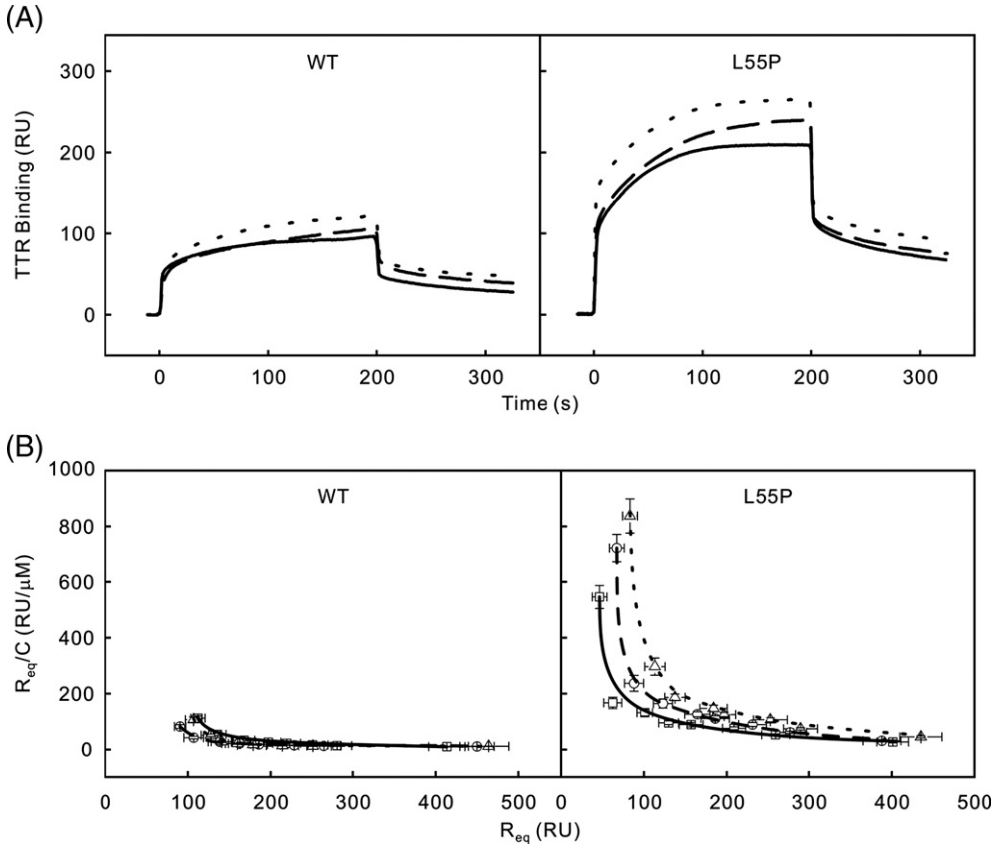

Fig. 1. (A) Sensograms of TTR (2.5 μM, aged at 37 °C for 7 days) binding to immobilized lipid bilayers made from lipid mixtures containing 0% (solid lines), 25% (dashed lines) and 50% cholesterol (dotted lines). TTR was applied for 200 s before a 10-min wash-off to allow for dissociation. The amount of binding was measured by changes in RU. (B) Scatchard plots of the binding between TTR (0.1–40 μM, aged at 37 °C for 7 days) and lipid bilayers made from lipid mixtures containing 0% (□, solid lines), 25% (○, dashed lines) and 50% (△, dotted lines) cholesterol. Values are means±SEM (n=4).

the binding of TTR to lipid membranes containing different cholesterol concentrations by an SPR biosensor system. Liposomes made from lipid mixtures containing 0%, 25% or 50% cholesterol were immobilized onto the surface of an L1 biosensor chip to create a lipid bilayer. TTR preparations (0.1–40 μM, aged at 37 °C for 7 days) were then applied to the lipid bilayers. The amount of binding at equilibrium (R_{eq}), which denotes the theoretical maximum amount of binding, was calculated from the known data as previously described [17].

Similar to the binding of TTR to the plasma membrane [17], TTR was found to bind reversibly to the lipid membranes (Fig. 1A). Scatchard plot analysis (R_{eq} vs. R_{eq}/C) of TTR binding (Fig. 1B) indicated the presence of both high- and low-affinity interactions between TTR and the

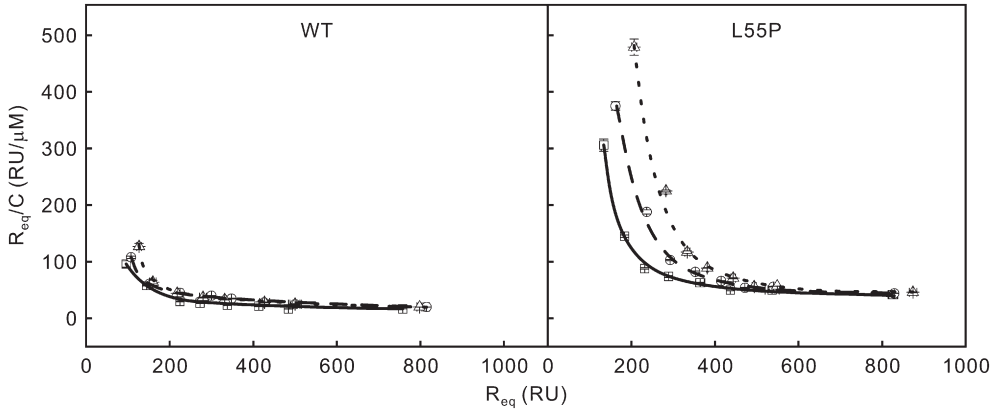

Fig. 2. Scatchard plots of the binding between TTR (0.3–40 μM, aged at 37 °C for 7 days) and homogeneous lipid bilayers made from PC (□, solid lines), PG (○, dashed lines) and PS (△, dotted lines). Values are means±SEM (n=4).

lipids. The equilibrium dissociation constants (K_D) of the low-affinity binding for all the TTR variants were in the order of 10^{-5} M. For the high-affinity binding, the K_D of WT TTR was in the order of 10^{-6} M, while the K_D of L55P TTR binding to the lipid bilayers was approximately 10^{-7} M (Fig. 1B). These K_D values were similar to those observed in our previous studies for TTR binding to the plasma membrane [17].

Scatchard plots of TTR binding to the lipid membranes also showed that varying membrane cholesterol concentration had no significant effect on the amount of binding for WT TTR. However, increasing the membrane cholesterol concentration was associated with greater amount of high-affinity binding for L55P TTR (Fig. 1B).

3.2. Binding between TTR and phospholipids

To examine the role of individual phospholipids in the binding of TTR to lipid membranes, homogenous phospholipid bilayers made from PS, PG or PC were used for binding assays. The binding of TTR (0.3–40 μM, aged at 37 °C for 7 days) to the phospholipid bilayers was examined. As observed with membranes made from the cholesterol-containing lipid mixtures, TTR bound to bilayers of individual phospholipids via both high- and low-affinity interactions (Fig. 2). The K_D values of high- and low-affinity interactions between TTR and the phospholipids were similar to those determined for the interactions between TTR and the cholesterol-containing lipid mixtures. For L55P TTR, a

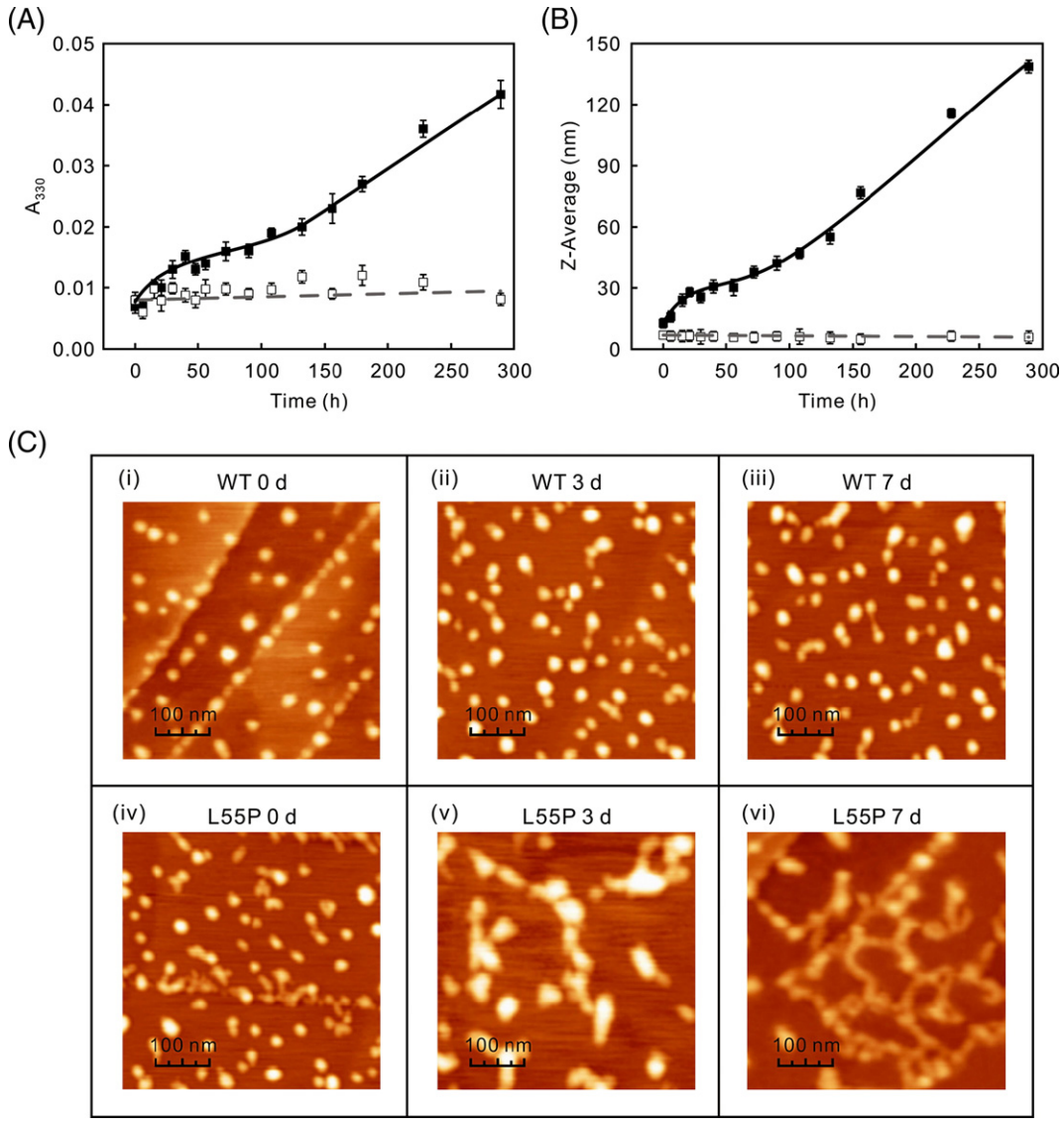

Fig. 3. Aggregation of TTR determined by solution turbidity measured as absorbance at a wavelength of 330 nm (A) and DLS (B). WT (□, grey dashed lines) and L55P (■, black solid lines) TTR (2 μM) were aged in 20 mM phosphate buffer (pH 7.4) containing 150 mM NaCl at 37 °C for 12 days. Values are mean±SEM (n=3–4). (C) Analysis of TTR aggregation by AFM. WT and L55P TTR were examined at 0, 3 and 7 days of ageing. For WT TTR, no significant changes in morphology were observed over the time of ageing. All the WT TTR preparations were predominantly comprised of globular structures ~20 nm in diameter (i, ii and iii). Before aging, the L55P TTR preparation contained similar structures to those in the WT TTR preparations (iv). Larger amorphous TTR aggregates with a diameter of approximately 50 nm were observed in the L55P TTR preparation after ageing for 3 days (v). After ageing for 7 days, fibrillar structures with an apparent diameter of ~20 nm were observed in the L55P TTR preparation along with the amorphous TTR aggregates (vi).

greater amount of high-affinity binding was observed to the membranes made from anionic phospholipids (PG and PS) than the membranes made from zwitterionic PC (Fig. 2). The amount of high-affinity binding of L55P TTR to the phospholipids showed a relative order of PS>PG>PC.

3.3. Effect of phospholipids on TTR aggregation

To explore the effect of phospholipids on the aggregation of TTR, aggregation of TTR in solution was studied first. TTR (2 μM) was incubated in 20 mM phosphate buffer (pH 7.4) containing 150 mM NaCl at 37 °C for up to 12 days. TTR aggregation was monitored by solution turbidity (absorbance at a wavelength of 330 nm, A_{330}) (Fig. 3A) and by DLS (Fig. 3B). No aggregation occurred in the WT TTR preparations during the time course of aging. For L55P TTR, both solution turbidity and DLS measurements showed a similar pattern of aggregation, including an initial increase in aggregation during the first day of ageing, followed by a stable phase lasting approximately 3 days in which there was little apparent increase in aggregation, and a continuous increase in aggregation thereafter. This biphasic pattern of aggregation is typical of a nucleation-dependent polymerization process which is common for the aggregation of many amyloidogenic proteins [13,34]. The average particle size in the stable phase as determined by DLS was approximately 30 nm.

TTR preparations at different times of aging were analysed by AFM to determine the morphological changes during aggregation (Fig. 3C). WT TTR exhibited a relatively constant morphology over the 7 days of aging. All the WT TTR preparations contained predominantly globular structures with an apparent diameter of approximately 20 nm, which are consistent in size with TTR tetramers [13]. Before aging, the L55P TTR preparation also predominantly contained structures with similar size to TTR tetramers. After aging for 3 days, irregular structures with a diameter of approximately 50 nm were observed in the L55P TTR preparation. Previous studies have shown that these amorphous aggregates are likely to be TTR oligomers [13]. After aging for 7 days, some fibrillar structures with an apparent diameter of approximately 20 nm were observed in the L55P TTR preparation in addition to the amorphous aggregates.

To examine whether binding of TTR to phospholipids influences TTR aggregation, WT and L55P TTR (10 μM) were aged at 37 °C for up to 7 days in 20 mM phosphate buffer (pH 7.4) containing 150 mM NaCl in the presence of liposomes (500 μM) made from PC, PG or PS. Since the presence of liposomes prevented accurate measurement of changes in the size of protein molecules by DLS or AFM (data not shown), aggregation of TTR was monitored by solution turbidity as measured by the absorbance at a wavelength of 330 nm (A_{330}) (Fig. 4).

No significant increase in solution turbidity was observed in the WT TTR preparation either in the absence or the presence of phospholipids. In the absence of phospholipids, L55P TTR aggregated via a similar pattern to that described above (at a shorter time scale due to the higher TTR concentration). Incubation with PC did not significantly change the aggregation kinetics of L55P TTR, whereas PG and PS promoted TTR aggregation by significantly accelerating the initial nucleation phase of aggregation. The effect on L55P TTR aggregation showed a relative order of PS> PG>PC (Fig. 4), consistent with their relative binding capacities for L55P TTR.

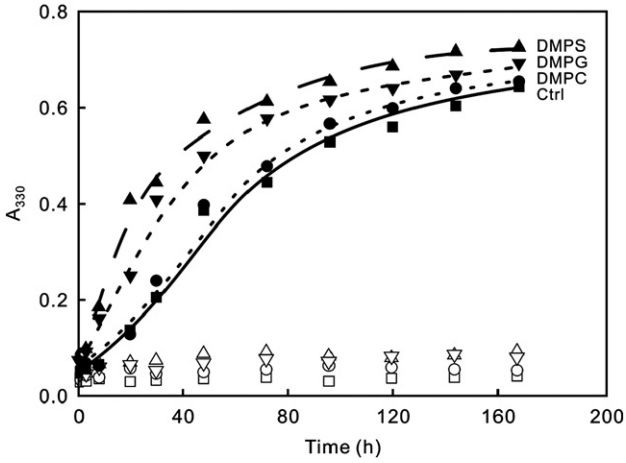

Fig. 4. Effect of phospholipids on TTR aggregation examined by solution turbidity measured as absorbance at a wavelength of 330 nm (A_{330}). WT (empty symbols) and L55P TTR (filled symbols) (10 μM) were aged at 37 °C over 7 days in the presence of liposomes made from DMPC (○ and ●), DMPG (▽ and ▼) and DMPS (△ and ▲) at a concentration of 500 μM. In the control incubation (Ctrl) TTR was aged in the absence of phospholipids (□ and ■). Values are means of multiple measurements (n=3–4, SEM smaller than 10% of the means).

4. Discussion

As binding of TTR to lipids in the plasma membrane is closely associated with its cytotoxic effects [17], the present study was undertaken to examine the role of individual lipids in the membrane binding and aggregation of TTR. This study demonstrates that TTR binding to lipid membranes is influenced by the composition of the membrane, suggesting that lipid composition may be important for the regulation of TTR aggregation and TTR-induced cytotoxicity.

Previous studies have reported that cholesterol concentration is correlated with the membrane binding of Aβ [19,25]. In the present study, binding between amyloidogenic TTR and lipid membranes containing different cholesterol concentrations showed that increasing the cholesterol concentration was associated with greater amount of high-affinity binding, indicating that an increase in membrane cholesterol concentration is associated with more high-affinity binding sites for TTR within the membrane. As TTR binds to membrane lipids through electrostatic interactions [17], the lack of charged groups in cholesterol makes it unlikely that cholesterol binds directly to TTR. As a modulator of membrane fluidity and rigidity [35], cholesterol is known to change the packing and orientation of phospholipids in the membrane [35,36]. Therefore, cholesterol could modulate the membrane binding of amyloidogenic proteins by changing the packing or orientation of phospholipids in the membrane [19,23,37,38]. In the case of TTR binding, cholesterol may promote the interaction between amyloidogenic TTR and phospholipids by altering the orientation

or packing of phospholipid head groups on the surface of the membrane.

The present study also show that a greater amount of L55P TTR bound via high-affinity to the anionic phospholipids (PG and PS) than to the zwitterionic phospholipid (PC), consistent with the view that TTR binds to the membrane via electrostatic interactions [17]. It has been suggested that electrostatic interactions are likely to occur between the negatively charged headgroups of anionic phospholipid and positively charged residues in TTR molecules [30]. The electrostatic interactions between anionic phospholipids and TTR may provide a favourable environment for amyloidogenesis. For example, anionic phospholipids may produce an acidic local environment [30], which can induce aggregation of the protein [39,40]. Anionic phospholipids have been reported to induce conformational conversion of Aβ [41] and PrP [31] to form β-sheet-enriched amyloidogenic structures.

Studies of TTR aggregation kinetics showed that TTR aggregated in a non-linear sigmoidal fashion, typical of a nucleation-dependent polymerization process where the initial stable phase corresponds to the nucleation phase [34,42]. In the present study, aging of L55P TTR in the presence of anionic phospholipids resulted in a significant acceleration of the initial nucleation phase of aggregation. Therefore, anionic phospholipids may promote TTR aggregation by increasing the rate of nucleation. Morphological studies using AFM showed that the nucleation phase of L55P TTR aggregation was associated with the formation of amorphous oligomeric structures, suggesting that the nucleation process may involve oligomerization of TTR. Formation of oligomers (or protofibrils) prior to the appearance of mature amyloid fibrils has also been reported for other amyloidogenic proteins [43,44]. Thus, anionic phospholipids may promote the formation of TTR oligomers.

The studies reported here indicate that the lipid composition of the membrane play an important role in TTR binding. Cholesterol is enriched in certain microdomains in the plasma membrane known as lipid rafts [45]. It is now understood that the organization of lipids in the membrane is important for signal transduction as membrane receptors and ion channels are preferentially located in these lipid rafts [46,47]. Previous studies have shown that binding of amyloidogenic proteins to lipid rafts may induce their aggregation [26,48,49]. Analysis of the lipids associated with different types of amyloid deposits has shown that the amyloids possess a similar lipid composition to that of lipid rafts [24]. In addition, studies of amyloid formation *in vitro* have shown that the association with lipid rafts only occurs at the early stages of amyloidogenesis [24], suggesting that binding to lipid rafts may contribute to amyloidogenesis by inducing conformational changes and aggregation of amyloidogenic proteins. Thus it is possible that TTR also binds preferentially to lipid rafts in the plasma membrane.

It is known that phospholipids are distributed across plasma membranes of eukaryotic cells in an asymmetrical manner, with PS predominantly present in the cytoplasmic leaflet [50]. The asymmetrical distribution of phospholipids is maintained by a variety of transporters including flippase, floppase and scramblase [51]. Although the transmembrane phospholipid asymmetry can be disrupted by membrane disruption, this is unlikely to be the mechanism by which TTR comes into contact with PS as our previous studies have shown that TTR does not induce membrane disruption [13]. Alternatively, it has been reported that surface exposure of PS can be induced by Ca^{2+} influx which alters the activities of transmembrane phospholipid transporters [52,53]. It is possible that surface exposure of PS is induced by Ca^{2+} influx in neuronal cells.

Previous studies have shown that oligomers are likely to be the major form of TTR causing cytotoxic effects [13,16]. Binding of TTR oligomers to lipid rafts could be involved in the induction of TTR cytotoxicity. Our group recently reported that TTR oligomers can disrupt intracellular Ca^{2+} homeostasis by inducing an influx of Ca^{2+} via voltage-gated Ca^{2+} channels (VGCCs) [13]. In addition, the negatively charged groups (e.g. phosphodiester) of anionic phospholipids have been shown to be involved in the regulation of the activity of VGCCs [54]. Therefore, we speculate that cholesterol and anionic phospholipids may be involved in the aggregation and cytotoxicity of amyloidogenic TTR by modulating its binding to lipid rafts.

In summary, the present study shows that cholesterol and anionic phospholipids can promote the membrane binding of amyloidogenic TTR. As binding to membrane lipids is likely to be a key step in the induction of TTR cytotoxicity, this study suggests that membrane lipid composition may influence TTR cytotoxicity. These findings also suggest that membrane lipids may be novel targets for the development of therapeutic strategies for FAP. For example, lowering cholesterol by inhibiting its synthesis using lovastatin has been shown to reduce not only the membrane binding of Aβ but its cytotoxic effects on neuronal cells [19]. Indeed, drugs that reduce cholesterol (e.g. lovastatin and simvastatin) have been shown to attenuate the neuropathological changes of Alzheimer's disease [55,56]. Whether these drugs might also be used for the treatment of FAP needs to be explored in future studies.

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