Clinica Chimica Acta 412 (2011) 2117–2121

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Correlation of misfolded transthyretin in abnormal vitreous and high myopia related ocular pathologies

Jun Shao [a,*,1], Yu Xin [b,1], Yong Yao [a]

[a] Department of Ophthalmology, Wuxi People's Hospital Affiliated to Nanjing Medical University, 299 Qing yang Road, Wuxi 214023, People's Republic of China
[b] School of Biotechnology, Jiangnan University, Key Laboratory of Industry Biotechnology, Ministry of Education, Wuxi 214036, Jiangsu, People's Republic of China

ARTICLE INFO

Article history:
Received 8 April 2011
Received in revised form 16 July 2011
Accepted 22 July 2011
Available online 29 July 2011

Keywords:
Transthyretin
Vitreous body
Macular hole
Macular detachment

ABSTRACT

Background: High myopia is usually defined as eyes with −6 dioptres (D) of myopia, or >26.0 mm in axial length. Progressive and excessive elongation of the eyeball leads to secondary ocular diseases such as macular detachment (MD) and macular hole (MH). Higher transthyretin (TTR) levels had been detected in abnormal serums, but the situation in vitreous humor and the mechanism are still unclear.
Method: Transthyretin concentrations in vitreous of 80 high myopia patients and 20 healthy controls were determined by ELISA. After affinity purification, SDS-PAGE, Western-blot, and circular dichroism (CD) spectroscopy were employed for the secondary structure research.
Results: Vitreous transthyretin concentration of MD patients was ~1.1 times higher than that of MH patients, and 4.1 times higher than that of healthy controls. TTR in MD patients' vitreous showed a much more stable tetrameric structure. In neutral condition, compared with the secondary structure of healthy TTR, purified TTR in MD and MH vitreous samples showed lower β-sheets content; ~50% of β-sheets of the TTR in MD vitreous samples were lost.
Conclusions: The results suggested that MD and MH patients had higher vitreous TTR concentrations; TTR in MD vitreous had an abnormally stable tetrameric structure, possibly due to misfolding.

1. Introduction

Transthyretin (TTR) is a plasma homotetrameric protein that acts as a fluid carrier for thyroxine (T4) [1] and retinol [2]. It is a major component of amyloid deposits in human tissues and so may cause a wide range of amyloid diseases, including vitreous amyloidosis [3] and familial amyloidotic polyneuropathy (FAP) [4]. Transthyretin exists in vivo mainly as a tetramer of four identical subunits and only a small amount of the monomer; each subunit consists of 125 to 136 amino acid residues, which are largely arranged into β-sheet structure (41% β-sheet and 5% α-helix). This high β-sheet content was believed to contribute to the extraordinary stability of the molecule and natural function [5,6].

In ophthalmology research, vitreous amyloid fibrils are the result of local synthesis of mutated TTR. Nuclear cataract is associated with low protein intake and low serum levels of TTR [7]. Immunohistochemical analysis of drusen in patients with age-related macular degeneration revealed the presence of TTR [8].

High myopia is the second most frequent cause of poor vision and blindness; it could be caused by either genetic and environmental factors (or their interaction); and it had been reported that the majority of genetic cases are associated with 6 identified gene mutants [9–11]. To date, there are fewer reports of TTR expression levels in clinical high myopia samples. One report demonstrated that TTR levels were increased in the aqueous humor of five high myopia patients [12]. In our previous study [13], significant increased TTR serum levels were detected in high myopia patients compared to healthy controls; TTR levels in several vitreous samples were also determined. But the relationships of vitreous TTR and high myopia related diseases were still unclear.

2. Materials and methods

2.1. Subjects

Samples of vitreous from 80 high myopia patients with MD and with MH were collected during vitrectomy surgery. The control group of normal human eyes with no known ocular diseases (n = 20) was donated for corneal transplant in accordance with the Standardized Rules for Development and Applications of Organ Transplants and obtained from the Eye Bank of Shanghai, China. Two hundred microliters of each sample was diluted with 600 μl of 20 mmol/l PBS, separated into 4 tubes (200 μl/tube) and stored at −20 °C for further analysis. A

* Corresponding author at: Department of Ophthalmology, Wuxi People's Hospital Affiliated to Nanjing Medical University, Qing yang Road 299, Wuxi 214023, People's Republic of China. Tel.: +86 13665138176.
E-mail address: shaojun1983@gmail.com (J. Shao).
[1] These authors contributed equally to this work.

0009-8981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2011.07.021

polyclonal antibody to TTR was from Dako Co. Ltd. (Denmark). Sepharose CL 4B was from GE Health care (Piscataway, NJ) and cyanogen bromide (CNBr) was from Fluka. Other chemicals were from local companies and were analytical grade or better.

2.2. ELISA

The concentrations of TTR in the vitreous samples from healthy individuals (n = 20), MH patients (n = 25), and MD patients (n = 55) were determined using enzyme linked immunosorbent assay (Groundwork Biotechnology Diagnosticate Ltd.). The test was operated in accordance with the protocol of the manufacturer. Briefly, 50 μL of standards and 50 μl of vitreous sample were added into antibody pre-coated wells in the holder; then, 100 μl of 5% BSA was added to each well. The wells were covered and incubated for 1 h at 37 °C. All wells were washed 5 times using distilled or de-ionized water. The HRP-coupled antibody was added and the wells were covered and incubated for another 1 h at 37 °C. All wells were then washed 5 times using distilled or de-ionized water. Tetramethylbenzidine (TMB) substrate (50 μl of a 0.1% solution) was added to each well. The wells were covered and incubate for 15 min at 20–25 °C, and then 50 μl of stop solution (2 M H_2SO_4) were added to each well. The OD_{450nm} was detected for each well using a microtiter plate reader within 30 min.

2.3. Preparation of antibody column

The purified antibodies were coupled with CNBr-activated Sepharose CL 4B particles using standard methods [14]. Briefly, 1.5 g CNBr was dissolved in 10 ml acetonitrile and this solution was mixed with 10 g Sepharose CL 4B in 100 ml of 0.1 M Na_2CO_3–$NaHCO_3$ (pH 8.0) at 0 °C and pH 11.0 for 15 min. Then the Sepharose was washed sequentially with 1 l pre-chilled dH_20 and 500 ml pre-chilled 0.1 mol/l Na_2CO_3–$NaHCO_3$ (pH 8.0); the polyclonal antibody to TTR (15 mg) was mixed with 1 ml activated Sepharose medium at 4 °C for 36 h; the mixture was then added to 0.5 ml ethanolamine and mixed at 4 °C for 5 h. After coupling, the medium with antibody-bound Sepharose was washed with dH_2O, and stored in 10 mmol/l PBS/0.01% Na_3N at 4 °C.

2.4. Purification of TTR in vitreous

About 1 ml of vitreous sample from 2 healthy individuals, 14 MD patients and 9 MH patients were adjusted to 4 ml with 1.5 ml of 10 mmol/l PBS (8 g NaCl, 0.2 g KCl, 2.9 g Na_2HPO_4•$12H_2O$, and 0.2 g KH_2PO_4 dissolved in 1 l dH_2O); 1 ml of each protein sample was applied onto 10 mmol/l PBS-equilibrated antibody medium. The TTR was captured and then eluted by 0.1 M HAc. The resulting samples were stored at 4 °C.

2.5. SDS-PAGE and Western blot analysis

Protein samples (10 μl) was mixed with 10 μl loading buffer (2% SDS, 350 mmol/l DTT, 25% (v/v) glycerol, 0.01% Bromophenol Blue in 62.4 mM Tris-HCl, pH 6.8) and incubated at 95 °C for 5–20 min prior to loading. Reducing SDS-PAGE (12%) was performed using a Miniprotean II system (Bio-Rad, CA, USA). Gels were stained with Coomassie® Brilliant Blue R, imaged on Gelpro Analyzer 3.0 software (Media Cybernetics, Inc.). The TTR concentrations on gel (12.5%) were compared by integration of the lane darkness.

TTR extracted from MD patients' vitreous showed 2 bands on gel (~14 kDa and 65 kDa); the higher band was identified by Western blot analysis [15]. The peptide bands on gels were transferred to PVDF membranes that were sequentially reacted with rabbit anti-human TTR antibodies, horseradish peroxidase (HRP)-labeled goat anti-rabbit antibodies, and diaminobenzidine (DAB).

2.6. Circular dichroism spectroscopy

The CD spectrum of 0.2 mg/ml purified TTR was scanned with a Jasco J-715 spectropolarimeter in a cell with a path-length of 1 mm in accordance with previously reported methods [16]. The CD spectrum was measured from 190 to 250 nm. The data from triplicate measurements was averaged and then analyzed by the "K2d online server" at (http://www.embl-heidelberg.de/~andrade/k2d.html) in order to estimate the percentages of different secondary structures.

2.7. Statistical analysis

Experimental data were expressed as means ± SD. Group means were compared by one-way ANOVA using the GraphPad Prism software system (GraphPad San Diego, CA) and the statistical software program SPSS 13.0 for windows (Chicago, IL). P values <0.05 were considered significant in all cases.

2.8. Ethics

The research followed the tenets of the Declaration of Helsinki for the use of human subjects. Informed consents were obtained from all subjects after verbal and written explanation of the nature and possible consequences of the study.

3. Results

3.1. ELISA analysis

Concentrations of TTR in the vitreous samples of healthy individuals, MD patients, and MH patients were determined using enzyme linked immunosorbent assay (Groundwork Biotechnology Diagnosticate Ltd.). The test was run in triplicate. As calculated, TTR concentrations in vitreous of healthy individuals, MD patients, and MH patients were 45.2 ± 1.0 mg/l, 230.0 ± 16.3 mg/l and 109.8 ± 19.0 mg/l ($p<0.05$) (Fig. 1).

3.2. SDS-PAGE and western blot analysis

The purified TTR in vitreous samples from healthy individuals, MH patients, and MD patients were analyzed by SDS-PAGE and Western blotting (Fig. 2 A–F). The protein samples were boiled with reducing solution for 5 min (Fig. 2 A–C). In Fig. 2 A, lane 1 was the total protein from the vitreous sample of one healthy individual, lane 2 was the flow-through fraction, lanes 3 and 4 were purified TTR from the two healthy vitreous samples, and lanes 5–9 were purified TTR from the MH vitreous samples. In Fig. 2 B, lanes 1–4 were purified TTR from the MH vitreous samples, lanes 5–9 were purified TTR from the MD vitreous samples. In Fig. 2 C, lanes 1–9 were purified TTR from the MD vitreous samples. In Fig. 2 D and E, proteins were boiled with reducing solution for 20 min; lanes 1–9 (Fig. 2 D) and lanes 1–5 (Fig. 2 E) were purified TTR from the MD vitreous samples.

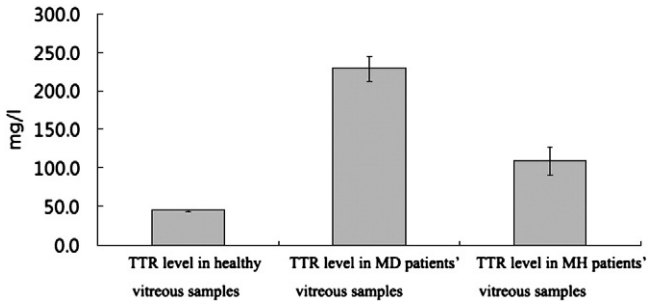

Fig. 1. ELISA analysis. TTR concentrations in vitreous of healthy individuals, MD patients and MH patients were 45.2 ± 1.0 mg/l, 230.0 ± 16.3 mg/l and 109.8 ± 19.0 mg/l, respectively.