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Transthyretin synthesis in rabbit ciliary pigment epithelium

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Abstract

Ocular symptoms of transthyretin (TTR)-related familial amyloidotic polyneuropathy (FAP) suggest that ciliary pigment epithelium (CPE) may synthesize TTR and its TTR may lead to amyloid formation in addition to TTR from vessels and retinal pigment epithelium (RPE). To clarify sites of TTR synthesis in ocular tissues, we performed in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR) for qualitative detection of TTR mRNA. In addition, we quantified levels of TTR mRNA expression by means of real-time quantitative RT-PCR. Furthermore, although TTR is an anti-acute phase protein in serum level, no reports on changes in TTR expression in ocular tissues during acute inflammation exist. To investigate changes in TTR expression in ocular tissues during inflammation, we induced uveitis by endotoxin challenge in rabbits and used real-time quantitative RT-PCR to examine changes in TTR mRNA expression in ocular tissues. In situ hybridization and RT-PCR qualitatively demonstrated TTR mRNA not only in RPE but also in CPE. Real-time quantitative RT-PCR showed that the level of TTR mRNA expression in the CPE was about one-third of that in the RPE. TTR mRNA expression in ocular tissues decreased as the degree of inflammation increased. These results suggest that TTR synthesized in the CPE may lead to ocular manifestations, especially glaucoma, in FAP. TTR mRNA also acts as an anti-acute phase reactant in ocular tissues.

Keywords: transthyretin; ciliary epithelium; familial amyloidotic polyneuropathy; glaucoma; anti-acute phase protein

1. Introduction

Transthyretin (TTR)-related familial amyloidotic polyneuropathy (FAP) is an autosomal dominant inherited disorder characterized by systemic accumulation of polymerized mutated TTR in the peripheral nerves and other organs, such as autonomic nervous system, choroid plexus, cardiovascular system, kidney, thyroid, gastrointestinal tract and eye (Ando et al., 1992). More than 100 different point mutations in the gene, most of which lead to production of amyloidogenic TTR (ATTR), have been identified in patients with FAP (Connors et al., 2003). In various types of FAP, ocular manifestations are commonly found although amyloid formation mechanism in ocular tissues as well as other systemic organs remains to be elucidated.

TTR is a 55-kDa tetramer protein in which each subunit is composed of 127 amino acids (Kanda et al., 1974). The main source of plasma TTR has been documented to be the liver (Felding and Fex, 1982), but the retinal pigment epithelium (RPE) (Martone et al., 1988; Cavallaro et al., 1990), the choroid plexus of the brain (Dickson et al., 1985; Soprano et al., 1985; Herbert et al., 1986) and the visceral yolk sac endoderm (Soprano et al., 1986) are also known to synthesize TTR. Furthermore, TTR protein had been detected in number of ocular tissues: RPE, retinal ganglion cells, nerve fiber layer of the retina, photoreceptor layer, ciliary epithelium, iris epithelium, lens capsule, corneal endothelium, and lacrimal glandular epithelium (Inada, 1988; Dwork et al., 1990). It plays an important role in plasma transport of thyroxin and, through its interaction with serum retinol-binding protein, of retinol (van Jaarsveld et al., 1973). The protein is an anti-acute phase protein whose serum levels decrease during acute inflammation, infection, and surgical stress. However, no reports on

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changes in TTR expression in ocular tissues in such pathologic conditions exist.

Most FAP patients with secondary glaucoma have vitreous opacities, dandruff-like substances on the lens surface or pupillary margin, and pigment deposition in the chamber angle (Kimura et al., 2003). This evidence led to the common belief that ocular amyloid deposition originating from the vessels and the RPE may result in obstruction of the aqueous outflow route (Silva-Araujo et al., 1993). However, because we had observed several FAP patients who had glaucoma with severe amyloid deposition on the pupil and pupil fringe with little or no vitreous opacity (Futa et al., 1984), thus making this hypothesis less likely. TTR synthesized by sites other than the RPE in ocular tissues, sites close to the chamber angle, may predominantly cause glaucoma with amyloid deposition on the pupil and pupil fringe in such cases. We believed that one possibility would be additional TTR expression in ciliary pigment epithelium (CPE) cells, which, like the RPE cells, are differentiated from the outer layer of the embryologic optic cup.

In the present study, we clarified sites of TTR synthesis in ocular tissues in addition to the RPE and investigated the effect of inflammation on the change in TTR expression in ocular tissues by inducing uveitis in endotoxin-challenged rabbits.

2. Materials and methods

2.1. Animals

Japanese adult albino male rabbits (Kyudo Co., Kumamoto, Japan), 12 weeks of age and each weighing about 2·0–2·5 kg, were used in this study. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Committee on Animal Research of Kumamoto University.

2.2. Tissue preparation

The animals were killed by using an intravenously injected overdose of pentobarbital. Ten eyes were enucleated immediately and fixed overnight in a mixture of 4% paraformaldehyde in 0·1 M phosphate buffer at 4°C. The eyes were cut circumferentially at the cornea (2–3 mm anterior from the limbus) to remove the cornea and lens to make posterior cups, and samples were embedded in paraffin. These samples were used for in situ hybridization, reverse transcription-polymerase chain reaction (RT-PCR) analysis and real-time quantitative RT-PCR analysis. Forty eyes were cut circumferentially at the sclera (1 mm posterior from the limbus) to make anterior cups without RPE and posterior cups. CPE and RPE were obtained via dissection directly from anterior cups and posterior cups, respectively. These fresh tissues were frozen immediately with liquid nitrogen and stored at −80°C until use for real-time quantitative RT-PCR analysis.

2.3. Preparation of probes

Rabbit TTR cDNA samples were amplified by use of RT-PCR with rabbit liver mRNA, and cDNA was cloned into pDrive Cloning Vector (Qiagen, Tokyo, Japan). Plasmid templates were linearized, and fluorescein-substituted antisense and sense RNA probes were transcribed by using Fluorescein RNA Labeling Mix (Roche Diagnostics GmbH, Penzberg, Germany) and DIG RNA Labeling Mix (Roche Diagnostics GmbH), according to the manufacturer's protocols.

2.4. In situ hybridization

In situ hybridization was performed with a tyramide signal amplification system with fluorescein-labeled probes (GenPoint Fluorescein; Dako, Carpinteria) according to the manufacturer's instructions with a slight modification. Before hybridization, paraffin-embedded sections were immersed in three changes of xylene for 5 min. Residual xylene was removed by immersing the sections in two changes of 99% ethanol, followed by two changes of 95% ethanol, for each 3 min. Sections were then rehydrated with several changes of water and were pretreated with proteinase K (20 $\mu g\,mL^{-1}$) at room temperature (RT) for 10 min. After sections were washed with Tris–buffered saline/Tween (TBST), they were passed through a graded ethanol series (70, 95, and 99%). Hybridization in mRNA in situ hybridization solution (Dako, Carpinteria), containing 0·2 $\mu g\,mL^{-1}$ fluorescein-labeled RNA probes, which was preheated at 80°C, continued overnight at 45°C. After the sections were washed with the stringent wash solution twice at 55°C for 20 min and with TBST for 5 min, they were covered by 3% hydrogen peroxide at RT for 5 min. Samples were washed with TBST twice for 3 min, and then the antibody was incubated in anti-fluorescein isothiocyanate (FITC)-horseradish peroxidase (HRP) solution (×100 dilution) for 30 min at RT. After sections were washed three times with TBST for 3 min, they were covered by fluorescyl tyramide solution at RT for 15 min. Sections were washed again three times with the TBST for 3 min, and then anti-FITC-HRP solution was added to the sections at RT for 30 min. Sections were then washed three times with TBST for another 3 min, after which the sections were covered for 15 sec with 3,3′-diaminobenzidine chromogen with buffered solution containing hydrogen peroxide. The reaction was stopped by immersing the sections in water for 1 min. Sections were then viewed under the light microscope and photographed.