CORNEAL DYSTROPHY WITH CALCIUM PHOSPHATE DEPOSITION IN *CHOLESTANOL*-FED MICE

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Abstract - In order to elucidate the effects of *cholestanol* feeding on serum and tissue concentrations and to establish pathological conditions resembling CTX, mice were fed a diet containing 1 % *cholestanol* for 8 months. 20 % of the animals developed degenerative lesions of cornea that resemble calcific band keratopathy and crystalline corneal dystrophy. Analysis of sterols in several organs were performed using high performance liquid chromatography. The concentrations of *cholestanol* in serum, liver and cornea of the corneal opacity bearing mice were 30-40 times higher than those of normal mice. On the other hand, brain *cholestanol* level increased only 7 times in the opacity group as compared with that of control group. The crystal particles were observed between epithelial basement membrane and superficial stroma by the electron microscopy. Energy dispersive analysis of particles revealed that the deposits were composed of calcium and phosphorus with other crystalline materials. These results suggest that the *cholestanol* may be deposited in the cornea from serum with elevated *cholestanol* level. Deposition of *cholestanol* in cornea and related area may be a cause of corneal dystrophy in CTX.

Key words *cholestanol* feeding, cerebrotendinous xanthomatosis (CTX), corneal dystrophy, calcium phosphate deposition.

Cerebrotendinous xanthomatosis (CTX) is a hereditary sterol storage disease. characterized by the increase in the *cholestanol* content in the serum and various organs. Clinically, this disorder causes a progressive neurologic dysfunction, tendon xanthomas, premature atherosclerosis, juvenile cataracts. Although various investigators have reported a storage of *cholestanol*, decreased synthesis of bile acids (particularly chenodeoxycholic acid), and accumulation of bile alcohols, the pathogenesis of this disease remains to be fully elucidated. Despite its structural resemblance to cholesterol (Fig. 1), the metabolism and physiological roles of *cholestanol* are not yet known with certainty. The present study was designated to examine the effects of *cholestanol* feeding on serum and tissue concentrations and to establish pathologic conditions resembling CTX, i.e. xanthomas or neurologic dysfunction development. We administered a diet containing 1 % *cholestanol* to mice for 8 months, and determined the concentrations of sterols in the serum and various organs using HPLC which is a more accurate and easier method than GC-MS (1), GC (2) and TLC (4). Histological examinations and qualitative elemental analysis were also performed.

EXPERIMENTAL

Benzoyl chloride reagent; The reagent for benzoyl derivatization was prepared before use by mixing 0.6 ml of benzoyl chloride with 20 ml of 1,2-dichloroethane and 2 ml of pyridine with continuous swirling and cooling. **Animals and diet;** Male BALB/c mice (final body weight of 25-30 g) obtained from single breeding parent colony were kept individually in metabolism cages to prevent coprophagy. They were fed a diet containing 1 % *cholestanol* for 8 months. The control mice were fed a standard diet.

Analysis of sterols;

Fig. 1 shows the structural difference of cholesterol and *cholestanol*. *Cholestanol* is a saturated sterol and differs from cholesterol by replacement of the 5 and 6 double bond with hydrogen atoms. The amount of *cholestanol* in serum is much lower than that of cholesterol, and accordingly the determination of *cholestanol*

Fig. 1 Structures of cholesterol and *cholestanol*

is difficult due to the similarity of both molecular structures. Fig. 2 shows the scheme of analytical procedure of serum sterols by HPLC. The sample preparation and analysis of sterols by HPLC were performed as described previously (5). 5β -Cholestan- 3α -ol ($10\mu\text{g}$; Sigma, St. Louis, MO, U.S.A.) as an internal standard was placed in a screw-capped test-tube, followed by 0.1 ml of serum and 10 volumes of 1 M etanolic KOH and hydrolyzed at 80°C for 1 h, followed by extraction twice with hexane. The solvent was evaporated under a stream of nitrogen and derivatized with benzoyl chloride reagent. The benzoyl compounds were analyzed by HPLC.

HPLC; Cholesterol and *cholestanol* were analyzed and quantitated by HPLC as described previously (5) on the LC-6A system, equipped with an autoinjector (SIL- 6A) and a data processor (Chromatopak C-R3A, Shimadzu, Kyoto). The column was packed with SBC-ODS (15 cm x 0.25 mm, Shimadzu) and maintained at 50°C during analysis.

Transmission electron microscopy ; The eyes from control and affected mice with corneal dystrophy were fixed in a 3 % glutaraldehyde solution. The corneas were removed, cut into 1 mm pieces, postfixed with osmium tetroxide. Samples were then dehydrated through a series of concentrations of alcohol, embedded in epoxy resin (Epok 812^R). Thick sections ($1\mu\text{m}$) were stained with Toluidine Blue O, and thin sections (70 nm) were stained with uranyl acetate and lead citrate and examined in an electron microscope (JEM 100).

Qualitative elemental analysis; Transmission electron microscopy/energy dispersive x-ray analysis (TEM/EDX) was used for analysis of minerals of the affected eyes. The eyes were postfixed in 2.5 % glutaraldehyde, then also fixed 1 % osmium tetroxide. Samples were then dehydrated with ethanol, embedded in epon araldite, sectioned and mounted on a nickel mesh.

Apparatus ; A H-7000 transmission electron microscope was integrated with an EMAX-3770 energy dispersive x-ray microanalyzer (Hitachi, Japan).

RESULTS

Determination of sterols by HPLC

The mice were divided into three groups, designated "opacity group" ; *cholestanol*-fed developing corneal dystrophy, "non-opacity group" ; *cholestanol* fed, but not developing corneal dystrophy, and "control group" ; fed with a standard diet. Fig. 3 shows the variations of sterols in serum and various tissues. *Cholestanol* concentrations in serum, liver and cornea of opacity group increased about 35 times as compared with control group, which is an agreement with the previous finding (6), whereas there was no significant difference between opacity and non-opacity groups (Fig.3A). In regard to *cholestanol* levels in cerebellum and cerebrum of opacity group were about 7 and 4 times higher than those in control group, and there is particularly more accumulation the cerebellum than in the cerebrum. That is the most prominent manifestation in CTX.

Fig. 2 Analysis of sterols by HPLC

3 Variations of sterols in serum and various tissues.

Fig. 4 shows HPLC chromatogram of corneal sterols. Sample preparation (7) and analysis of sterols by HPLC were performed as described previously (5). The chromatography profiles of corneal sterols revealed that the peak of *cholestanol* was detected in opacity group (Fig.4A). However, in control group, prominent *cholestanol* peak scarcely detected (Fig.4B). The most characteristic and striking observation in the present study is two kinds of corneal dystrophies, histologically resembling calcific band keratopathy and Schnyder's crystalline dystrophy in humans.

Qualitative elemental analysis

Fig. 5 shows computerized energy dispersive x-ray analysis of a case of corneal calcific band keratopathy. The electron dense crystal particles were present between subepithelium and superficial stroma, but were not in epithelial cell. The crystalline particles of the eye of two affected mice and one normal mouse were examined by a transmission electron microscopy/energy dispersive x-ray analysis (TEM/EDX). An energy dispersive x-ray analysis revealed prominent peaks of calcium and phosphorus deposition in electron dense portion. However they were never detected in the background. The peak designated Ni means the presence of nickel which was used as a grid.

Fig. 4 HPLC chromatogram of corneal sterols.

Fig. 5 computerized energy dispersive x-ray analysis of corneal crystal deposits (arrows). The height of peaks represents the relative quantity of minerals. B*; background, Ca; calcium, P; phosphorus, Os; osmium, Ni; nickel (grid).

DISCUSSION

The present results show the development of ophthalmic abnormalities in mice fed with *cholestanol*-rich diet. When the diet containing a 1 % *cholestanol* was fed to mice for 8 months, the *cholestanol* concentrations in serum, liver and cornea of opacity group were increased about 35 - fold as compared with those of control group. The most characteristic and striking observation in the present study is the development two kinds of corneal dystrophies, histologically resembling the calcific band keratopathy and Schnyder's crystalline dystrophy in humans. Although we were not yet able to elucidate the fine mechanism of formation of the depositions of calcium and phosphorus in cornea, it is possible to speculate that the increased serum *cholestanol* concentration causes disturbance of calcium metabolism in cornea, resulting in local deposition of calcium. Our results suggested that the crystalline-like deposits in the cornea are probably *cholestanol*. Though this report is the result of preliminary studies on the effects of *cholestanol* feeding on tissue sterols and corneal opacity in mice, it may be of great interest in connection with the pathology in CTX which is a lipid storage disease with predominant accumulation of *cholestanol* in serum and tissues.

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