Enhanced Susceptibility of LDL to Oxidative Modification in a CTX Patient: — Role of Chenodeoxycholic Acid in Xanthoma Formation —

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Cerebrotendinous xanthomatosis (CTX) is a rare familial sterol storage disease, causing multiple xanthomas in tendons and the brain. The underlying biochemical defect is a lack of the hepatic mitochondrial cholesterol 27-hydroxylase involved in the normal biosynthesis of bile acid, resulting in reduced biosynthesis of chenodeoxycholic acid (CDCA). It has been reported that administration of CDCA to CTX patients improves neurological disorders and xanthomas of the Achilles tendon. The present study investigated the effect of CDCA on the mechanism of cholesterol accumulation in macrophages, the major cells in xanthoma. The LDL from the patients in this study was significantly more susceptible to oxidative modification than normal LDL, and supplement therapy with CDCA resulted in an improvement in the susceptibility to oxidative modification. In the incubation of CDCA with plasma, 13% of the CDCA added to serum was recovered in the LDL fraction. In addition, supplementation with CDCA enhanced cholesteryl ester transfer protein (CETP) activity and reduced high-density-lipoprotein cholesterol levels in the plasma. This evidence suggests that the multiple xanthomas observed in CTX may be induced by increased oxidized LDL and the low activity of CETP, both of which are caused by a lack of CDCA. J Atheroscler Thromb, 2004; 11: 167–172.

Key words: Cerebrotendinous xanthomatosis, Macrophage, Oxidized LDL, Reverse cholesterol transport

Introduction

Cerebrotendinous xanthomatosis (CTX) is a rare familial sterol storage disease characterized by the accumulation of cholesterol and cholesteryl in xanthomas and the brain (1). The underlying biochemical defect is a lack of hepatic mitochondrial cholesterol 27-hydroxylase, which is involved in the normal biosynthesis of bile acid, resulting in a reduced biosynthesis of chenodeoxycholic acid (CDCA) (2). However, the relationship between the biochemical defect and xanthoma formation in CTX patients is not well understood.

It has been reported that xanthomas observed in CTX patients are composed of foam cells, which are composed of many cholesterol ester-laden macrophages (1). In the formation of foam cells, denatured low-density lipoprotein (LDL), such as oxidized LDL, is considered to be a major source of cholesterol ester, which is deposited in macrophages via scavenger receptors (3–5). On the other hand, reverse cholesterol transport is known to prevent foam cell formation (6, 7). Cholesteryl ester transfer protein (CETP) plays a key role in reverse cholesterol transport, and the activation of CETP may reduce cholesterol ester deposition in macrophages (8). It has been
reported that the administration of CDCA improves neurological disorders and xanthomas of the Achilles tendon in CTX patients (9). This evidence suggests that CDCA may affect the production of oxidized LDL and/or reverse cholesterol transport in CTX patients.

The present study investigated the effects of CDCA on the susceptibility of LDL to oxidative modification and on the activity of CETP in CTX patients.

**Materials and Methods**

**Materials**

CDCA sodium salt, cholesterol, cholesteryl oleate, and sodium cholate were purchased from Sigma (St. Louis, MO). [Carboxyl-\(^{14}\)C] CDCA was purchased from NEN (Wilmington, DE) and \([1-^{14}\)C]-cholesteryl oleate and \([1,2(n)-^{3}\)H]\) -cholesteryl linolate were purchased from Amersham (Buckinghamshire, England). Sephadex G25 was obtained from Pharmacia Fine Chem (Uppsala, Sweden).

**Diagnosis of CTX**

Three patients with CTX participated in this study under informed consent. CTX was diagnosed based on a ratio of cholestanol over cholesterol of higher than 0.3%, and a diminution of sterol 27-hydroxylase activity in leukocytes, as reported by Kasama et al. (10). The plasma concentration of cholestanol was measured by the method of Ishikawa et al. (11), and sterol 27-hydroxylase activity in leukocytes was measured by the method of Skrede et al. (12). The ratio of cholestanol to cholesterol and the sterol 27-hydroxylase activity in the leukocytes of the three CTX patients are shown in Table 1.

**Susceptibility of LDL to oxidative modification**

CDCA (400 mg/day) was orally administered to Patient A in Table 1 for 2 months, and vitamin E (tocopherol nicotinate: 300 mg/day) was administered for 1 month under informed consent. LDL was collected from serum at the beginning and end of administration of each drug, and the susceptibility of LDL to oxidative modification was determined.

This susceptibility to oxidative modification was measured by the method of Esterbauer et al. (13). Briefly, LDL was collected from serum, which was collected in a fasting state in the presence of EDTA, using step-wise ultracentrifugation at a density of 1.020–1.050 g/ml (14). The LDL was then dialyzed against phosphate-buffered saline (PBS, \(\mu = 0.17\), pH 7.4). After the protein moiety of the LDL was adjusted to 150 \(\mu\)g/ml by PBS, 1.66 \(\mu\)mol/ml of \(\text{Cu}^{++}\) was added to the LDL. While the LDL was incubated at 37°C, production of conjugated diene was monitored by observing the absorbance at 234 nm. From the production curve of the conjugated diene, the lag phase and propagation phase were determined, and the susceptibility of LDL to oxidative modification was determined based on the duration of the lag phase (13).

**Retinyl-palmitate absorbance test**

As a fat absorption test, a retinyl-palmitate loading test was performed on Patient A. Retinyl-palmitate (60,000 U/m\(^2\)) was administered orally with cream (50 g/m\(^2\)), and the plasma retinyl-palmitate concentration was measured at the indicated time after administration by high performance liquid chromatography (HPLC) (15).

**Binding of \(^{14}\)C-CDCA to lipoproteins**

One milliliter of human plasma obtained from a healthy volunteer in a fasting state was incubated with \(^{14}\)C-CDCA (37 kBq) for 1 hour at 37°C. After the total lipoprotein fraction (TLF) was collected from the plasma by ultracentrifugation at a density lower than 1.21 g/ml (14), the TLF was applied to the Sephadex G25 column to separate free \(^{14}\)C-CDCA from lipoprotein-bound \(^{14}\)C-CDCA. The lipoprotein-containing fraction, which appeared at void volume, was applied to non-denaturing polyacrylamide gel (2–33.8%), and the lipoproteins were separated by electrophoresis. After electrophoresis, the gel was stained with Coomassie brilliant blue, and the radioactivity was detected by BAS 2000 (Fuji Film Co, Tokyo, Japan).

**Other methods**

Plasma lipids were determined enzymatically. The plasma CETP activity was measured as described previously (16). The plasma concentrations of vitamin E and CDCA were measured by HPLC (17, 18).

**Results**

**Susceptibility of LDL from CTX patients to oxidative modification**

The susceptibility to oxidative modification of the LDL from the three CTX patients was determined (Fig. 1). The
mean of the duration of the lag phase, which represents the susceptibility of LDL to oxidative modification, was significantly shorter than that for healthy controls (38.8 min (Patient A), 60.1 min (B) and 52.0 min (C) versus 72.3 \pm 1.2 min (control: n = 4); p < 0.05), indicating that LDL from CTX patients is more susceptible to oxidation.

**Effects of administration of CDCA to CTX patients**

Administration of CDCA (400 mg/day) to Patient A dramatically improved the susceptibility to oxidative modification of LDL (Fig. 2). Although the administration of CDCA was discontinued after 2 months, its effects continued for an additional 3 months.

Though the CTX patient continuously showed an increased level of high-density-lipoprotein (HDL)-cholesterol, administration of CDCA induced a decrease in the HDL-cholesterol level to about 75%. On the other hand, CETP activity, which was 58% of the normal control before treatment with CDCA, increased to 105–118% of the normal control after the administration of CDCA (Fig. 3).

In the CTX patient, the retinyl-palmitate loading test showed a retardation of fat absorption to about 10% of that of the normal control. After CDCA administration for 3 months, fat absorption improved, but only to 20% of that of the normal control (results not shown). In spite of the retarded fat absorption of the CTX patient, the plasma concentration of vitamin E, a fat-soluble vitamin, was in normal range (9.47 \mu g/ml) before the administration of CDCA and did not change during the administration of CDCA. Vitamin E administration (300 mg/day) to the CTX patient improved the susceptibility to oxidative modification of LDL along with the plasma vitamin E concentration (Fig. 4).

**In vitro binding of CDCA to lipoproteins**

To elucidate the mechanism by which the susceptibility of LDL to oxidative modification is improved by CDCA, the binding of CDCA to lipoproteins was examined. After incubation of \(^{14}C\)-CDCA with plasma, the TLF was collected by ultracentrifugation. Twenty-two percent of the total radioactivity was recovered in the TLF. The TLF was then applied to a Sephadex G25 column to separate the...

**Fig. 1.** Measurement of the susceptibility of LDL to oxidative modification by monitoring conjugated dienes.

Susceptibility of LDL to oxidative modification was measured in three CTX patients and four normal controls as described in the Methods section. The results obtained from one CTX patient (Patient A; open triangle) and one normal control (open circle) are shown. Arrows indicate the lag phase of two LDLs. The lag phases of the three CTX patients were 38.8 min (patient A), 60.1 min (B), and 52.0 min (C). That of the four normal controls was 72.3 \pm 1.2 min.

**Fig. 2.** Changes in the susceptibility of LDL to oxidative modification before, during, and after CDCA administration.

CDCA (400 mg/day) was administered to Patient A for 8 weeks, and the change in the susceptibility of LDL to oxidative modification was monitored (open circle: lag phase). The plasma CDCA concentration is indicated by a closed circle.

**Fig. 3.** Effect of CDCA on CETP activity and HDL cholesterol concentration.

CDCA was administered to Patient A (same period as Fig. 2). Plasma CETP activity (open square) and HDL cholesterol (closed triangle) were monitored during CDCA administration. The plasma CDCA concentration is indicated by an open circle.
lipoprotein-bound CDCA from the free CDCA. Fifty-nine percent of the \(^{14}\)C-CDCA was bound to lipoproteins (about 13% of the initial activity), whereas 41% was free from lipoproteins. By analyzing the lipoprotein-bound \(^{14}\)C-CDCA by non-denaturing polyacrylamide gel electrophoresis, it was revealed that \(^{14}\)C-CDCA was bound mainly to LDL but not to HDL or VLDL (Fig. 5).

**Discussion**

CTX is characterized by multiple xanthomatosis, especially in the Achilles tendon. Additionally, neurological disorders which are observed in CTX patients have been reported to be due to xanthomas in the brain (19). Therefore, it is essential to explore the mechanism of xanthoma formation for the treatment of CTX patients. It is known that xanthoma formation is initiated by the deposition of cholesteryl ester (CE) in macrophages in an area of atherosclerosis, and oxidized LDL is the main source of CE accumulated in macrophages (4, 5).

The underlying biochemical defect of CTX is a lack of hepatic mitochondrial cholesterol 27-hydroxylase, resulting in reduced biosynthesis of CDCA (1). As it has already been reported that the administration of CDCA improves neurological disorders and xanthomas of the Achilles tendon in CTX patients (9), it is possible that CDCA may prevent CE deposition to macrophages.

To study the mechanism by which CDCA improves neurological disorders and xanthomas of the Achilles tendon in CTX patients, we first evaluated the effects of CDCA on the susceptibility of LDL to oxidative modification in CTX patients.

As shown in Fig. 1, the LDL from the CTX patients was more susceptible to oxidative modification than that of the normal controls. After the administration of CDCA for 8 weeks, susceptibility to the oxidative modification of LDL from the CTX patients improved to a normal range (Fig. 2). This improvement disappeared after the withdrawal of CDCA administration, suggesting that CDCA was responsible for the effect. This change in the susceptibility of LDL to oxidative modification might be due to the change in the cholestanol content of the lipoproteins. However, there was no significant change in the cholestanol content of the plasma during the 8 weeks. Because it has been reported that small dense LDL is more susceptible to oxidative modification (20), the LDL sizes of the CTX patients were determined before and during CDCA treatment. There was no difference in the size of the LDL during treatment (results not shown). Therefore, it is possible that CDCA directly improved the susceptibility of the LDL from the CTX patients to oxidative modification. It is not clear why, after 3 months of suspension of CDCA administration, the susceptibility to oxidative modification of LDL was still improved, while CDCA concentration was low. It is possible that trace amounts of CDCA may improve the susceptibility to oxidative modification of LDL. Further study will be required to elucidate this mechanism.

Incubation of \(^{14}\)C-CDCA with plasma revealed that \(^{14}\)C-CDCA bound to LDL with specificity (Fig. 5), indicating that the lack of CDCA on LDL enhances the susceptibility of LDL to oxidative modification, which can lead to the accumulation of CE in macrophages in CTX patients.
This also indicates that CDCA may act as an antioxidant by binding with lipoproteins, especially with LDL.

Because these patients lacked CDCA in the bile, it is clear that they experiencing fat malabsorption. Although this can lead to malabsorption of vitamin E, which is a fat-soluble vitamin and is known as an antioxidant, the plasma level of vitamin E was within normal limits and did not change during the treatment with CDCA. Despite the patients having a normal concentration of vitamin E in plasma, vitamin E administration to the CTX patients improved the susceptibility of LDL to oxidative modification (Fig. 4). It was also revealed that the size of the LDL did not change during the administration of vitamin E, as was observed with the administration of CDCA.

CDCA administration to CTX patients enhanced CETP activity in the plasma and led to a decrease in the HDL cholesterol (Fig. 3). Before CDCA administration, the CTX patients had about half the level of CETP activity of the control. It is well known that CETP plays an important role in reverse cholesterol transport (6–8), and a low level of CETP activity may lead to atherosclerosis, despite a high HDL cholesterol level (21, 22). Therefore, this low activity of CETP in CTX patients might cause CE accumulation in macrophages, and this disorder could be corrected by the administration of CDCA. As there are many CTX patients who do not show an elevated HDL cholesterol level, our finding of the relationship between CDCA and CETP activity may be limited only to the patients who show a low level of CETP activity and a high HDL cholesterol level.

Additionally, Bahr et al. reported that macrophages have sterol 27-hydroxylase activity (23). A lack of sterol 27-hydroxylase in macrophages is one of the mechanisms of xanthoma formation in CTX patients.

In this study, we elucidated the mechanism of CE accumulation in macrophages that forms xanthomas in CTX patients. Our findings suggest that the multiple xanthomas observed in CTX may be induced by an increased susceptibility of LDL to oxidative modification and, in part, by a low level of CETP activity, which are both caused by a lack of CDCA. As these disorders can be corrected by the administration of CDCA, CDCA is a good tool for decreasing xanthoma development in CTX patients. Additionally, the administration of vitamin E to CTX patients may prevent xanthoma formation by improving the susceptibility of LDL to oxidative modification.

References


