Cytotoxicity of Cholestane 3β,5α,6β-Triol on Cultured Intestinal Epithelial Crypt Cells (IEC-6)

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The effects of cholestane 3β,5α,6β-triol on intestinal epithelial crypt cells were investigated using the IEC-6 cell line. Cholestane 3β,5α,6β-triol decreased SH groups (glutathione and protein SH) in the cell, and showed cytotoxicity in a time-dependent manner. Although the concentration of cholestane 3β,5α,6β-triol used in this study (100 μM) was very high compared with that in plasma of experimental animals, cholestane 3β,5α,6β-triol did not show any cytotoxicity on IEC-6 cells without fetal calf serum (FCS). The level of cytotoxicity was dependent upon the concentration of FCS in the culture medium. Unknown components in FSC (not VLDL or LDL) were suggested to be associated with the cytotoxicity of cholestane 3β,5α,6β-triol. Moreover, the fact that even heat-treated FCS (100°C for 30 min) still mediated the cytotoxicity suggested the participation of non-protein components.

Key words: cholesterol oxide; cholestane 3β,5α,6β-triol; IEC-6 cell; intestinal epithelial crypt cell; cytotoxicity

Intestinal epithelial cells, the most rapidly proliferating tissues in the body, are important not only as the place of absorption of various kinds of nutrients but as the first barrier to protect us from xenobiotics.

Cholesterol, one of the important nutrients, is easily oxidized to produce various oxides. In fact, several cholesterol oxides have been identified in processed foods.1) and in animal tissues.2) We also identified some kinds of cholesterol oxides in Japanese traditional processed marine food.3)

Intestinal epithelial cells incorporate their membrane cholesterol from several sources, luminal cholesterol from the diet and from bile, and circulating low- and high-density lipoprotein cholesterol. Intestinal epithelial cells display receptors for low and high density lipoproteins.4–8)

Watabe et al.9) have reported that a cholesterol diet increases the concentration of cholestane epoxide and cholestane 3β,5α,6β-triol in the plasma and liver. Several cholesterol oxides have various adverse effects on cellular function, which may be involved in human diseases such as atherosclerosis10,11) and cancer.12,13) Boissonneault et al.14) have reported that cholestane 3β,5α,6β-triol compromises the selective barrier function of cultured vascular endothelial cell monolayers, resulting in the initiation of atherosclerotic lesions.

In this study, the uptake and the biological effects of cholestane 3β,5α,6β-triol, one of the most cytotoxic cholesterol oxides, were investigated using cultured monolayers of intestinal epithelial crypt cells (IEC-6), because the effects of cholesterol oxides on intestinal epithelial cells were not well defined.

Materials and Methods

Reagents. Cholesterol, cholestane 3β,5α,6β-triol, and glutathione (reduced form) were purchased from Sigma (St. Louis, U.S.A.). Glutathione (oxidized form), DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), and o-phthalaldehyde were from Wako Pure Chemical Industries (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) was from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum (FCS) was purchased from CSL Limited (Victoria, Australia) and bovine serum was purchased from the Research Institute for Microbial Diseases, Osaka University. Streptomycin sulfate and Penicillin G potassium were from Meiji (Kanagawa, Japan).

Preparation of cells. The rat intestinal epithelial crypt cell line, IEC-6, was obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded at a density of 1 × 10^5 cells/mL of Dulbecco’s modified Eagle’s medium containing 0.025% glucose, 4 mM glutamine, 10% FCS immobilized at 55°C for 30 min, streptomycin (0.1 g/100 ml), and penicillin (10,000 units/100 ml) in 100-mm plastic dishes. Cells were used at or below passage 23.

Cell culture. Cells were seeded at a density of 1 × 10^5 cells/mL of DMEM and cultured in a humidified incubator with 5% CO2:95% air at 37°C. The cells used for experiments were at 4 days after plating. Culture medium was replaced with fresh DMEM containing 100 μM of cholesterol or cholestane 3β,5α,6β-triol. Cholesterol or cholestane 3β,5α,6β-triol was dissolved in ethanol.

The purity of cholestane 3β,5α,6β-triol was confirmed by gas chromatography as the TMS derivative. No other oxysterol was detected. All experiments were done on the cells cultured with cholesterol as a control, because we confirmed that 100 μM cholesterol was not cytotoxic to IEC-6 cells.

Analytical methods. Protein content was measured by the Lowry–Folin method15) after dissolving the cultured cells in 1 M NaOH at 37°C for 1 h. Cell viability was measured by the neutral red assay.16) The culture medium was replaced with 500 μl of diluted neutral red (NR) (0.4% NR: sodium bicarbonate and phenol red-free Hanks’ balanced salt solution, pH 7.0, diluted 1:80). Cultured monolayers of IEC-6 cells were incubated with NR at 37°C for 2 h to allow the lysosomes of viable cells to absorb the dye. The NR was then removed and the cells were washed rapidly with 1% formaldehyde-1% calcium chloride. A mixture of 1% acetic acid–50% ethanol was added to the cell monolayers to extract the NR from lysosomes at room temperature for 30 min. The extracted dye was measured spectrophotometrically at 540 nm.

Lactate dehydrogenase (LDH) activity in the cultured medium was

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measured as a marker of the destruction of the cell membrane by a UV-assay. The volume activity was calculated from the value of the mean extinction change in the absorbance at 340 nm in 50 mM phosphate buffer, pH 7.5, at 25°C.

Protein sulfhydryl groups were measured as described by Monte et al. using reduced glutathione as the standard. The cell pellet was precipitated, washed twice with 6.5% trichloroacetic acid, and finally suspended in 2 ml of 0.5 M Tris-HCl, pH 7.6. DTNB (100 µM final concentration) was added to the cell suspension. After 20 min, the absorbance at 412 nm was measured.

Reduced and oxidized glutathione were measured by a fluorescence assay (ε-phthalaldehyde method).

Cholesterol 3β,5α,6β-triol incorporated into cells was also measured by GLC analysis as TMS derivatives. We confirmed the same molar response of TMS derivatives of cholesterol and cholestane 3β,5α,6β-triol. A crude lipid fraction was extracted from cultured cells with chloroform-methanol (2:1) after sonication in methanol, and then saponified with 1 M KOH at 40°C for 1 h. The unsaponified fraction extracted with hexane was trimethylsilylated with pyridine-hexamethyldisilazane-methyltrichlorosilane (10:3:2). GLC was done at 300°C with helium gas as a carrier. The column used was a fused silica capillary column DB-5 (0.25 mm x 50 m).

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Statistical analysis. All data are from each 3 or 4 measurements for each experiment and each is shown as means ± SD. The paired Student's t-test for mean separation was used where appropriate.

Results

Cytotoxicity of cholestane 3β,5α,6β-triol

Cholestane 3β,5α,6β-triol was added to the subconfluent cells (4 days after plating). Figure 1 shows the relative viability of intestinal epithelial crypt cells after incubation with cholesterol or cholestane 3β,5α,6β-triol. The cell viability added 100 µM cholestane 3β,5α,6β-triol was significantly lower than the control (p < 0.05) and decreased time-dependently (p < 0.01). The LDH activity in the culture medium, which indicates the destruction of the cell membrane, was also measured. As shown in Fig. 2, the LDH activity of the culture medium containing 75 or 100 µM of cholestane 3β,5α,6β-triol, was significantly higher than that of culture medium containing 100 µM of cholesterol (p < 0.05) even 30 min after the addition, and increased gradually throughout the incubation period. These results indicate that the decrease of their viability induced by cholestane 3β,5α,6β-triol was due to the destruction of the cell membrane.

Effects of serum on the cytotoxicity

Serum lipoprotein may reduce the cytotoxicity of cholesterol oxide by scavenging it in one case, and increase the cytotoxicity by producing the denatured LDL in another case. Then, the effects of FCS on the cytotoxicity of cholestane 3β,5α,6β-triol were examined. As shown in Fig. 3, when IEC-6 cells were cultured in the FCS-depleted medium containing 100 µM of cholestane 3β,5α,6β-triol, cell viability was not decreased. Viability was maintained at about 90% of the control group. However, the cell viability was decreased by FCS in a dose-dependent manner. This result indicated that only a small amount of FCS (1%) was essential for the expression of the cytotoxicity of cholestane 3β,5α,6β-triol. To examine the roles of LDL and VLDL in the expression of the cytotoxicity of cholestane 3β,5α,6β-triol, cells were cultured in a medium containing the VLDL- and LDL-depleted FCS, which was obtained by treating FCS with heparin and 1 M MnCl₂ at 4°C for 30 min. However, the cell viability was still low (p < 0.05) as shown in Fig. 4, which indicated that LDL and VLDL were not concerned with the expression of cytotoxicity of cholestane 3β,5α,6β-triol. The effects of other types of serum or plasma, for example immobilized rat serum, rat plasma,
and bovine serum were also examined. However, cholestane 3β,5α,6β-triol did not show such a strong cytotoxicity on IEC-6 cells as in the medium containing FCS, indicating that unknown components especially rich in fetal serum might be involved with the expression of cytotoxicity. HDL was also reported to have high affinity with the epithelial cells of human small intestine. 4 However, we could not identify the role of HDL in the expression of the cytotoxicity of cholestane 3β,5α,6β-triol directly in this study, because we could not prepare HDL-depleted FCS. When heat-treated FCS at 65, 80, or 100°C for 30 min was examined, the cell viability in the medium containing the heat-treated FCS even at 100°C for 30 min was still significantly (p < 0.01) low, as shown in Fig. 5. This result indicated that the heat stable unknown factors in FCS that mediated the cytotoxicity were not protein. Furthermore, to obtain information on these unknown components, FCS was fractionated by an ultra filter unit-centrifuge type (USY-1, 5 and 20; Advantec Toyo, 10K; Eyela) into different molecular sizes, > 2.0 x 10^5, 2.0 x 10^5-5.0 x 10^4, 5.0 x 10^4-1.0 x 10^4, and < 1.0 x 10^4. The result shown in Fig. 6 indicated that the molecular size of the unknown components were large.

Incorporation of cholestane 3β,5α,6β-triol into cell

The incorporation of cholestane 3β,5α,6β-triol into IEC-6 cells was examined by using gas chromatography. When IEC-6 cells were cultured in the medium containing FCS, cholestane 3β,5α,6β-triol was incorporated into cells easily. After 4 hours of incubation, the molar ratio of cholesterol to cholestane 3β,5α,6β-triol was 1.0:1.2. However, when FCS was not added to the medium, cholestane 3β,5α,6β-triol was not detected.

Effects of cholestane 3β,5α,6β-triol on cellular metabolism

The transmembrane ion gradient is known to be disordered by cholestane 3β,5α,6β-triol, and the disordered ion gradient might oxidize SH-compounds in the cell. Therefore, we examined the effects of cholestane oxide on
the content of protein SH and reduced glutathione. As shown in Fig. 7, cholestane 3β,5α,6β-triol decreased SH-groups in the cell during 1 h of incubation; at that time cell viability of the experimental group was not very different from the control group, although the LDH activity in the culture medium was already higher in the experimental group than in the control.

**Discussion**

IEC-6 is a cell line derived from a normal rat intestine. It has several features characteristic of normal cells in culture, is nontumorigenic, and retains the undifferentiated nature of epithelial stem cells. Cultured cells principally derive membrane cholesterol from endogenous synthesis or uptake of exogenous cholesterol. Cholesterol oxide decreases cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase), and inhibits cholesterol esterification, resulting in a change of cell morphology and membrane function. 142 The lack of cholesterol would result in defective membrane formation, reduce cell growth, or induce cell death. However, it is also reported that the correlation between the ability of cholesterol oxides to inhibit cholesterol biosynthesis and their cytotoxicity is inconsistent. 22

Cholestane 3β,5α,6β-triol has a strong polar group on one end and a hydrophobic group on the other, suggesting that it easily penetrates the cell membrane. Sevanian et al. 23 reported that cholestane 3β,5α,6β-triol was incorporated not only in a dose-dependent manner but in a time-dependent manner into rabbit aortic endothelial cells as denaturated LDL through the LDL receptor. In addition, it is also reported that HDL has a high affinity for isolated epithelial cells of human small intestine 43 and that large amounts of HDL are degraded by intestinal cells in the rat. 7 Then, the mechanism to incorporate cholestane 3β,5α,6β-triol into the cell membrane was thought to be in 2 ways; the direct incorporation through its chemical properties according to its detergent action, or receptor-mediated incorporation through the LDL and HDL receptors. In this study, cholestane 3β,5α,6β-triol could enter into cells easily in the presence of FCS, but not in the absence of FCS. Although the concentration of cholestane 3β,5α,6β-triol used in this study, 100 μM, was very high compared with its concentration in plasma of experimental animals, 9 0.2-0.8 μM, cholestane 3β,5α,6β-triol was not cytotoxic for IEC-6 cells without FCS. To express the cytotoxicity of cholestane 3β,5α,6β-triol, a small amount of FCS must be necessary. The results of this study suggested that the essential component in FCS for the expression of cytotoxicity might not be LDL and VLDL. In addition, the fact that heat-treated FCS at 100°C for 30 min could still mediate the expression of cytotoxicity of cholestane 3β,5α,6β-triol indicated that the unknown components were not HDL, of which the protein content is about 40 to 60%, and the unknown components must be heat stable.

Cholestane 3β,5α,6β-triol incorporated into cells might compromise the selective barrier function of the cell membrane and the ability of the membrane to maintain the various functions necessary for continued cell viability, such as a transmembrane ion gradient, resulting in the reduction of SH group in the cell. 20 It is also reported that cholestane 3β,5α,6β-triol elevates albumin transfer in vascular endothelial cell monolayers 143 by compromising the selective barrier function. In this study, SH group in the cell was decreased by the addition of cholestane 3β,5α,6β-triol. Although much more investigation is necessary, the reduction of SH groups might be not only from the compromising the membrane function but from the partial destruction of the cell membrane. The destruction of the cell membrane would induced cell death.

The difference in the mechanism of cytotoxicity of cholestane 3β,5α,6β-triol in vivo and in vitro and unknown components in FCS that mediate the expression of cytotoxicity in vitro are now under investigation.

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**References**