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Title page

Effect of Ursodeoxycholic Acid and Chenodeoxycholic Acid on Major Histocompatibility Complex Class I Gene Expression.

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A short title : Effect of UDCA on MHC class I mRNA

Footnote Page

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Abstract

We investigated effect of ursodeoxycholic acid on major histocompatibility complex class I gene expression in cultured human hepatoma cells. Ursodeoxycholic acid, which has recently been used for treatment of various autoimmune liver diseases, paradoxically increased mRNA level of major histocompatibility complex class I. However, endogenous bile acids, for example, chenodeoxycholic acid, more strongly increased major histocompatibility complex class I mRNA expression when compared with ursodeoxycholic acid. Concerning interplay between ursodeoxycholic acid and chenodeoxycholic acid, these bile acids additively induced major histocompatibility complex class I mRNA expression. In contrast, when total concentration of ursodeoxycholic acid and chenodeoxycholic acid was kept constant, expression of major histocompatibility complex class I mRNA appeared to be rather decreased in a dose-dependent manner along with increasing ratio of ursodeoxycholic acid. These data might indicate that the beneficial action of ursodeoxycholic acid is related to this relative decrease in major histocompatibility complex class I gene expression.

Introduction

Primary biliary cirrhosis (PBC) is a chronic and progressive cholestatic disease predominantly affecting middle-aged women.^{1, 2} In patients with PBC, an up-regulated expression of major histocompatibility complex (MHC) class I antigens on hepatocyte membranes and an aberrant expression of major histocompatibility complex (MHC) class II antigens on bile duct epithelium were reported, as opposed to normal subjects.³⁻⁵ Especially, increased MHC class I antigens have been shown to be expressed in areas of piecemeal necrosis, and to be seen in the later stages of PBC.⁶ In contrast, it is known that infiltrating lymphocytes in the lesions showing piecemeal necrosis and/or intralobular necrosis were predominantly cytotoxic T lymphocytes in patients with PBC.^{7, 8} Thus, increase in MHC class I expression may have direct association with hepatocyte injury which is mediated by cytotoxic T lymphocytes.

In general, it is well known that hydrophobic bile acids such as chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) are hepatic toxins, as evidenced in laboratory animals *in vivo*,⁹⁻¹¹ in perfused livers,¹² and in isolated hepatocytes from human beings.^{13, 14} Therefore, it is possible that endogenous bile acids, e.g., CDCA, DCA, and LCA, cause secondary hepatocellular injury by hepatocytotoxic and/or immune-mediated mechanisms.⁵ In animal experiments, it is also shown that cholestasis itself, which is induced by ligation of the extrahepatic bile duct, leads to an increased expression of MHC class I antigens on hepatocytes in rats.¹⁵ In the other hand, ursodeoxycholic acid (UDCA) is considered to be a nonhepatotoxic hydrophilic bile acid that may reverse the potential hepatotoxicity of endogenous bile acids, and is often used in patients with cholestasis to improve liver dysfunction.¹⁶⁻¹⁸ Notably, a beneficial effect of UDCA in patients with PBC has been demonstrated in double-blind controlled studies.¹⁹⁻²¹ In those studies, UDCA has been shown to improve biochemical parameters.¹⁹⁻²¹ Calmus et al reported that UDCA significantly decreased MHC class I molecules on hepatocytes in patients with PBC using an immunocytochemistic techniques.⁵ Terasaki et al reported that treatment of UDCA not only decreased the hepatocellular expression of MHC class I antigens, and also reduced the hepatocellular infiltration of activated T lymphocytes in patients with PBC.²² However, experimental evidence is completely lacking to confirm these hypothesis. Since UDCA is an isomer of CDCA, these mutual exclusive interaction between these two bile acids appears to be extremely interesting. Recently, we demonstrated that CDCA, which is one of major constituents of bile acids in cholestasis,⁵ directly increased both immunoreactivities and mRNA levels of MHC class I in human cultured hepatoma cells,²³ indicating that cholestasis itself may influence on immune-regulation in hepatobiliary system. Moreover, Hillaire et al

recently confirmed our finding by studying normal human hepatocytes instead.²⁴ We more recently showed that MHC class I mRNA expression was induced by CDCA in cultured human hepatoma cells via activation of protein kinase C-dependent pathway.²⁵ Given this background, we studied effect of UDCA on MHC class I gene expression. We describe here that in HepG2 cells UDCA induces MHC class I mRNA expression as well as other bile acids, but relatively decreases CDCA-induced MHC class I mRNA expression.

Materials and Methods

Cells.

Human hepatoma cell line HepG2 was cultured in the Dulbecco's modified Eagle's medium (GIBCO BRL, New York, U.S.A.) supplemented with 0.1mM non-essential amino acids, 10% fetal calf serum (GIBCO BRL), 100 · g/ml penicillin, and 100 U/ml streptomycin at 37 · C, in a humidified atmosphere of 5% CO₂ in air. Serum steroids were stripped with dextran-coated charcoal before use.

Ligands.

Sodium salts for UDCA and CDCA were kindly gifted from Tokyo Tanabe Co. (Tokyo, Japan). These bile acids were dissolved in ethanol. Gaschromatography demonstrated that composition of all bile acids was a purity of at least 99.5%. These bile acids were used at final concentrations of 10 · M to 250 · M, because the serum concentration of UDCA in patients with PBC undergoing UDCA treatment was reported to be around 10 · M to 100 · M.²⁶⁻²⁸

RNA isolation.

Total RNA was isolated according to the method of acid guanidinium thiocyanate phenol chloroform (AGPC) extraction.²⁹ Briefly, HepG2 cells were cultured in 100-mm diameter plastic dishes (IWAKI GLASS) in the absence or presence of bile acids for the indicated time periods. After washing twice with PBS, cells were then lysed with a solution that contained 4 M Fluka-purum grade guanidinium thiocyanate (Tridom, Inc., New York, U.S.A.), 25 mM sodium citrate, pH 7.0, 0.5 % sodium N-lauroylsarcosine, and 0.1 M 2-mercaptoethanol. RNA was further purified by phenol/chloroform extraction and then precipitated in isopropanol. RNA pellets were solubilized in diethylpyrocarbonate-treated filtered water and the concentration of RNA was determined spectrophotometrically.

Northern blot hybridization.

Northern blot analysis was done as described previously.^{23, 25} In brief, 20 μ g of total RNA was denatured in a loading buffer by heating at 70 $^{\circ}$ C for 5 min and was separated on a 1% formaldehyde-agarose gel. The fractionated RNA was immobilized on a charge-modified PVDF membrane (Immobilon-N⁺, Millipore, Massachusetts, U.S.A.) using a pressure blot apparatus (Stratagene, California, U.S.A.), and RNA was cross-linked by means of UV irradiation. The plasmid pDP001 was kindly provided by Japanese Cancer Research Resources Bank (JCRB) and the 0.4-kb Pvu II-Pst I DNA fragment was used as the cDNA probe for HLA-B7 as described.^{23, 25, 30} The cDNA probe for β -actin was from Cleveland et al.³¹ These cDNA probes (25-50 ng) were labeled with [α -³²P]dCTP (3,000 Ci/mmol, Amersham, Buckinghamshire, U.K.) using BcaBESTTM labeling kit (TaKaRa, Tokyo, Japan) according to the manufacturers' protocol. The membrane was prehybridized with a QuikHybTM hybridization solution (Stratagene, California, U.S.A.) for 15 min at 68 $^{\circ}$ C, and was hybridized to 2 x 10⁶ cpm/ml of ³²P-labeled probes for 60 min at 68 $^{\circ}$ C. After the membrane was washed for 30 min in 0.1% SDS with 2 x SSC at 25 $^{\circ}$ C, and for additional 30 min in 0.1% SDS with 0.1 x SSC at 60 $^{\circ}$ C, radioactivity on the membrane was quantified with the BIO-IMAGING ANALYZER BAS2000 (FUJIX, Tokyo, Japan) and then the membrane was exposed to X-ray film (HyperfilmTM-MP, Amersham) with intensifying screens (KODAK, Tokyo, Japan) at -70 $^{\circ}$ C overnight.

Statistical analysis.

All values in this study were expressed as means \pm SE. Comparisons were made using analysis of variance, followed by two-tailed Student's t-test. Differences were considered significant if P values were less than 0.05.

Results

Northern blot analysis of MHC class I gene expression.

We have already demonstrated that CDCA increased MHC class I mRNA. (ref. 23 and also see Fig. 3). To assess whether UDCA induces MHC class I mRNA expression as well, Northern blot analysis was performed. Treatment with the increasing concentrations of UDCA revealed an apparent elevation of MHC class I mRNA (Fig. 1). Such UDCA effect appeared to be dose-dependent up to 100 μ M (Fig. 1), however, at 250 μ M of UDCA, the

expression of MHC class I mRNA was slightly decreased, most possibly due to a decrease in cell viability (data not shown). Time course study showed that, the level of MHC class I mRNA seemed to be significantly increased in a time-dependent manner (Fig. 2). Maximum effect of UDCA on MHC class I mRNA expression was observed at 48 h (Fig. 2). At 72 h of treatment with UDCA, MHC class I mRNA expression was rather decreased, although cell viability was intact (Fig. 2). Collectively, we may present that UDCA induced MHC class I mRNA expression in a dose- and time-dependent manner, nearly as well as CDCA (Fig. 3, and ref. 23). However, quantitative analysis demonstrated that at $100 \cdot \text{M}$, UDCA is revealed to be a weaker inducer of MHC class I mRNA than CDCA (Fig. 3).

Effect of UDCA and CDCA on MHC class I mRNA expression.

The fact that UDCA, as well as CDCA, induces MHC class I mRNA expression appears to be contradictory to already-confirmed clinical effectiveness of the drug. Then, to address this issue, we studied effect of these two bile acids on MHC class I mRNA expression. First, effect of various concentrations of UDCA was examined on CDCA-induced levels of MHC class I mRNA. As shown in Fig. 4, UDCA additively and dose-dependently enhanced MHC class I mRNA expression in the presence of various concentrations of CDCA. However, UDCA effect was relatively weak and, for example, $10 \cdot \text{M}$ of CDCA showed stronger effect on MHC class I mRNA expression when compared with $100 \cdot \text{M}$ of UDCA (Fig. 4). Second, effect of bile acid composition was studied keeping total bile acid concentration constant. Along with increasing ratio of UDCA, MHC class I mRNA expression was decreased (Fig. 5). Therefore, we may suggest that once replacement between UDCA and CDCA would occur in vivo, UDCA could relatively reduce MHC class I mRNA expression.

Discussion

In the present study we for the first time presented the evidence that, as well as CDCA, UDCA increases mRNA level of MHC class I in HepG2 cells. We used unconjugated forms of the bile acids at concentrations of $0\text{-}250 \cdot \text{M}$, covering the range of tissue bile acid concentrations commonly observed during chronic cholestasis.³² Although hepatocytes are also exposed to conjugated forms of bile acids, which have less marked toxic effects than unconjugated forms,^{33, 34} our results can reasonably be extrapolated to the in vivo situation, because the contact time between hepatoma cells and bile acids was much shorter in our in vitro conditions than during cholestasis.¹⁵ In addition, we showed that UDCA enhanced

CDCA-induced MHC class I mRNA expression in our experimental system in HepG2 cells. Along with increasing ratio of UDCA, MHC class I mRNA expression was decreased when total bile acid concentration of CDCA and UDCA in cultured medium was kept constant. Thus, a relative decrease of MHC class I mRNA expression by UDCA in HepG2 cells may result not only from a reduction in the intrahepatic accumulation of cytotoxic bile acids such as CDCA, but also from a reduction in immunological injury. However, we cannot rule out the possibility that the net degree of hydrophobic-hydrophilic balance are reduced by means of coating hydrophobic site of CDCA molecules with UDCA in cultured medium, and that MHC class I expression is rather differently regulated in non-malignant cells. For further confirmation, studies using primary hepatocyte culture would be clearly merited.

Recently we reported that the ascending order of MHC class I mRNA inducibility by bile acids was as follows : UDCA, CA, CDCA, DCA, and LCA.²⁵ In addition, inducibility of MHC class I mRNA was clearly correlated with the hydrophobicity of the corresponding bile acids.²⁵ Quist et al have also reported that histamine release from mast cell is highly correlated with the hydrophobicity of bile acids.³⁵ They showed that the order of potency for histamine release for CDCA and DCA was unconjugated > glycine conjugated > taurine conjugated.³⁵ Thus, those reports strongly support that the notion that the hydrophobicity of bile acids may be related to alteration in certain biological function including MHC class I gene regulation. We also need to further study inducibility of MHC class I mRNA by conjugated forms of UDCA and CDCA.

From clinical viewpoints, it is of extreme importance to assess whether UDCA could act as an immune modulator in vivo. Batta et al investigated the effect of UDCA on bile acid metabolism both in patients with PBC and in healthy subjects.³⁶ In this report, serum mean concentration of total bile acids by treatment with 900 mg/day of UDCA was $8 \cdot M$ in healthy subjects, while was $78 \cdot M$ in patients with PBC.³⁶ We showed that, at $10 \cdot M$ of UDCA, levels of MHC class I mRNA were only marginally changed when compared with basal levels. Therefore, it is unlikely that UDCA affects the expression of MHC class I antigen in healthy subjects. On the contrary, in patients with cholestasis, it is expected that endogenous bile acids markedly increase expression of MHC class I antigen, since serum mean concentration of total bile acids in patients with PBC is almost comparable with the concentration of bile acids which induced MHC class I mRNA expression. It is speculated that useful effects of long term administration of UDCA in patients with PBC is related to the replacement of hydrophobic endogenous bile acids to a hydrophilic UDCA in patient's sera.³⁷ Batta et al examined the effect of UDCA on the urinary and serum bile acids in patients with PBC.²⁶ In this report, they demonstrated that the beneficial effect of UDCA

may be due to reduction of hydroxylated derivatives of endogenous bile acids together with the appearance of hydroxylated derivative of UDCA, or may be due to displacement of the more hydrophobic endogenous bile acids by the hydrophilic UDCA.²⁶ In addition, they indicated that serum concentration of total bile acids after UDCA treatment in patients with PBC was rather decreased when compared with serum concentration before UDCA treatment, although urinary concentration of total bile acids after UDCA treatment in patients with PBC was significantly increased.²⁶ Crosignani et al reported that, in patients with PBC, UDCA modified the composition of the bile acid pool by inhibiting the ileal absorption of endogenous bile acids.²⁸ They suggested that the substitution of CA with UDCA would explain a decrease in the relative hepatotoxicity index of the bile acid pool after UDCA administration, although serum total bile acid concentrations were unchanged after treatment with UDCA.²⁸ In our *in vitro* conditions, UDCA additively increased CDCA effect on MHC class I mRNA expression, however, increasing ratio of UDCA relatively decreased MHC class I mRNA expression, when the total bile acid concentration was constant. Therefore, it is suggested that the beneficial effect of UDCA in clinical situations is not ascribed to a competition between endogenous bile acids and UDCA at the target cell level, but would rather be related to a relative reduction in the endogenous bile acid concentration after long-term administration of UDCA.^{26, 36}

Recently, several groups have investigated the effect of long-term administration of UDCA on symptoms, serum markers of cholestasis, histology and the need for liver transplantation in patients with PBC.³⁸⁻⁴⁰ Poupon and the UDCA-PBC study group reported that long-term UDCA therapy slowed the progression of PBC and reduced the need for liver transplantation, although they postulated that further studies were required to assess the additional benefit of combined medical treatments, such as UDCA plus immunosuppressive agents.³⁸ On the contrary, Lindor et al and Heathcote et al reported that UDCA in patients with PBC did not significantly improve patient survival and need for liver transplantation.^{39, 40} Those reports suggested that a combination therapy with UDCA and other agents, e.g., immunosuppressive drugs, are needed for patients with PBC, although UDCA therapy might slow the progression of PBC. Thus, combinational effects of UDCA with other drugs should be examined in further studies.

In conclusion, we demonstrated that mRNA level of MHC class I was up-regulated by UDCA. Since UDCA had weaker effect on MHC class I mRNA expression than CDCA, we might indicate that one of the beneficial action of UDCA in patients with PBC is related to a relative decrease in MHC class I expression, most possibly, due to replacement the hepatotoxic hydrophobic endogenous bile acids.

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Figure Legends

Fig. 1.

MHC class I mRNA expression after UDCA treatment. The cells were cultured in the presence of the indicated concentrations of UDCA for 48 h. Northern blot hybridization was performed as described in Materials and Methods.

Fig. 2.

Kinetics of MHC class I mRNA expression. The cells were cultured in the presence of 100 μ M of UDCA for the indicated time periods. Northern blot hybridization was performed as described in Materials and Methods.

Fig. 3.

Quantitative analysis of MHC class I mRNA expression. The cells were cultured in the presence of the indicated concentrations of UDCA and CDCA for 48 h. Northern blot hybridization was performed as described in Materials and Methods. Radioactivity on the membrane was quantified with a phosphorimage analyzer. Results were presented as the ratio of MHC class I mRNA to β -actin mRNA, and were expressed as mean \pm SE of four different experiments. *, $p < 0.05$ v.s. 0 μ M of UDCA, $p < 0.01$ v.s. 0 μ M CDCA, Δ , $p < 0.01$ v.s. 100 μ M of UDCA by two-tailed Student's t -test.

Fig. 4.

UDCA additively increased CDCA effect on MHC class I mRNA expression. The cells were cultured in the absence or presence of the indicated concentrations of CDCA and/or UDCA for 48 h. Northern blot hybridization was performed as described in Materials and Methods. Radioactivity on the membrane was quantified with a phosphorimage analyzer. Results were presented as the ratio of MHC class I mRNA to β -actin mRNA, and were expressed as mean \pm SE of four different experiments. *, $p < 0.05$, **, $p < 0.01$ v.s. 0 μ M of CDCA, Δ , $p < 0.05$, $\Delta\Delta$, $p < 0.01$ v.s. 10 μ M of CDCA, and $\Delta\Delta\Delta$, $p < 0.01$ v.s. 100 μ M of CDCA by two-tailed Student's t -test.

Fig. 5.

Effects of bile acid composition on MHC class I mRNA. The cells were cultured in the absence or presence of the indicated concentrations of CDCA and/or UDCA for 48 h (total concentration of bile acids was kept constant at 50 μ M). Northern blot hybridization was

performed as described in Materials and Methods. Radioactivity on the membrane was quantified with a phosphorimage analyzer. Results were presented as the ratio of MHC class I mRNA to β -actin mRNA, and were expressed as mean \pm SE of four different experiments. *, $p < 0.05$, **, $p < 0.01$ v.s. 50 μ M of CDCA by two-tailed Student's t -test.