Secondary bile acids (SBAs) with high hydrophobicity are abundant in the colonic lumen. However, both aggravating and protective roles of SBAs have been proposed in the pathogenesis of inflammatory bowel diseases (IBDs). We observed that oral administration of hyodeoxycholic acid (HDCA), a hydrophilic bile acid, prevented the development of dextran sulfate sodium (DSS)-induced colitis in mice. We then examined the individual effects of DSS and HDCA as well as their combined effects on fecal bile acid profile in mice. HDCA treatment increased the levels of most of fecal bile acids, whereas DSS treatment had limited effects on the levels of fecal bile acids. The combined treatment with DSS and HDCA synergistically increased the levels of fecal chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) in feces, which are potent activators of the farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR5). The overall hydrophobicity of fecal bile acids was not modified by any treatments. Our data suggest that the preventive effect of HDCA on DSS-induced colitis in mice is due to the synergism between DSS and HDCA in increasing the levels of the fecal bile acids with potencies to activate FXR and TGR5.

Key words  
chenodeoxycholic acid; deoxycholic acid; farnesoid X receptor; hydrophobicity; Takeda G-protein-coupled receptor 5

INTRODUCTION

Primary bile acids (PBAs) are synthesized from cholesterol in the liver as their conjugated forms and then secreted into the intestinal lumen. Most of the luminal bile acids are absorbed in the ileum and then returned to the liver for their recycling. However, portions of the intestinal conjugated PBAs spill over into the colon and then undergo deconjugation and conversion into secondary bile acids (SBAs) such as deoxycholic acid (DCA) and lithocholic acid (LCA) by gut bacteria. These SBAs as well as chenodeoxycholic acid (CDCA) are highly hydrophobic and possess high potencies to induce mucosal injury by inducing oxidative stress and impairing the tight junctions of intestinal epithelial cells. The overall hydrophobicity of fecal bile acids has also been reported to positively correlate with the severity of experimental colitis induced by dextran sulfate sodium (DSS), an experimental model of inflammatory bowel diseases (IBDs). On the other hand, CDCA, DCA, and LCA have been reported to be the ligands of the farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR5), which can elicit anti-inflammatory responses. Furthermore, recent studies have demonstrated that the activation of FXR or TGR5 attenuates the enhancement of permeability in the colonic epithelial cells during DSS-induced colitis by strengthening tight junction and mucus barrier. Thus, both aggravating and protective roles of intraluminal bile acids are proposed in the pathogenesis of IBD.

Hyodeoxycholic acid (HDCA) is a hydrophilic bile acid and abundant in pig bile. A study indicated that HDCA lowered the hydrophobicity of intestinal bile acids and suppressed the intestinal absorption of cholesterol by inhibiting its micellar solubilization. In addition, HDCA protected cultured intestinal epithelial cells from cytotoxic hydrophobic bile acids. On the other hand, HDCA has been reported to have limited potencies to activate FXR or TGR5 in vitro, although our previous study has showed a possibility that HDCA increases hepatic FXR activity in mice by decreasing the levels of the endogenous FXR antagonist, β-muricholic acid (MCA), in the liver. Thus, we have proposed an indirect pathway for FXR activation by HDCA in the liver; however, we have not examined its effect on bile acid profiles in terms of the modulations of bile acid hydrophobicity and bile acid receptor responses in the colon. In this study, we demonstrated that HDCA prevented DSS-induced colitis in mice. Then, we examined to explore the correlation between the changes in bile acid profiles and the preventive effect of HDCA on DSS-induced colitis. For this purpose, we evaluated the individual effects of HDCA and DSS as well as their combined effects on the profile of fecal bile acids in mice. Furthermore, we determined the potencies of the fecal bile acids whose levels were increased by the combined treatment with DSS and HDCA to activate FXR and TGR5 in vitro. Results of these evaluations suggest that the interaction between DSS and HDCA increase the levels of fecal bile acids and play significant roles in the preventive effect of HDCA on DSS-induced colitis in mice.

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MATERIALS AND METHODS

Animal Treatments We first carried out Experiment 1 to evaluate the effect of HDCA on DSS-induced colitis in mice (Fig. 1a). Forty-five six-week-old female BALB/c mice were purchased from SLC (Shizuoka, Japan) and acclimated for one week to the environment of the animal room under a daily light (08:00–20:00)–dark cycle at room temperature in the range of 23–25°C. A pelleted diet (CE-2, Nippon Clea, Tokyo, Japan) was given ad libitum to the mice throughout the experimental period. After the acclimation period, the mice were divided into three groups, each composed of a number of mice indicated in Fig. 1a. Distilled water was given to the untreated group of mice and 3% (w/w) DSS (MW: 36000–50000, MP Biomedicals, Inc., Tokyo, Japan) in distilled water was given to the other two groups of mice for 7 d. During this period, 0.5% carboxymethylcellulose sodium (CMC-Na) was orally administered at 0.01 mL/g body weight once a day to the mice of the untreated and DSS groups. HDCA (Wako Pure Chemical Corporation, Osaka, Japan) suspended in 0.5% (w/v) CMC-Na was orally administered to the DSS-treated mice at 500 mg/kg: 50 mg/mL of HDCA solution at 0.01 mL/g body weight, which was designated as the DSS/HDCA group. On the following day, the mice were fasted from 08:00 to 13:00 and then sacrificed under anesthesia induced with 3% (w/v) isoflurane in air to obtain the colons, each of which was divided into three portions and used for the assessments of inflammatory responses induced by DSS-induced colitis. Samples of one of the three colon portions were fixed in formalin. Those of the second portion of the colon were frozen in liquid nitrogen for the assessments of myeloperoxidase (MPO) activity. Samples of the third portion were stored at −60°C after fixation with RNAlater (Life Technologies, Carlsbad, CA, U.S.A.) for the estimation of the expression levels of inflammatory cytokine mRNAs. Then Experiment 2 was carried out to examine the individual effects of DSS and HDCA as well as their combined effects on the fecal levels of bile acids. Twenty-four six-week-old Balb/c mice at 6 weeks age mice were divided into four groups composed of six mice as shown in Fig. 1b. The protocols for animal treatments with DSS and HDCA were the identical to those in Experiment 1. On day 3, the mice were housed individually and the feces excreted within following 24 h were harvested. We recognized that the mice treated with DSS excreted wet and bloody feces from day 5 in the Exp. 1, which were inappropriate for the determination of their bile acid levels. Therefore, we harvested the feces obtained on day 4 after DSS treatment and used to determine their bile acid levels. The animal treatments performed in Experiments 1 and 2 were in accordance with the Guidelines for Proper Conduct of Animal Experiments established by the Science Council of Japan (June 1, 2006) (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf) and was approved by the Committee for Animal Care and Experiments of the University of Toyama (Approval No. A2014INM-3).

Assessment of Colitic Symptoms The severity of colitic symptoms was assessed on the basis of the disease activity index (DAI) calculated from the scores for relative body weight loss, stool consistency, and bleeding during the DSS treatment.16) The MPO activity in the colon tissue

Fig. 1. Schedules of Experiments 1 and 2
samples was determined using the methods described by Kim et al. 17) The RNAlater-fixed colon tissues were processed for the determination of the mRNA expression levels of interleukin (IL)-1β and IL-6 by real-time PCR as described previously. 18) 18S ribosomal RNA was used as an external standard to estimate the relative expression levels of the above mRNAs in each sample.

**Determination of Fecal Bile Acid Levels** Dried feces obtained in Experiment 2 were weighed and then pulverized using mortar and pestle. Fecal powder (50 mg) was used to extract bile acids as described previously. 18) The levels of bile acids from the fecal samples were determined by liquid-chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) as described previously. 19)

**Calculation of Hydrophobicity Index of Fecal Bile Acids** The proportions of individual bile acids were calculated as molar factions in the total fecal bile acids, which were multiplied by the hydrophobicity index (HI) of respective bile acids reported by Heuman 20) and then summed to obtain the overall HIs of fecal bile acids.

**Luminescence Reporter Assays of the Activation of Mouse-Derived FXR and TGR5 by Bile Acids** The luciferase reporter assay of FXR transactivation by HDCA, CDCA and DCA was conducted using Hepa 1–6 cells, a mouse hepatoma reporter assay system (Promega) as described in our previous report. 21)

**Results**

**Effect of HDCA on the Development of Colonic Inflammation in DSS-Treated Mice** The activity of MPO, a specific marker of neutrophils, as well as the mRNA expression levels of IL-1β and IL-6 in the colon tissues were much higher in the DSS group than in the untreated group on days 6 and 7 (p<0.01 and p<0.05, respectively). The final body weight of the DSS group was slightly but significantly lower than that of the untreated group (p<0.01, Fig. 2b), whereas that of the DSS/HDCA group tended to be higher than that of the DSS group (p=0.068) and not significantly different from that of the untreated group. The colons from the DSS group were markedly shorter than those from the normal group (p<0.001), but the extent of difference in colon length was attenuated in the DSS/HDCA group (p<0.001) (Fig. 2c).

Mice were treated as shown in the schedule of Experiment 1 and disease activity index (DAI) was assessed daily during DSS treatment (a). On the final day, body weight (b) and colon length were measured (c). Columns and bars indicate mean values and standard error of the mean (S.E.M.), respectively, from 17 mice in the untreated and DSS control groups and 11 mice in the DSS/HDCA group. Statistical analyses were performed by one-way ANOVA followed by the Newman–Keuls multiple comparison test. The statistically significant differences from the DSS control group are shown as p values.
ed infiltration of inflammatory cells into the colonic wall associated with erosion and/or ulceration were observed in the tissue section from the mice in the DSS group. These inflammatory features were attenuated in the tissue sections from the mice in the HDCA/DSS group. The extents of inflammatory cell infiltration into the mucosal layers and mucosal damage in the colon sections were scored and represented as Fig. 3e, which supported the preventive effect of HDCA on the development of inflammatory responses in DSS-induced colitis.

**Effects of DSS and HDCA on Fecal Bile Acid Levels**

**Fig. 3. Effects of Hyodeoxycholic Acid on Colonic Inflammation in Dextran Sulfate Sodium-Treated Mice**

Colonic tissues obtained from the mice on day 8 after DSS treatment were used for the measurements of MPO activity (a), the expression levels of IL-1β (b) and IL-6 mRNAs (c) relative to those of 18s RNA. The typical representatives of photographs of the colon tissue sections from the mice in untreated, DSS and HDCA/DSS groups (d) and histological evaluations of mucosal inflammation/ulceration (e) were shown. Columns and bars indicate mean values and SEM, respectively, from 17 mice in the untreated and DSS control groups and 11 mice in DSS/HDCA groups. Statistical analyses were performed by one-way ANOVA followed by the Newman–Keuls multiple comparison test. The statistically significant differences from the DSS control group are shown as p values.

**Fig. 4. Effects of Hyodeoxycholic Acid and DSS on Fecal Bile Acid Levels**

Feces obtained in Experiment 2 were processed to determine fecal bile acid levels as described in Materials and Methods. Columns and bars indicate mean values and S.E.M., respectively, from six mice in each group. Statistical analysis was performed by two-way ANOVA for the estimation of significance of the effect of HDCA or DSS as well as their combined treatment. The results of two-way ANOVA are shown in the inserted table. Tukey’s multiple comparison test was used to determine the significance of difference in fecal bile acid levels between two groups. When the p value was lower than 0.05, the difference was considered significant and shown with an asterisk (*)
Two-way ANOVA revealed that HDCA treatment induced significant effects on the levels of all the fecal bile acid although DSS treatment significantly changed the levels of limited types of fecal bile acid such as βMCA, HDCA, and LCA (Fig. 4). In addition, significant interactions between HDCA and DSS treatments were observed in the fecal levels of αMCA, βMCA, HDCA, and DCA. In following multiple comparison tests, we confirmed that the levels of all the fecal bile acids except ωMCA, CDCA and LCA were significantly higher in the HDCA group than in the untreated group. The levels of ωMCA and LCA in feces were significantly lower in the HDCA group than in the untreated group. The fecal level of LCA was significantly lower in the DSS group than in the untreated group. The fecal levels of αMCA, βMCA, CA, UDCA and DCA were significantly higher in the HDCA/DSS group than in the DSS group, although the fecal levels of ωMCA and ωMCA were not significantly different between these groups. In addition, the levels of all the bile acids except ωMCA and LCA were not significantly different between these groups. These data indicate that HDCA treatment elevated to greater extents the fecal levels of αMCA, βMCA, HDCA, CDCA and DCA in the presence of DSS than in its absence.

**Effects of DSS and HDCA on Overall Hydrophobicity of Fecal Bile Acids**

There was no significant difference in the overall hydrophobicity of the fecal bile acids among the four experimental groups as determined by two-way ANOVA (Fig. 5).

**Potencies of HDCA to Activate Mouse-Derived FXR and TGR5**

HDCA was shown to be an inert bile acid in activating FXR in a study using the cells expressing human FXR. On the other hand, HDCA was shown to possess a low agonistic activity (EC50 at 31.6µM) in cells expressing human TGR5. However, there has been no report indicating the potencies of HDCA to activate mFXR or mTGR5. We determined that the potencies of HDCA at a high concentration to activate mFXR were much lower than those of CDCA, although DCA exhibited a moderate level of FXR activation (Fig. 6a). In addition, HDCA showed a much lower potency to activate mTGR5 than LCA, although the potency of DCA to activate mTGR5 was slightly lower than that of LCA (Fig. 6b). The potency of HDCA to activate mTGR5 estimated in our system was lower than those in the human system reported earlier.

**DISCUSSION**

Since HDCA is a hydrophilic bile acid, its increase in feces was expected to lower the overall hydrophobicity of fecal bile acids. However, HDCA treatment, unexpectedly, did not change the overall hydrophobicity of fecal bile acids (Fig. 5). Note that HDCA administration increased its molar fraction but decreased those of three types of MCA in the total fecal bile acids. Since MCAs are highly hydrophilic bile acids, their decreases in proportions in the total fecal bile acids increased hydrophobicity of bile acid and mostly countered the decrease in HI due to the increase in the proportion of HDCA. Thus, our data cannot confirm the aggravating role of hydrophobic bile acids, as reported by Stenman et al., in the severity of DSS-induced colitis in mice.

The fecal levels of HDCA and, in addition, those of αMCA, βMCA, CA, UDCA and DCA were significantly higher in the HDCA group than in the untreated group (Fig. 4). These increases might reflect the overflow of small intestinal bile acids into the colon due to the suppression of their intestinal absorption. The estimation of the expression levels of intesti-
nal bile acid transporters would help elucidate the mechanism of the increases in the levels of fecal bile acids induced by HDCA treatment. The decreases in the fecal levels of αMCA and LCA by HDCA might be due to the attenuation of their synthesis from precursor bile acids via gut bacteria.\textsuperscript{22-24} However, DSS treatment lowered the levels of a limited number of fecal bile acids such as LCA (Fig. 4), as similarly observed in HDCA treatment. Since LCA is generated from CDCA or UDCA through secondary metabolism by intestinal bacteria,\textsuperscript{22,23} DSS might change the levels of intestinal bacteria having the abilities to generate LCA. Thus, HDCA and DSS exerted distinct and overlapped actions in the modifications of fecal bile acid levels. In Exp. 2, we harvested the fecal samples on day 4 after the start of treatments (Fig. 1b); no colitic symptom was observed in the mice treated with DSS at this time point (Fig. 2). However, the inflammatory responses upon DSS treatment could alter the fecal bile acid levels even on day 4 after the start of treatments. Therefore, the elucidation of the mechanism of increases in the levels of selected fecal bile acids synergistically induced by HDCA and DSS treatment requires extensive analyses from many viewpoints including intestinal absorption and secondary metabolism of bile acids by gut bacteria as well as the influences of inflammatory responses.

DSS and HDCA synergistically increased the fecal levels of αMCA, βMCA, HDCA, and DCA in mice; we assume that these bile acids play a significant role in the preventive effect of HDCA on DSS-induced colitis in mice. A recent study has shown that deficiency of intraluminal SBAs due to dysbiosis promotes intestinal inflammation in three different mouse models, which was improved by the application of DCA through enema.\textsuperscript{25} Our observations supported the protective effects of the bile acids with a high hydrophobicity such as DCA in a mouse model of DSS-induced colitis. We also confirmed that DCA possesses significant potencies to activate FXR and TGR5 as shown by their reporter assays (Fig. 6). The increase in the level of fecal DCA by HDCA in the DSS-treated mice (Fig. 4) is assumed to contribute to the activation of FXR (Fig. 6) and TGR5,\textsuperscript{14} we cannot exclude the role of CDCA in the preventive effect of HDCA on DSS-induced colitis. Ward \textit{et al.}\textsuperscript{26} and Lajczak-McGinley \textit{et al.}\textsuperscript{27} demonstrated that UDCA, a hydrophilic bile acid, ameliorated DSS-induced colitis in mice. The beneficial effect of UDCA was mediated by LCA generated from UDCA via microbial transformation\textsuperscript{22,23}; LCA is a potent agonist of TGR5.\textsuperscript{14} Our previous study showed that bear bile (BB) rich in UDCA and CDCA prevented the development of DSS-induced colitis in mice, which was associated with the increase in cecal LCA levels.\textsuperscript{6} On the other hand, HDCA did not increase the levels of fecal LCA in DSS-treated mice (Fig. 4), indicating its limited role in the preventive effect of HDCA on DSS-induced colitis. Furthermore, Van den Bossche \textit{et al.} demonstrated the suppressive effects of UDCA and its taurine- or glycine-conjugated species on DSS-induced colitis in mice and their involvement in the improvement of colitogenic dysbiosis.\textsuperscript{28} As shown by the above-mentioned reports, the interactions between the activation of bile acid receptors and the secondary metabolism of bile acids by gut bacteria play crucial roles in the beneficial effects of SBAs on DSS-induced colitis.

Our reporter assays using the cells expressing \textit{mFXR} or \textit{mTGR5} revealed that HDCA at the concentration range tested showed only limited potencies to activate these receptors compared with those of CDCA or LCA, respectively (Fig. 6). The cecal concentration of total unconjugated bile acids in human is reported to be close to 0.4 mM.\textsuperscript{29} Considering this observation, the concentration of HDCA in the colonic lumen of the mice co-treated with DSS and HDCA is assumed to be higher than the concentration rage of HDCA used in the reporter assays for \textit{mFXR} and \textit{mTGR5} (Fig. 6), Therefore, HDCA in the colon from the mice co-treated with DSS and HDCA could exert a protective effect on DSS-induced colitis. HDCA and DSS synergistically increased the levels of αMCA and βMCA in feces; however, the levels of these bile acids were much smaller than that of HDCA in the feces from the mice co-treated with DSS and HDCA (Fig. 4). In addition, we previously reported that αMCA and βMCA are inert ligands for mouse FXR.\textsuperscript{30} Thus, we cannot assume the significant roles of αMCA and βMCA in the preventive effect of HDCA on DSS-induced colitis.

Our data showed for the first time the preventive effect of HDCA on DSS-induced colitis in mice. In addition, we found the interaction between DSS and HDCA in increasing the fecal levels of DCA, which was proposed as the most probable bile acid responsible for the preventive effect of HDCA on DSS-induced colitis in mice. HDCA may also contribute to the preventive effect of HDCA on DSS-induced colitis. However, the fecal levels of DCA and HDCA were concomitantly increased by HDCA in the DSS-treated mice (Fig. 4). Therefore, we should further study to discriminate the roles of HDCA and DCA in the preventive effect by HDCA on IBD by means of pharmacological agents or other experimental models. In addition, further studies using other approaches are necessary to elucidate the mechanism of the interaction between DSS and HDCA in increasing the levels of selected types of bile acid in feces. In addition, we should determine whether HDCA is effective for different types of IBD model. These approaches are helpful for evaluating the potential of HDCA as a novel therapeutic agent for IBDs.

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\textbf{Conflict of Interest} The authors declare no conflict of interest.

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