Bile Acid Transformation in Gnotobiotic Mice Associated with Human Intestinal Flora

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Bile acids are transformed by intestinal microflora. Secondary bile acids including deoxycholic acid and lithocholic acid appear to show a strong correlation with colorectal cancer. However, the role of intestinal bacteria in the transformation of bile acids *in vivo* is still not clear. We attempted to evaluate which bacterial groups are responsible for bile acid transformation *in vivo*. First, we orally inoculated germfree (GF) mice with human intestinal bacteria that have the ability to transform bile acids *in vitro*, and showed that it is not necessarily true that bacterial transforming ability of bile acids *in vitro* is reflected *in vivo*. Then GF mice were orally inoculated with human fecal dilution or various components of human feces. In the cecal contents of ex-GF mice associated with human fecal dilutions of 10^{-2} or 10^{-6} , or the anaerobic growth from a dilution of 10^{-6} , free-form bile acids accounted for more than 80% and DCA for about 20% of total bile acid. When GF mice were associated only with clostridia, free-form bile acids made up less than 40% of total bile acids, but the percentage of secondary bile acids was the same as in the other groups. These results indicate that predominant bacteria—mainly bacteroides in human feces, which are the main bacterial group for deconjugation of bile acids—and clostridia may play an important role in 7α -dehydroxylation of free-form primary bile acids in the intestine. The combination of five strains of bile acid-deconjugating bacteroides and five strains of bile acid 7α -dehydroxylating clostridia isolated from ex-GF mice converted tauro-conjugated cholic acid into DCA both *in vitro* and in the intestine. This GB mouse model should be useful in studies of bile acid metabolism by human intestinal bacteria *in vivo*.

Key words: cecal bile acids; ex-germfree mice; gnotobiotic mice; human intestinal bacteria

INTRODUCTION

In humans and animals, intestinal flora are indispensable for bile acid transformation. Bile acids in humans are synthesized from cholesterol in the liver and conjugated to either glycine or taurine. They are secreted into the small intestine as a component of bile and more than 90% of the bile acids are reabsorbed mostly in the ileum and returned to the liver via the portal blood. The rest of them enter the large intestine and the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are deconjugated and 7α -dehydroxylated to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. There are many reports that implicate the correlation between colorectal cancer and secondary bile acids *in vitro* (34, 48) and in animal studies (35, 39, 40). In epidemiological studies, it is reported that fecal 7α -dehydroxylase activity in colorectal cancer patients was significantly higher than that in controls

(30). It is essential to understand which bacterial groups are responsible for bile acid metabolism *in vivo* in order to control bile acid transformation in the intestine.

Deconjugating activity of conjugated bile acids is widely observed in many kinds of intestinal bacteria (1, 31, 44), especially in the predominant intestinal bacteria. 7α -dehydroxylating activity has also been reported in certain groups of intestinal bacteria (5). However, the results for some of the species are contradictory. Some reports indicate that bacterial strains of *Bifidobacterium* (3), *Bacteroides* (3) and *Lactobacillus* (13, 32) showed 7α -dehydroxylating activity. In contrast, other groups showed negative results for the activity of *Bifidobacterium* strains (11, 45), *Bacteroides* strains (16, 42), or *Lactobacillus* strains (7, 45). Only certain strains belonging to *Eubacterium* and *Clostridium* have been specifically reported (2, 10, 15, 19, 43). Many reports indicated that the difficulties of isolating 7α -dehydroxylating intestinal bacteria arise from the small populations of these bacteria in the intestine (18, 47). However, most of the primary bile acids in the intestine are deconjugated and subsequently transformed into secondary bile acids. The precise

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mechanism of transformation of bile acids by intestinal bacteria in the intestine is still unknown.

Although we inoculated human intestinal bacteria with the ability to transform bile acids *in vitro* into germfree (GF) mice, only a small amount of secondary bile acids was observed in their cecal contents. These data indicate that bacterial transforming ability of bile acids *in vitro* is not necessarily reflected *in vivo* (37, 38). Therefore we produced various groups of ex-GF mice associated with human fecal dilution, with anaerobic growth from a dilution of 10^{-6} , or with only human fecal clostridia (36). The aim of our study was to clarify which bacterial groups harbored in the human intestine are responsible for bile acid transformation in the intestine, and also to produce a good gnotobiotic (GB) mouse model for the analysis of bile acid metabolism by intestinal bacteria *in vivo*.

BILE ACID TRANSFORMATION BY HUMAN INTESTINAL BACTERIA *IN VITRO* AND *IN VIVO* —GNOTOBIOTIC STUDY—

There are several reports of establishment of gnotobiotic mice or rats and evaluation of secondary bile acids in their intestines (6, 24, 27–29). However, in most studies bacteria of animal origin were used, and it is still unclear if bacteria isolated from the human intestine act in the same way as those from animals *in vivo*. We orally inoculated human intestinal bacteria for which the ability to transform bile acids was confirmed by *in vitro* screening, and evaluated cecal bile acid composition in the intestine.

In the *in vitro* assay, *Clostridium ramosum* strain R-18 deconjugated taurocholic acid (TCA) to CA, but did not dehydroxylate or dehydrogenate CA. *Eubacterium lentum*-like c-25 did not deconjugate TCA, but transformed CA to DCA (30%) and 7-oxo-deoxycholic acid (7=O DCA) (10%). Unidentified Gram-positive rod strain HD-17 (now *Clostridium hiranonis*) converted TCA to CA and DCA, and dehydroxylated CA to DCA. *Clostridium* sp. strain M-7 also deconjugated TCA to CA to some extent, but only dehydrogenated CA to 7=O DCA.

Three weeks after inoculation of the bacteria into GF mice, all the strains colonized in the intestine at greater than 10^8 CFU. The predominant bile acids in GF mouse ceca were CA and β -muricholic acid (MCA), and all bile acids were taurine-conjugated in the intestine. Free-form bile acids were detected in the cecal contents of gnotobiotic mice associated with deconjugating bacteria, *C. ramosum* R-18 (above 10%) or *Clostridium* sp. M-7 (3.6%). *E. lentum*-like c-25 did not form free bile

acids. GB mice associated with strain R-18 formed both unconjugated CA and MCA, while in the cecal contents of GB mice associated with strain M-7, almost all unconjugated forms were in the CA group. The deconjugating activity of bacteria varied in accordance with both bacterial strains and bile acid structures as reported by Chikai et al. (6).

However, GB mice showed the ability to deconjugate only 15% of bile acids. The results were similar to those of previous reports (7, 14) and the rate of deconjugation was lower than that of Kayahara et al. (24), which was more than 30%. Dickinson et al. (7) indicated that deconjugation *in vitro* did not reflect the potential of deconjugation of microorganisms growing in the intestine. The correspondence of deconjugating activity *in vitro* and *in vivo* should be clarified by investigating more bacterial strains.

DCA was observed only in the cecal contents of gnotobiotic mice associated with a combination of deconjugating and 7 α -dehydroxylating bacteria, i.e. strain R-18 and *Eubacterium lentum* like c-25 (4.3%) or a combination of strain R-18 and strain HD-17 (1.1%). 7=O DCA was detected in the cecal contents of gnotobiotic mice associated with strain M-7 (1.3%) or strain R-18 plus strain M-7 (2.4%). All secondary bile acids detected were in the unconjugated form, suggesting that secondary bile acids may be mainly formed from unconjugated primary bile acid in the intestine, which is consistent with the *in vitro* study (4). However, the percentage of DCA in total bile acid was very low (Fig. 1).

In another of our reports, no secondary bile acid was detected in the intestine of GB mice monoassociated with either *Eubacterium* sp. strain 36S (now *Clostridium scindens* 36S (25), which possesses dehydroxylating activity but no deconjugating activity for taurine-conjugated bile acids *in vitro* (47), nor *Clostridium* sp. strain TO-931 (now *Clostridium hiranonis* (26), which has both deconjugating activity for taurine- or glycine-conjugated cholic acid and high dehydroxylating activity for bile acids (8). Although all of the strains used were colonized in the intestine of the GB mice, no DCA was detected in any of the intestinal contents of the GB mouse group.

There are several reasons for the inconsistency of bile acid transformation by intestinal bacteria *in vitro* and *in vivo*. Intestinal conditions such as pH of the intestinal contents or normal ability of the mucosal epithelium of the intestine may be important factors in transformation of bile acids by bacteria. Koopman et al. (28, 29) compared bile acid compositions in various

Fig. 1. Bile acid composition in the cecal contents of gnotobiotic mice (Ref. 37).

kinds of GB mice and evaluated normalization using a number of parameters including fecal bile acids. They reported that very complex intestinal microflora was needed to normalize germfree mice. Itoh et al. (22) revealed that a combination of bacteroides, lactobacilli, and clostridia was necessary to convert the intestinal characteristics of GF mice to the normal state. In our study, cecal sizes of the GB mice were more than 5% of total body weight, far from the normal state.

Another possible reason for the discrepancy in bacterial bile acid transformation *in vitro* and *in vivo* is that enhancement of the transformation of microflora in the intestine required cooperative action by different bacteria. Hirano and Masuda (17) investigated the transforming ability of strain c-25, which is enhanced not only by bacteroides in mixed culture, but also by addition to the medium of cell extracts from bacteroides. Takamine and Imamura reported that *Bacteroides distasonis* strain K-5 has no ability to deconjugate conjugated cholic acid or to dehydroxylate, but this strain can stimulate 7β -dehydroxylation by other bacteria (46). Although strain K-5 did not stimulate 7α -dehydroxylation by other bacteria *in vivo* in our previous study (38), the possibility of cooperative action by different bacteria in the transformation ability of microflora in the intestine still can not be excluded (50).

In conclusion, the results of these studies indicate that factors other than bacterial transformation of bile acids, such as the intestinal conditions of host animals and cooperation from other bacteria reported by Eysen

and Robben (9), are required to establish a gnotobiotic model presenting secondary bile acids in the intestine.

COMPOSITION OF CECAL BILE ACIDS IN EX-GERMFREE MICE INOCULATED WITH HUMAN INTESTINAL BACTERIA

GF ICR female mice were orally inoculated with various suspensions or components of human fecal microflora. Samples were obtained from a 47-year-old healthy male donor. In these fecal samples, 99.6% of the bile acids were deconjugated, and 96% of the unconjugated bile acids were transformed to secondary bile acids. Fresh feces (1 g) was immediately placed in 9 ml of an anaerobic diluent under oxygen-free CO_2 gas and a portion was transferred to an anaerobic chamber and serially diluted with anaerobic broth. The 10^{-2} and 10^{-6} diluted fecal suspensions were prepared for inoculation into GF mice to produce ex-GF mice of group A and group B, respectively. Another 0.1 ml of 10^{-6} diluted solution was inoculated on Eggerth Gagnon (EG) agar plates (33) in an anaerobic chamber and incubated at 37°C for 48 hrs. A further 0.1 ml of 10^{-6} solution was inoculated on mEG agar (Nissui, Japan) plates and incubated in an anaerobic steel wool jar filled with 100% CO_2 gas at 37°C for 48 hrs. From all colonies which developed on each plate, cells were scratched and suspended in anaerobic broth for group C and group D. The remaining portion of the 10^{-1} dilution of human feces was further diluted to 10^{-2} and treated with chloroform according to the method of Itoh and Mitsuoka

Fig. 2. Production of ex-germfree mice and gnotobiotic mice.

(21) for group E. Two weeks after the inoculation, fecal bile acid composition in ex-GF mice of groups A to E was examined. From the feces of ex-GF mice of groups B and E in which DCA was detected, we prepared further groups of bacterial combinations. For ex-GF group F and G, we prepared a mixed fecal suspension of B (10^{-2} and 10^{-6} , respectively) together with E (10^{-2}). Feces of group B mice were diluted to 10^{-8} and inoculated on EG agar plates. The colonies that developed were isolated, purified and subcultured. Then the predominant bacterial suspension was inoculated into GF mice for group I. From the feces of ex-GF mice of group E, we also obtained the predominant bacterial suspension for group J. Clostridia from feces of ex-GF group B were also inoculated into GF mice as group H (Fig. 2).

Among the ex-GF mice in groups A, B, C, and D, bacteroidaceae were detected at the highest levels, and eubacteria and clostridia were also detected in high numbers. Among aerobic bacteria, only enterobacteriaceae and streptococci were detected in the feces of the four ex-GF groups, and staphylococci were detected only in the feces of group A mice. In feces of group E mice, which were inoculated with chloroform-treated fecal suspension, only clostridia were observed.

In the cecal contents of SPF mice, 44.1% of MCA

was transformed into ω -MCA, but transformation to ω -MCA did not occur in the cecal contents of any group of ex-GF or GB mice associated with human fecal bacteria. This result is consistent with that of Sacquet et al. (41), indicating that human intestinal bacteria have no ability to transform α - or β -MCA to ω -MCA. It appears that human intestinal bacteria react differently, especially to the CDCA group, from murine intestinal bacteria.

In groups A to D, the deconjugating ratio was 80–90% of total bile acids. However, in group E, in which intestinal bacteria consisted of only clostridia, the percentage of unconjugated bile acids was significantly lower than those in groups A to D ($p < 0.05$). The conversion ratios from cholic acid to DCA and from CDCA to LCA were significantly higher in SPF mice than that in ex-GF mice. Although the difference was not statistically significant, the cecal contents of ex-GF mice in group E tended to show a slightly higher conversion ratio from cholic acid to DCA than those in groups of A, B, C and D (Fig. 3).

DCA was observed in the cecal contents of ex-GF mice in groups of A, B, C, D and E. The percentage of DCA in total bile acids was about 20%, and no significant difference was detected among the 5 groups (Fig. 4). These results indicate that a 10^{-6} fecal dilution of

Fig. 3. Bile acid composition in the cecal contents of ex-germfree mice associated with various components of human intestinal flora (Ref. 36).

Fig. 4. Deconjugation and conversion ratio of total bile acids in the cecal contents of ex-germfree mice (Ref. 36).

human feces can transform bile acids into the secondary form in the intestines of mice. In the human intestine, the bacterial count reaches the order of 10^{11} CFU per gram. It was suggested that the count of 7α -dehydroxylating bacteria in the human feces of this

sample may be at least 10^6 CFU per g feces, which is a little higher than those in previous reports (12, 43). Takamine and Imamura (47) reported that 7α -dehydroxylation was detected in dilutions of human feces below 10^{-5} . In the ex-GF A, B, C and D groups,

Fig. 5. Bile acid composition in the cecal contents of ex-germfree mice and gnotobiotic mice.

with fecal bacteria comprising mainly anaerobic bacteria, including bacteroidaceae, eubacteria and clostridia, about 90% of the cecal bile acids were deconjugated. In contrast, in the cecal contents of ex-GF mice of group E, only 40% of total bile acids were deconjugated. These results indicate that deconjugation of cecal bile acid mainly depends on bacteria belonging to bacteroidaceae or eubacteria, as reported in *in vitro* experiments (6, 23, 31, 44), and that bile acid transforming bacteria are culturable on EG agar plates and do not require extreme anaerobic conditions.

In mice and rats, clostridia (fusiform-shaped bacteria) are one of the key bacterial groups for the normalization of GF animals (22, 27). Recently, Uchida et al. (49) reported that clostridia isolated from rat cecum play an important role in the formation of secondary bile acids in rats. In the cecal contents of ex-GF mice associated with chloroform-treated human fecal suspension, DCA was detected at a level equivalent to that of SPF mice, even though the deconjugating ratio was significantly lower than that in other ex-GF mice. It is assumed that clostridia transformed almost all unconjugated cholic acid to the secondary form. It is strongly suspected from the results of our study that clostridia also exert an effect on the formation of secondary bile acids in human intestines.

In the cecal contents of ex-GF mice associated with a combination of feces from B and E, total and secondary bile acids exhibited higher concentration than those of other ex-GF mice (group F and group G). Fecal flora

in these mice consisted of large numbers (10^{11} CFU/g feces) of bacteroidaceae, followed by clostridia (10^{10} CFU/g feces) and eubacteria (10^9 CFU/g feces). However, DCA was not detected in the cecal contents of ex-GF mice in group H, which were inoculated with clostridia from ex-GF mice group B. No DCA was detected in the cecal contents of GB mice in groups I and J. They were inoculated with predominant bacteria isolated from ex-GF mice in groups B or E, respectively, in which a considerable amount of DCA was detected in the cecal contents (Fig. 5). One of the reasons for the absence of DCA in groups I and J is that 7α -dehydroxylating bacteria are not present in large numbers in the intestine of group B or E, and bacterial combinations isolated from a 10^{-8} fecal dilution of a group B or E mouse may not have 7α -dehydroxylating activity. Another possible explanation for the absence of DCA in the cecal contents of these mice may be the lower percentage of deconjugated bile acids in group I or Batta et al. (4) reported that prior deconjugation of bile acids is required for further bacterial 7α -dehydroxylation *in vitro*. It was suggested that a large amount of free-form cholic acid is necessary for the concomitant 7α -dehydroxylation.

In only a few years, two new species with bile acid 7α -dehydroxylating activity were phylogenetically proposed in the genus *Clostridium* (25, 26). *Clostridium hiranonis* includes strain HD-17 and TO-931, both of which have high bile acid 7α -dehydroxylation activity (8), while *Clostridium hylemonae* has relatively low

7 α -dehydroxylation activity. Furthermore, the strain formerly called *Eubacterium* sp. VPI 12708 (52), which is known for its high 7 α -dehydroxylation activity *in vitro* and the most precise report of the multistep bile acid 7 α -dehydroxylation pathway (20) together with strain TO-931 (51), was recognized as a member of *Clostridium scindens* (25). These studies strongly support the hypothesis that clostridial strains are responsible for secondary bile acid formation in the human intestine.

In summary, our results indicate that bacteroides in human feces are the main bacterial group for deconjugation of bile acids, and clostridia may play an important role in 7 α -dehydroxylation of free-form primary bile acids in the intestine.

PRODUCTION OF GNOTOBIOTE MICE ASSOCIATED WITH HUMAN INTESTINAL BACTERIA

Ex-GF mouse intestinal microflora associated with a human fecal dilution of 10⁻⁶ (group B) showed high deconjugating ability for taurine-conjugated bile acids. We inoculated the feces of these ex-GF mice onto NBGT selective agar plates and isolated 24 strains of bacteria considered to be members of genus *Bacteroides*. Among these strains, we selected 5 strains with high deconjugating activity for TCA *in vitro*. The deconjugating ability of TCA *in vivo* was maintained at high levels when GF mice were associated with these strains. In contrast, selected clostridia from the feces of ex-GF mice group B deconjugated only 10% of total bile acids.

We also isolated clostridial strains with 7 α -dehydroxylation activity for primary bile acids from ex-GF mice associated with human intestinal clostridia (ex-GF mice group E) according to the method of Takamine and Imamura (47) with minor modifications. One loopful of 10-fold diluted mouse feces from ex-GF mice group E was directly streaked onto EG agar plates. Finally, we isolated 5 strains of clostridia which showed 7 α -dehydroxylation activity for CA.

Germfree mice associated with five strains of bile acid-deconjugating bacteroides and five strains of bile acid 7 α -dehydroxylating clostridia converted TCA into DCA in their intestine, and the transforming activity reflected the results *in vitro*.

CONCLUSION

Gnotobiotic techniques make it possible to simplify the complicated ecosystem in the intestine, and to understand the interaction between defined bacteria and the host animal, although we should be aware that there

are obvious differences between humans and the animal models. To our knowledge, this is the first report of a gnotobiotic animal model associated with human intestinal bacteria that can convert TCA to DCA. These gnotobiotic mice could be useful in studies of bile acid metabolism by human intestinal bacteria *in vivo*, and may provide clues concerning the regulation of metabolism by controlling these bacteria in the human intestine.

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