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学 位 論 文 題 名

Bile acid is a host factor that regulates the composition and metabolism of rat cecal microbiota

（胆汁酸は宿主因子としてラット盲腸内細菌叢の組成および代謝を制御している）

学位論文内容の要旨

The complex microbial community (Gut Microbiota) colonizing throughout human gastrointestinal tract contributes a lot to a multiple set of functions. Recently, the idea that gut microbiota influences host health has become popular. It has been argued that an imbalanced bacterial population affects the development of metabolic diseases. Consequently, much emphasis has been put on assessing changes in the gut microbiota associated with high-fat diets and/or diseases and on how such reshaped gut microbiota mediates the development of metabolic diseases. However, the most important questions about why and how high-fat diets and/or diseases induce changes in the bacterial population remain to be elucidated. This seems to be due to a relative lack of knowledge about the effect of host factors on gut microbiota formation *in vivo*.

In this context, we hypothesized that the host factor; bile acids, the main component of bile, might be a good candidates that have not yet been well characterized. By virtue of their detergent effect bile acids are bactericidal and thus seem to exert strong selective pressure on the gut microbiota. Therefore, to examine the possibility that the gut microbiota population is controlled by bile acids *in vivo*, rats were fed a basal diet or a cholic acid (CA; a primary bile acid) supplemented diet, and their cecal microbiota were analyzed by 16S rRNA gene clone library sequencing and fluorescence in situ hybridization (FISH). The composition of fecal bile acids and cecal organic acids were analyzed, as were some host responses. The overall results revealed remarkable effects of CA on both the gut microbiota population and their metabolism.

1. Effect of cholic acid supplemented diet on microbiota composition; a clone library approach. Cecal microbiota composition following administration of cholic acid supplemented diet was investigated in 3 groups (namely, control group, M-CA group and H-CA group fed basal, 1.25 mmol CA/kg supplemented and 5 mmol CA/kg supplemented diet, respectively) of male WKAH/HkmSlc rats. Analysis of the 16S rRNA gene clone library sequences revealed striking alterations in the bacterial populations of both the M-CA and H-CA groups. The control microbiota was dominated by Firmicutes (54.1%) and Bacteroidetes (30.7%), together with minority populations such as Proteobacteria (6.1%) and Actinobacteria (2.6%). In contrast, in the CA-fed groups, Firmicutes expanded significantly, to approximately 95% of the total clones, at the expense of Bacteroidetes and Actinobacteria. The increase in Firmicutes resulted from expansion of the Clostridia class in particular, as well as the Erysipelotrichi class. Within these two classes, the genus *Blautia* and the genus *Allobaculum* dominated, and accounted for approximately 60% and 15% of the total clones, respectively. In Proteobacteria, the class Gammaproteobacteria expanded with increasing CA concentration, and the remaining classes nearly disappeared in the CA groups. Analysis of OTUs (Operational Taxonomic Unit; using 97% identity as a cutoff) identified 123 OTUs and revealed that specific bacteria at the genus and species levels were highly concentrated in the CA groups. These bacteria seemed to be highly

resistant to bile acid. The diversity of the OTUs in the M-CA and H-CA groups, expressed as Shannon indices (2.936 and 3.591, respectively), was lower than that of the control group (5.852). In particular, the changes in the gut microbiota were similar to those observed in mouse models fed high-fat diets.

2. Impact of cholic acid on gut microbiota composition as revealed by DNA staining and Fluorescence in-situ hybridization (FISH). Because biases are inherent in all molecular methods employed for microbial ecology studies, we used DNA staining by DAPI and FISH technologies to further confirm the observed alterations in the bacterial populations. DAPI staining revealed higher variety of morphologies; from cocci to long rods in control group, whereas the CA-fed groups showed much less variety and primarily displayed cocci and short rods. Total bacterial counts were also significantly decreased with increasing dietary CA concentration indicating alteration of bacterial community. In FISH technology, we deployed Eub338, Erec482 and Bac719 probes to detect total bacteria and major bacterial groups in Firmicutes and Bacteroidetes, respectively. FISH results revealed that although Bac719-positive cells remained detectable in the CA groups, these populations were significantly smaller than those in the control group and decreased significantly as the dietary CA concentration increased. Simultaneously, a significant lower total count by Eub338 but a significantly higher proportion of Firmicutes in CA fed groups was also observed. Overall, the results obtained by FISH agreed well with those obtained using the clone library method, and thus we conclude that CA in the diet increases the relative abundance of Firmicutes and decreases that of Bacteroidetes in the rat cecal microbiota.

3. Impact of Cholic acid administration on rat cecal fermentation and host's parameters. To study the overall effect of cholic acid on host, some host's parameters were also monitored. Though other parameters weren't affected much, cholic acid significantly affected epididymal adipose tissue weight, serum cholesterol and adiponectin in one or both of the CA-fed groups. Fecal bile acid analysis clearly indicated that CA in the diet was extensively transformed into DCA (Deoxycholic acid) by bacterial 7α-dehydroxylation reaction. The average cecal DCA concentration (0.07 mM) in the control group increased as high as 2.55 mM in a CA group suggesting them high enough to exert great environmental stress upon the cecal microbiota. Consistently, the total bile acid concentration for the M-CA and H-CA groups increased 6-fold and 20-fold, respectively, compared to the control group. As expected, bile acid stress affected the fermentative metabolism of the gut microbiota. The concentrations of short-chain fatty acids (SCFAs) decreased as the CA concentration in the diet increased. Among the SCFAs, acetate and butyrate showed marked and significant reductions. In response to these changes, the pH of the cecal contents became significantly higher in the H-CA group. These changes suggested that the metabolic activities of the gut microbiota are repressed and altered as a result of the antimicrobial activity of DCA.

In conclusion, using simple rat model, for the first time we have clearly demonstrated the hitherto unexplored role of bile acid as a host factor that controls the gut microbiota population and their metabolism *in vivo*. Moreover, bile acid appeared to affect host health by modulating the secretion of adiponectin, which contributes to the pathophysiology of obesity-linked diseases. We deeply believe that our study findings not only open a new door to the better understanding of the host factors regulating gut microbiota but also providing a new insight into the understanding of the metabolic diseases and their pathophysiology in relation to gut microbiota composition.

学位論文審査の要旨

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本論文は英文179頁，図22，表12，10章からなり，参考論文1編が付されている．

ヒトの腸内細菌叢には様々な機能が報告されてきた．近年，特に宿主の健康と腸内細菌叢の関連が注目されており，高脂肪食の摂取による腸内細菌叢バランスの変動と肥満を含む様々な代謝病やメタボリックシンドロームの発症との因果関係が疑われている．現在，高脂肪食の摂取や代謝疾患による腸内細菌叢の変化，また，変化した腸内細菌叢による代謝疾患誘発の機構について，活発な研究が行われている．しかし，高脂肪食摂取や代謝疾患が，なぜ，どのようにして腸内細菌叢を変化させるのか，その機構については全く報告がない．これは腸内細菌叢を制御する宿主側の因子についての知識が乏しいことに原因がある．本研究はこの点を明らかにすべく，宿主因子の有力な候補として胆汁の主成分である胆汁酸を想定し，腸内細菌叢の構成に及ぼす胆汁酸の影響を検討したものである．胆汁酸は強力な界面活性作用により食品中の脂質の分解と吸収を補助しているが，一方で腸内細菌の細胞膜を損傷するため，強力な殺菌作用がある．従って，胆汁酸は腸内細菌に対して強力な環境ストレス因子となっている可能性があるが，実際に胆汁酸が腸内細菌叢の組成を制御しているか否かについては不明である．

そこで，ヒトやラットの代表的な胆汁酸であるコール酸（CA）を基本食に添加した食餌を，雄 WKAH/HkmSlc ラットに10日間摂取させ，基本食群に対して盲腸内細菌叢の組成，盲腸内有機酸発酵，胆汁酸代謝，さらにはいくつかの宿主の生理的マーカーがどのように変化するか，を調べた．CA 摂取群は基本食に 1.25 mmol/kg の CA を添加した中濃度 CA 摂取群（M-CA 群），5 mmol/kg の CA を添加した高濃度 CA 摂取群（H-CA 群）の2群とした．

1)　ラット盲腸内発酵に及ぼすコール酸摂取の影響

M-CA 群および H-CA 群の生育，食餌摂取量，体重増加量等は基本食群と差がなかったが，特に H-CA 群で，脂肪組織の重量および血中 adiponectin 濃度が基本食群に比べて有意に低下した．H-CA 群では盲腸内有機酸濃度は基本食群の約半分に低下した．糞中胆汁酸濃度の測定により，投与された CA は主としてデオキシコー

ル酸（DCA）に変換されたことが示され，計算による盲腸内 DCA 推定濃度は，基本食群の 0.07 mM に対し，M-CA 群では 0.98 mM，H-CA 群では 2.55 mM に増大した．DCA は CA に比べて抗菌活性が 10 倍程強く，種々の腸内細菌に対する生育阻害実験において，1mM で強い阻害が報告されている．従って，CA 摂取群では DCA が盲腸内細菌叢に強い生育阻害を及ぼし，有機酸発酵を抑制していることが考えられた．

2) ラット盲腸内細菌叢の組成に及ぼすコール酸摂取の影響

16S rRNA 遺伝子クローンライブラリーの配列決定により，各群の盲腸内細菌叢を調べたところ，M-CA 群及び H-CA 群に門レベルでの大きな菌叢の再編成が検出された．基本食群では Firmicutes (全体のクローン数の 54.1%)と Bacteroidetes (同 30.7%)を中心とする組成であったものが，CA 摂取群では Bacteroidetes が消失し，ほぼ Firmicutes のみからなる菌叢構成に変化した．Firmicutes の中でもクラスレベルで見ると，CA 摂取群では特に Clostridia が増大し（属レベルで見ると *Blautia* 属が全体のクローン数の約 60%まで増大），次いで Erysipelotrichi が増大した（属レベル見ると *Allobaculum* 属が同約 15%まで増大）．このように CA 摂食群では *Blautia* 属と *Allobaculum* 属だけで，全体のクローン数の約 75%を占めていた．16S rRNA 遺伝子の同一性が 97%以上を同じグループ（operational taxonomic unit; OUT）と見なしてグルーピングすると，全体で 117 の OTU が検出され，CA 摂取群には属や種レベルで特定の OTU が高度に濃縮されており，OTU の多様性が低下していた．また，確認のため fluorescence *in situ* hybridization (FISH)法によって門レベルでの菌叢変動を調べたが，16S rRNA 遺伝子クローンライブラリー法とほぼ同様な結果が得られた．以上の結果から，CA はラット盲腸内細菌叢において，Firmicutes/Bacteroidetes 比を増大させることが明らかになった．このような変化の直接の原因は，抗菌活性の強力な DCA の濃度上昇であると考えられたため，盲腸内容物から培養可能な細菌を分離し，それらの DCA 感受性を比較した．その結果，取得された範囲では，Firmicutes に属する OTU の分離株は Bacteroidetes に属する OTU の分離株よりも高い DCA 耐性を示した．このことから，Firmicutes/Bacteroidetes 比の増大は，DCA 感受性の差によることが示唆された．

以上，本研究では，CA が宿主因子としてラット盲腸内細菌叢の構成および発酵代謝を制御することを明らかにし，それが CA から生成された DCA の抗菌活性によることを示唆した．また，CA 摂取が adiponectin の分泌や脂肪細胞の重量等，宿主の生理に影響することも示した．胆汁酸の抗菌活性については，分離菌株を用いた生育実験の報告は多数あるが，菌叢全体の構成に与える影響は報告されていない．本論文はこの点を明らかにした点で重要であり，さらに腸内細菌叢とメタボリックシンドローム発症の関係を理解する上でも大きく貢献するものと考えられる．

よって審査員一同は，K. B. M. Saiful Islam が博士（農学）の学位を受けるのに十分な資格を有するものと認めた．