Graduate School of Advanced Science and Engineering
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博 士 論 文 概 要
Doctoral Thesis Synopsis

論 文 題 目
Thesis Theme

Exploring the intestinal bacteria involved in the regulation of farnesoid X receptor

Farnesoid X receptor の制御に関わる腸内細菌の探索

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Since the discovery of farnesoid X receptor (FXR) as a transcriptional sensor of bile acids, FXR has displayed a key role in regulation of bile acid homeostasis, cholesterol, glucose, lipid metabolism, hepatic regeneration, inflammation response. Therefore, FXR is a potential drug therapeutic target for a number of metabolic disorders. Up to now, many synthetic or natural extracts have been turned out to be FXR ligands or modulators, which have shown proofs in regulation of FXR relevant diseases. However, the underlying FXR function mechanism is still complicated. Indeed, many apparent discrepancies or even complete opposites often appeared due to the varied experimental methods. Consequently, FXR activation may control metabolic pathways though disparate modulations. FXR modulation has discrepant effects on certain metabolic pathways. The uses of FXR agonists for clinic treatment are still under estimate. The development of tissue-specific or gene-selective FXR modulators which can be easily absorbed after oral administration, may provide the appropriate applications to determine the physiological benefit of FXR modulators, and elucidate the complex FXR action pathways.

It is widely realized gut bacteria protect against obesity and insulin resistance, attenuate inflammation and restore colon homeostasis. Mouse studies indicated that there is a connection between gut microbiota and FXR function. Bile acid levels were reduced in the gallbladder and small intestine in the presence of gut microbiota compared to germ free mouse. Intestinal microbiome regulates bile acid homeostasis by altering bile acids composition resulting in FXR activation in intestine and liver. In addition, a recent study has shown a functional FXR activity is necessary for the probiotic VSL#3 to exert its activity on bile acid excretion and neo-synthesis in mouse. Asking how intestinal microbiome affects relevant diseases via FXR activation, the work would be easier by using individual bacterial strains due to complexity of whole gut microbiome.

Thus, our goal is to identify individual bacterial strains or their products which may act as FXR modulators. Meanwhile, by screening FXR stimulating bacteria, better understanding in FXR action pathways and mechanism of bacteria-host crosstalk can be expected.

In chapter 1, the background and previous studies related with this study were summarized, and the purpose of this study was described.

In chapter 2, in order to screen FXR modulators, a stable FXR reporter gene system was obtained. First, a FXR expression vector EX-T0601-M02 was transfected into human colorectal carcinoma cell line SW480. Then, a DNA fragment containing four copies of the FXR element (FXRE) from the phospholipid transfer protein promoter was ligated into a pGL4.27 vector to form pGL4-4×FXRE-*luc* vector. Finally, it was further transfected into SW480 FXR expression cells to construct FXR reporter cell line under G418 and hygromycin B selection. Luciferase activity of each colony was determined by administration of GW4064 as a FXR synthetic agonist. The stable FXR reporter cell colony was picked up based on high fluorescence and low background. Then, candidates for FXR reporter cells were exposed to different concentrations of GW4064 for characterizing agonist dose-response. The results showed that the level of FXR reporter activity increased in a dose-dependent manner. Furthermore, this study determined whether stimulation with FXR agonist is able to transactivate the FXR target genes in the FXR reporter cells. The results suggested that GW4064 induced the mRNA expression

of ileal bile acid binding protein (IBABP), organic solute transporter α (OSTα) and fibroblast growth factor 19 (FGF19) in a dose-dependent manner. Taken together, the FXR reporter system was successfully obtained.

In chapter 3, by using the FXR reporter system constructed in chapter 2, a total of 38 bacterial strains derived from the intestine or dairy foods were evaluated to check whether they can be modulators for FXR activation. In order to understand the FXR activity inducement by different parts of bacteria, bacteria samples in the case of intact bacteria, mechanical disrupted bacteria, heat-killed bacteria and bacterial culture supernatants were assessed by luciferase assay. The results presented that some bacterial cell forms slightly induced FXR activity less than two-fold compared to control group which only DMEM medium was added. Culture supernatants of *Bacteroides dorei* and *Eubacterium limosum* can intensely stimulate FXR activity.

To further determine if the culture supernatants of *B. dorei* and *E. limosum* intervene in bile acids homeostasis, expression levels of FXR target genes including IBABP, OSTα and FGF19 mRNA, were examined by real-time PCR in stable FXR reporter system. Culture supernatants of *B.dorei* and *E.limosum* at 10% level significantly induced FXR target gene IBABP and OSTα mRNA expression. But neither of these two culture supernatants affected FGF19 mRNA expression. These results reveled that both of two bacterial culture supernatants selectively modulated expression of FXR target genes involved in bile acids metabolism in stable FXR reporter cells.

Activation of FXR by its ligands plays an important role in inflammatory processes as well. Before investigation of two bacterial culture supernatants, the anti-inflammatory effect of GW4064 was confirmed in the FXR reporter system. GW4064 repressed tumor necrosis factors α (TNFα) induced inflammatory cytokine interleukin 8 (IL8) mRNA expression at 2 μM. However, neither *B.dorei* nor *E.limosun* deduced TNFα induced IL8 expression when pretreated with culture supernatants for 18 hours. However, IL8 expression decreased when given lower concentration of bacterial culture supernatants, which implied that endotoxin contributing to IL8 expression may exist in the supernatants.

In chapter 4, to investigate whether two FXR-stimulatory bacteria confer the anti-obesity effect, the bacterial culture supernatants of *B. dorei* or *E. limosum* were administrated to high fat diet (HFD)-fed mice by the intragastric gavage for 11 weeks. The results showed HFD dramatically elevated mice body weight compared to standard diet (STD). Since 6 weeks of *B.dorei* derived culture supernatant administration, the mice showed lower body weight compared with mice that received PBS only, indicating that *B.dorei* cultural metabolites may help mice to be resistant to the body weight gain. However, the mice that received *E.limosum* did not show any changes in body weight. In the meantime, HFD feeding mice displayed increased liver weight compared to STD control group. *E.limosum* administration reduced mice liver weight, while *B.dorei* feeding mice showed a little lower liver weight without significant difference.

Serum biochemical analysis gave the results that increased activities of liver function markers, including serum alanine aminotransferase (ALT), cholesterol and glucose, indicating pathological changes in HFD feeding mice. The levels of ALT and aspartate aminotransferase (AST) were down-regulated when mice received *B.dorei* derived culture supernatant for 11 weeks. On the other hand, the mice received *E.limosum* derived

culture supernatant reduced levels of ALT, cholesterol and triglycerides. Thus, the results revealed that two bacterial metabolites might be effective in suppressing the development of HFD-induced fatty liver diseases.

To investigate the role of two bacterial culture supernatants on bile acids homeostasis in diet induced obesity mouse model, total RNA were obtained from the ileum, colon and liver for real-time PCR analysis. Upon HFD administration, the expression of *Shp* mRNA was up-regulated in the ileum, whereas other genes were not affected. When mice were fed with *B.dorei* or *E.limosum* derived culture supernatants, the levels of *Fxr* increased by 1.6-fold and 2.1-fold, respectively, which confirmed the *in vitro* findings. Transporter *Ibat* was up-regulated with 1.9-fold and 2.5-fold. In addition, *E.limosum* down-regulated target gene *Shp* expression in the ileum. Gene *Fgf15*, *Ibabp* or *Ostα* were unaffected by two bacteria culture supernatants. These results revealed gene-selective regulation of two bacterial culture supernatants in the ileum.

In the colon, feeding of HFD did not provide significant impact on *Fxr* activity, while level of *Shp* was up-regulated by 6-fold, *Ibabp* and *Fgf15* were reduced with 2.7-fold and 5-fold, respectively. However, the differences of three gene expression levels were not significant, due to the big error. The culture supernatants derived from *B.dorei* or *E.limosum* did not affect the expression of *Fxr* and its target genes in the colon.

In the liver, HFD administration induced significant increase of gene *Shp* and *Bsep,* while *Fxr* level was not affected. However, *Fxr* expression was elevated when mice were fed with two bacteria derived culture supernatants, which showed increase of 1.3-fold and 1.4 fold, respectively. The mice fed with *E. limosum* derived culture supernatant displayed significant *Shp* reduction and increased *Ntcp* expression. On the other hand, *B. dorei* culture supernatant did not regulate *Fxr* target genes involved in bile acids metabolism in the liver.

Feeding mice with a HFD always induce impaired liver damage in the liver. With hematoxylin and eosin (HE) stain, marked fat accumulation and histology were observed when mice were fed with HFD, which was characterized by macrovesicular steatosis with large and small fat droplet, mixed inflammatory cells infiltration and hepatocyte ballooning. Treatment with *B.dorei* derived culture supernatants slightly alleviated the severity of hepatic steatosis, while *E.limosum* treatment did not enhance liver steatosis. Picrosirius red stain was used to observe collagen in liver as well. More collagen was found around and diffusing along the vein when mice received a HFD. However, two bacterial culture supernatants did not alleviate the hepatic fibrosis.

To sum up in chapter 5, this study for the first time discovered culture supernatants of *B.dorei* and *E.limosum* can be FXR direct modulators by using a stable FXR reporter system. The *in vivo* assessment revealed the bile acid regulation by two bacteria is both gene- and tissue- specific. The findings of this study expand our current knowledge of FXR modulators and bile acids metabolism alteration by bacteria in HFD fed mice. They may provide a new direction to clarify both FXR action pathway and molecular mechanisms of microbe-host interactions. Probiotics are currently used as therapeutic options for many diseases, and thus *B.dorei* and *E.limosum* could be applied as a therapy for bile acids disorders through intestinal specific activation.

早稲田大学　博士（工学）　学位申請　研究業績書

(List of research achievements for application of doctorate (Dr. of Engineering), Waseda University)

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2	Xianqin Zhang, Toshifumi Osaka, Satoshi Tsuneda. The interaction between host and commensal bacteria through the farnesoid X receptor. Japanese Society for Bacteriology. 2015.03. Gifu, Japan. (Poster presentation)