Prevention of hepatic fibrosis by probiotics in mice

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Hepatic fibrosis is the final destination of all types of chronic liver injuries including chronic viral hepatitis, alcoholic liver diseases, non-alcoholic fatty liver diseases, and autoimmune hepatitis. It may lead to irreversible cirrhosis with portal hypertension, resulting in development of gastroesophageal varices, ascites, and hepatic encephalopathy, unless intervened at early stages.\(^1\)

Hepatic fibrosis may occur predominantly in portal or periportal areas (e.g., chronic viral hepatitis) or central areas (e.g., alcoholic liver disease) according to their etiologies. Although relative distribution of extracellular matrix (ECM) within the hepatic lobules varies according to the type of the insults, hepatic fibrosis is characterized by increased deposition and altered composition of ECM components such as collagens I, III, and IV. It is well known that hepatic stellate cells (HSCs, also known as lipocytes, fat-storing, or Ito cells) are central to the process of hepatic fibrogenesis as they are the main source of ECM proteins.\(^2\) In the normal liver, HSCs produce large quantities of cytokines such as prostanoids (prostaglandin (PG) F\(_{2\alpha}\), PGD2, PGI2, PGE2; leukotriene(LT)-C4, LT-B4), leukocyte mediators (macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1), platelet-activating factor (PAF)), acute phase components (\(\alpha_2\)-macroglobulin, interleukin-6), mitogens (hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), stem cell factor (SCF), insulin-like growth factor (IGF)-I and II, \(\alpha\)-fibroblast growth factor (\(\alpha\)FGF)), adhesion molecules (I-CAM-1, V-CAM-1, N-CAM), vasoactive mediators (endothelin-1 (ET-1), nitric oxide (NO)), fibrogenic compounds (transforming growth factor (TGF)-\(\beta\)1, 2, 3, and connective tissue growth factor (CTGF). HSCs also have other major functions such as expression of membrane receptors, cell matrix synthesis and degradation, and regulation of hepatic sinusoidal blood flow owing to their contractility. In particular, HSCs play a critical role in hepatic fibrogenesis during chronic liver injury by transforming into proliferating, fibrogenic myofibroblast-like cells. Once activated, they typically express \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) and over-produce collagens, leading to hepatic fibrosis.\(^3,4\)

Hepatic fibrogenesis and activation of hepatic stellate cells (HSCs), the main fibrogenic cell type in the liver, almost exclusively occur in an inflammatory environment. Proinflammatory signaling...
pathways contribute to fibrogenesis by activating survival pathways in HSCs and by promoting the recruitment of leukocytes. LPS is one of the strongest known inducers of inflammation and an important contributor to hepatic injury and inflammation. It has recently shown that lipopolysaccharide (LPS) and its receptor toll-like receptor 4 (TLR4) are required for hepatic fibrogenesis and that HSCs are highly responsive to LPS. Hepatic fibrosis is associated with elevated portal and systemic levels of LPS in patients as well as in mouse models. Elevations of LPS are caused by abnormalities in the intestinal mucosal structure, intestinal motility as well as changes in the bacterial flora and subsequent increases in bacterial translocation. A significant proportion of patients with hepatic fibrosis display increased levels of lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria, that is among the strongest inducer for proinflammatory signaling cascades. The increased levels of LPS are due to abnormalities in the coordinated motor function of the small bowel and the resulting decreased intestinal motility, intestinal overgrowth, and the subsequent bacterial translocation. Previous studies demonstrated that gut-derived LPS and its receptor Toll-like receptor 4 (TLR4) are required for hepatic fibrogenesis. Taken together, HSCs, the main fibrogenic cell type of the injured liver, express abundant TLR4 on the cell surface and are, therefore, highly responsive to LPS, leading to liver fibrogenesis.

On the other hand, probiotics have shown the beneficial effects for irritable bowel disease, ulcerative colitis of inflammatory bowel diseases, and even some liver diseases such as non-alcoholic fatty liver diseases, alcoholic liver diseases, and complications of decompensated liver cirrhosis. In addition to the effect of reduction in intraintestinal inflammation through preventing the overgrowth of Gram negative bacteria competitively, probiotic bacteria may not only repress the bacterial translocation of pathogenic ones but also suppress the LPS movement to extraintestinal tissues. Theoretically, liver fibrogenesis can be in part prevented by probiotic treatment. To date, there is no study about the effects of probiotics in the early stage of liver fibrogenesis.

Based on the previous data that showed a crucial role for TLR4 and its signal LPS in early hepatic fibrogenesis, it was hypothesized that probiotics are more effective in preventing hepatic fibrosis than treating complications of established fibrosis. In the present study, it was investigated whether probiotics may prevent hepatic inflammation by preventing bacterial translocation before the onset and at early time points of fibrogenesis. This study also investigated probiotics as a potential treatment option for liver fibrosis. If so, probiotics will be a feasible and safe treatment option in patients.

Male Balb/c mice and C57BL/6 (8 weeks old from the Jackson Laboratory; Bar Harbor, ME, USA.) were housed in a specific pathogen-free, climate-controlled animal facility under a 12-hour light-dark cycle. Ten mice were used for each group. After a week of acclimatization, hepatic fibrogenesis was induced by ligation of the common bile duct (BDL). Control mice underwent sham operation. Briefly, mice were anesthesized with xylazine and ketamine. After midline laparotomy, the common bile duct was ligated twice with 4-0 silk and transected between the two ligations. Sham operations were performed similarly with the exception of ligating and transecting the bile duct. Ten
mice were used for each condition and blood and liver samples were obtained. Mice were sacrificed 5 days, 14 days, or 21 days after BDL. As probiotic drugs, VSL#3 was used, which was composed of eight Gram positive bacteria; *Bifidobacterium longum Y10, Bifidobacterium infantis Y1, Bifidobacterium breve Y8, Streptococcus salivarius* subsp. *Thermophilus MB455, Lactobacillus plantarum MB452, Lactobacillus delbrueckii* subsp. *Bulgarcus MB453, Lactobacillus acidophilus MB443*, and *Lactobacillus casei MB451; 9×10^{11} colony-forming units (=2.7g of lyophilized bacteria) per sachet (VSL Pharmaceuticals Inc., Towson, MD, USA.); four species of *Lactobacilli*: 3.1% (wt/wt) of *L. plantarum*, 7.3% of *L. acidophilus*, 16% of *L. casei*, and 8.4% of *L. delbrueckii* subsp. *Bulgarcus*; three species of *Bifidobacteria: B. infantis, B. breve*, and *B. longum*, representing 17.7% of the mixture; and 47.5% of *Streptococcus salivarius* subsp. *Thermofilus*. The placebo control was composed of cornstarch (2.7 g per sachet) but did not differ in taste. One sachet of VSL#3 (450 billion colonies/packet) was dissolved in 1L of autoclaved distilled water and provided to mice instead of drinking water freshly made daily.

Mice will be fed the probiotics dissolved in sterile distilled water freshly made daily without any restrictions, starting 15 days before and during the induction of experimental fibrosis until they were sacrificed. To determine whether antibiotic pretreatment enhances intestinal colonization, additional groups of mice received a cocktail of non-absorbable oral antibiotics consisting of neomycin (1 g/L), ampicillin (1 g/L), metronidazole (1 g/L) and vancomycin (0.5 g/L) in drinking water for 1 week followed by treatment with probiotics or no treatment.

Intestinal colonization was analyzed by cecal extraction. The effects of probiotics on hepatic inflammation and hepatic fibrosis were determined at each time point after BDL. Haematoxylin and eosin (H&E)-stained sections was used for histologic diagnosis, and picrosirius-stained sections for morphometric analysis. Liver samples were also fresh frozen for isolation of mRNA, western blot analysis, and measurement of hydroxyproline. One liver sample can be used to measure all these parameters.

Hepatic inflammation was detected by histologic evidence of infiltration in H&E sections, serum ALT levels, and by quantitative real time PCR for markers of ongoing inflammation. Electrophoresis of protein extracts and blotting were performed as described previously. The blots were incubated with primary antibody to α-SMA (Abcam plc., Cambridge, MA, USA) at a dilution of 1:5,000. The ratio was expressed as a fold over β-actin. The intensity of the bands was assessed using ImageJ 1.39u (NIH, Bethesda, MD, USA).

Probiotic used, VSL#3, was confirmed to have all 8 bacteriae on PCR using specific primers. Real-time PCR revealed that mRNA expression of both proinflammation (F4/80) and HSC activation (α-SMA) was reduced significantly in the mice group treated with probiotics, compared to the group treated with vehicle alone. Additionally, H&E staining showed the similar benefits on hepatic inflammation. In the respects of final products by fibrosis, there was a high variability of range in results among species, mice, and liver lobes. Accordingly, a portion of mice or some liver lobes of each mouse tended to show a preventive effect of probiotics on fibrosis at early fibrogenesis.
evaluated by Sirius red staining, Western blotting, and hydroxyproline assay, though statistically insignificant because of high variability.

Hepatic fibrogenesis is regarded as a type of repair process, leading to an accumulation of extracellular matrix including collagens. In the liver, HSCs play a critical role in the pathogenesis of hepatic fibrosis as they are major sources of ECM proteins. HSCs are activated by paracrine stimuli from injured hepatocytes, Kupffer cells, endothelial cells, and platelets. They subsequently produce abundant ECMs through the autocrine or paracrine actions of fibrogenic cytokines including TGF-β1, which is the most fibrogenic cytokine. Any injury, such as hepatotropic viral infection or due to reactive oxygen species, may induce fibrogenesis in the liver through direct or indirect mechanisms. After injury or inflammation around the hepatocytes in the liver, HSCs undergo phenotypic changes and convert into myofibroblast-like cells, a process known as "activation". Although various injuries may cause liver damage by different pathways, TLR4-signaling pathway, the interaction between pathogen-associated molecular pattern (PAMP) and TLR4-receptor, is regarded to be one of the most important ones. It means that PAMP such as LPS is considered one of the strongest stimuli for liver fibrogenesis.

In conclusion, it is probable that probiotics actively administered at early stage of liver fibrogenesis may suppress the overgrowth of pathogens in the intestine competitively. Subsequently, TLR4-induced fibrogenic signaling in HSCs may remain under inactivation. Therefore, probiotics may be used as an effective or at least a very safe therapeutic option for liver fibrosis.

REFERENCES