Reintroduction of gut microbiota improves liver regeneration in germ-free mice

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Abstract. The gut microbiota has been implicated in liver regeneration, evident by delayed liver regeneration in mice with sterile gut (germ-free mice) and mice subjected to antibiotic treatment. Here we investigated the involvement of gut microbiota in liver regeneration by observing liver regeneration process in germ-free mice which have been reconstituted with normal gut microbiota (ex-germ-free mice). Ex-germ-free (XGF), germ-free (GF) and normal wild-type (WT), C57BL/6 mice underwent two-thirds partial hepatectomy (PHx). Early regenerative response of the liver was assessed by cytokine and growth factor tissue concentration and gene expression at 3-hours post PHx, while liver mass restoration was assessed by liver/body weight ratio (LW/BW) ratio at 72-hour post PHx. At 3-hours post PHx, GF mice demonstrated significantly lower concentrations of IL6R and lower expressions cytokine receptors *Il6ra* and *Tnfrsf1a,* and growth factor *Hgf* genes compared to control mice. Signifying reduced early regenerative response in the remnant liver of GF mice. Similarly, LW/BW ratio and normalised liver growth percentage of GF mice were significantly lower compared to control mice at 72-hours post PHx, signifying reduced liver mass restoration. Conversely, early regenerative response and liver mass restoration indices in XGF mice were similar to that of controls, with no significant differences observed. Therefore, signifying normal liver regeneration in XGF mice. The study demonstrated that reconstitution of normal gut microbiota in GF mice ameliorates liver regeneration following partial hepatectomy in GF mice, evident by normal liver mass restoration and early cytokine and growth factor response in XGF mice. Taken together, our findings indicate that gut microbiota is involved in the early regenerative response of the liver and that reconstitution of gut microbiota may improve liver regeneration.

Keyword: Gut microbiota, Liver regeneration, Germ-free mice

Introduction

The gut microbiome is known to affect the pathophysiology of the liver via the gut-liver-axis[1]. Changes in the gut microbial composition has been implicated in various liver diseases, including non-alcoholic fatty liver disease[2], alcoholic liver disease[3], primary sclerosing cholangitis[4], and hepatocellular carcinoma[5]. Furthermore, gut microbiota has been shown to affect the regenerative capability of the liver. Liver regeneration is delayed in mice with sterile gut (germ-free mice) and mice subjected to antibiotic treatment[6]. Recent studies have demonstrated positive effects of reconstitution of gut microbiota in the treatment of liver diseases associated with gut microbiota dysbiosis. Reconstitution of normal gut microbiota normalises gut microbial dysbiosis associated with these conditions, leading to improved disease outcome[7].

As recent studies have demonstrated positive effects of reconstitution of gut microbiota in treating various liver diseases, we would like to investigate the effect of reconstitution of gut microbiota on liver regeneration. Germ-free mice were reconstituted with normal gut microbiota and liver regeneration was assessed following 70% partial hepatectomy. Liver mass restoration and hepatocyte proliferation was assessed by liver weight/body weight (LW/BW) ratio, while the

regenerative response was assessed by tissue concentrations and expression of tumour necrosis factor (TNF), interleukin-6 (IL6), and hepatocyte growth factor (HGF) together with their respective receptors, TNF receptor-1 (TNFR1) and IL6 receptor (IL6R).

Hypothesis and objectives

We hypothesise that reconstitution of gut microbiota in sterile mice will improve liver regeneration mice following partial hepatectomy (PHx) in the germ-free mice.

Therefore, the objectives of the study were (1) to investigate the regenerative process in germ-free mice that have been reconstituted with normal gut microbiota (ex-germ-free mice) and (2) to investigate whether reconstitution of normal gut microbiota improves liver regeneration by comparing liver regeneration between ex-germ-free, germ-free and control mice.

Material and methods

Male mice, 8-10 weeks old were used in the study. Germ-free mice were reconstituted with normal gut microbiota by co-habitation with wild-type (WT) mice. Since mice are coprophagic, transfer of gut microbiota occurs via the faecal-oral route. Germ-free mice were also included in the study, while WT, C57BL/6 mice were used as controls. Number of animals per group = 3-4.

Liver mass restoration was assessed by liver weight to body weight (LW/BW) ratio and normalised liver growth percentage at 72 hours after PHx. Early regenerative response was assessed by cytokine and growth factor tissue concentration and gene expression levels at 3 hours after PHx.

Partial hepatectomy and sampling of tissues

Mice underwent two-thirds PHx with the removal of right medial, left medial, and left lateral lobes which constitute 70% percent of the total liver volume[8]. Control mice underwent gentle manipulation of the exposed liver lobes.

Sample of the remnant liver was done at 0, 3, and 72 hours following initial surgery.

Liver weight/body weight ratio (LW/BW)

Liver mass restoration was estimated by the animal's LW/BW ratio. The regenerating liver was removed en bloc, and the liver weight was measured. The LW/BW ratio was calculated as follows:
LW/BW ratio (%) = 100 × (regenerating liver weight/body weight).

Protein assay

Protein extraction was done using the Tissue Protein Extraction Reagent (T-PER) (Thermo Scientific, MA, USA) according to manufacturer's protocol. For protein assay, the Mouse Premixed Multi-Analyte Kit (R&D Systems, Inc. USA) was used, according to manufacturer's protocol, and the Luminex MAGPIX Analyzer was used to read the microparticle beads.

RNA-sequencing

RNA extraction was done using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. RNA integrity number (RIN) was evaluated using an Agilent 2100 Bioanalyzer (Agilent, CA, USA), and samples with RIN >7 were selected for sequencing.

Library preparation was done using TruSeq RNA Sample Preparation Kit (Illumina, CA, USA). Sequencing was performed using Illumina HiSeq 2500 system (Illumina, CA, USA).

Statistical analysis

All results were expressed as mean values ± standard deviation (SD). Data were analysed using Mann-Whitney and Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's multiple comparisons tests, using GraphPad Prism version 5.00 for Windows. (GraphPad Software, San Diego California USA). P-value of <0.05 was considered statistically significant.

Results and discussions

Liver mass restoration and hepatocyte proliferation is reduced in germ-free mice but not in ex-germ-free mice

LW/BW ratio in GF mice was significantly lower compared to WT mice at both 0-hour (p= 0.0436) and 72-hours post PHx (p=0.0422). Normalised liver growth by percentage calculated from LW/BW ratio demonstrated significantly lower liver growth of GF compared to WT mice (p=0.0107) at 72-hours after hepatectomy. Although Lower LW/BW ratio at both 0-hour and 72-hours post PHx, may indicate GF mice to be phenotypically smaller than normal WT mice, the lower percentage of liver growth in GF mice suggests a reduced liver regeneration.

LW/BW ratio and liver growth percentage of XGF mice were similar to control WT mice, therefore suggesting XGF mice to be phenotypically similar to WT mice, while also demonstrating normal liver regeneration.

Delayed liver regenerative response in germ-free mice but not in ex-germ-free mice

At 3-hours post PHx, GF mice demonstrated significant lower tissue concentration levels of IL6R (p=0.0338) compared to WT mice. While statistically not significant, concentration levels of the other proteins including TNF, TNFR1, IL6 and HGF were also lower in GF compared to WT mice.

Expression of the respective encoding genes showed that expression of *Tnfrsf1a* (p=0.0006), *Il6ra* (p=0.0006), and *Hgf* (p=0.0047) genes were significantly lower in GF compared to WT mice. The lower protein concentration and gene expression levels at 3-hours post PHx suggest a reduced regenerative response in GF mice.

Tissue protein concentration and gene expression levels in XGF were similar to WT controls. No significant differences between the groups were detected. Suggesting normal regenerative response in XGF mice.

Clustering of mouse groups by gene expression levels showed that XGF and WT mice have similar expression patterns of early cytokines and growth factors involved in liver regeneration. Expression pattern of GF mice was distinct to that of the XGF and WT mice.

Conclusions

The reduced liver mass restoration and early cytokine responses in GF mice confirms the role of gut microbiota in liver regeneration. Normal indices of liver regeneration in XGF mice suggests that reconstitution of gut microbiota improves liver regeneration, hence, further confirming the involvement of gut microbiota in the regenerative process of the liver.

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