Liver cirrhosis and hepatocellular carcinoma (HCC) represent the endstage of most chronic liver diseases and are a major global health burden. It has been consistently shown that both liver cirrhosis and HCC are triggered by inflammatory processes, but the molecular mechanisms linking chronic hepatitis with cirrhosis and HCC are only poorly understood. Recent studies suggested that the intestinal microflora as a main source of portal-vein LPS might play a critical role in this process. Here we summarize the available literature on the role of the gut microbiome in hepatofibrogenesis and -carcinogenesis. Such knowledge might help to develop novel, innovative strategies for the prevention and therapy of liver disease.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary carcinoma of the liver.1 Up to 85% of cases arise in chronically inflamed and subsequently cirrhotic livers. Drug abuse, uptake of liver toxins (e.g., Aflatoxin B1), alcohol consumption, autoimmunity, or infections with Hepatitis B- (HBV) and Hepatitis C-virus (HCV) are major risk-factors for hepatocarcinogenesis.2,3 In the last years, the epidemic spread of HBV and HCV has led to more than 500 million people chronically infected, which caused a strong rise in HCC incidence. At present, HCC represent the fifth most common cancer in men and the seventh in women.2,3 Of note, the global distribution of HCC incidence and death is far from being homogeneous: in some African or Asian countries HCC is the most common cause for cancer related morbidity.2,3 In the past, 85% of patients were reported from developing countries with highest prevalence of HBV and HCV. However, in the last two decades the rise in other pathogenic entities like obesity and fatty liver disease, which are much more frequent in high income countries, has led to an increase in HCC frequency also in these regions.4-6

In the last years advances in supportive treatment modalities have led to a continuous improvement in prognosis for patients with liver cirrhosis. However, in the meantime mortality rates for HCC even increased and, at present the incidence of HCC still nearly equals its mortality rate.7,9 Curation can only be achieved for a small subgroup of about 25% of HCC-patients, whose tumors are resectable or suitable for loco regional ablation or liver transplantation at the time-point of diagnosis.10 For these patients, five-year survival rates of up to 70% are achievable.11-13 However, for patients with more advanced stage of disease treatment options are still limited. Classical anti-cancer agents have been demonstrated to be ineffective in a large number of clinical trials and for most patients with cirrhosis-related liver dysfunction this treatment is related to unacceptable toxicity.10 In this setting the introduction of small inhibitor molecules such as Sorafenib represented a major breakthrough. Sorafenib inhibits pro-tumorigenic as well as pro-angiogenic and receptor tyrosine kinases. Encouraging preclinical results and a large phase 2 trial including 137 patients with advanced HCC led to a multi-center trial with a randomized, placebo-controlled design.7 This phase 3 Sorafenib HCC Assessment Randomized Protocol (SHARP) trial demonstrated a 31% decrease in risk of death with a median survival of 10.7 months for the sorafenib arm vs. 7.9 months for placebo.10 After sorafenib, various substances have been tested for their potential as therapeutic agents in HCC- therapy. However, all of these new therapeutics do not provide curation and new approaches in systemic therapy are needed.14

As outlined above, it has been well established that chronic inflammation and fibrosis precedes hepatocarcinogenesis. In patients with viral hepatitis, it was shown that an antiviral treatment against Hepatitis B and Hepatitis C might be beneficial to prevent chronic inflammation and development of HCC.15,16 Although these specific anti-inflammatory treatment might be an option to prevent HCC development in a subgroup of cirrhosis patients, it is presently not possible to prevent the development of primary liver cancer in the large majority of patients with hepatic cirrhosis.14 Of note, deeper insights into basic molecular mechanisms linking inflammation with fibrosis and cancer in the liver represent a prerequisite for the development of a chemopreventive strategy against HCC in patients with chronic liver diseases.14

Chronic liver diseases are associated with an increased translocation of intestinal bacteria and, subsequently, with...
increased frequency of bacterial infections in these patients. Spontaneous bacterial peritonitis (SBP), pneumonia, and urinary tract infections are the most frequent infections in cirrhotics.\textsuperscript{17}\textsuperscript{20} Impairment of mucosal immunity and barrier function as well as a systemic immune deficiency\textsuperscript{20}\textsuperscript{21} represent major risk factors for bacterial infections in patients with chronic hepatic diseases. In line with these findings, 40% of cirrhotic rats with ascites and 80% of cirrhotic rats with SBP displayed a bacterial translocation into their mesenteric lymph nodes.\textsuperscript{22} Interestingly bacterial strains isolated from mesenteric lymph nodes were shown to be genetically identical to those isolated from ascites of rats with SBP in the same animal, demonstrating a causal relationship between bacterial translocation and infectious complications.\textsuperscript{20}

In line with these experimental studies, Cirera and colleagues as well as other groups found enteric organisms in mesenteric lymph nodes of approximately 10% of human cirrhosis patients.\textsuperscript{17}\textsuperscript{23} Interestingly, similar to the results from mice, a higher frequency of bacterial translocation was also found in patients with more advanced disease stage (30.8% in patients with a Child-Pugh score C)\textsuperscript{17} when compared with patients with earlier disease stages.\textsuperscript{17}\textsuperscript{23}

In addition to translocation of viable microorganisms, translocation of bacterial components termed pathogen-associated molecular patterns (PAMPs) have been demonstrated in different rodent models of liver disease as well as in patients with chronic liver diseases. In a recently published collective of 169 patients with chronic hepatic disease, elevated levels of LPS were found in 27%, 85%, and 41% of patients with chronic hepatitis, chronic hepatitis with acute exacerbation, and cirrhosis, respectively.\textsuperscript{24} Moreover, plasma LPS concentrations correlated with the degree of liver dysfunction according to Child-Pugh scores, highlighting that the progression of hepatic failure represents the driving force behind the development of endotoxemia in patients with chronic liver diseases.\textsuperscript{24}

The Role of Gut Microbiota in Development of Liver Cirrhosis

Although inflammation is a common feature of chronic hepatic diseases and correlates with the development and progression to fibrosis and cancer, the molecular link between these different steps of chronic liver injury have remained poorly understood. Inflammatory stimuli via translocated PAMPs such as LPS might represent a potential cellular link in this setting. In a recent publication, Seki and colleagues demonstrated that the intestinal microflora as the main source for portal LPS represent an important prerequisite for the development of liver fibrosis during chronic liver injury.\textsuperscript{25} Mice treated with a cocktail of antibiotics demonstrated an impaired increase in plasma LPS levels after undergoing the bile duct ligation (BDL) procedure and were protected from developing liver fibrosis and/or cirrhosis, when compared with mice with an intact intestinal microflora. In addition, livers of these mice showed an impaired infiltration with macrophages, suggesting that intestinally derived LPS crucially influences immunological processes involved in the development of liver fibrosis.

Toll-like receptors (TLRs) represent a conserved family of receptors that are involved in recognition of PAMPs and act as receptors for LPS, which is among the strongest known inducers of inflammation.\textsuperscript{26} As mentioned above, during the course of chronic liver injury, LPS levels increase both in the systemic and in the portal circulation. Within the liver, it is well known that Kupffer cells express TLR4 and might thus be a target for LPS. Moreover, it was recently shown that activated hepatic stellate cells representing the source for collagen production during liver fibrosis express TLR4 and are highly responsive to even low concentrations of LPS, providing a potential cellular link between intestinal derived LPS and liver fibrosis.\textsuperscript{25}\textsuperscript{27} In the above mentioned study, Seki and colleagues recently demonstrated that TLR4-mutant mice displayed significantly less fibrosis after BDL-surgery, when compared with wild type mice. Interestingly, this effect could be attributed to the knockout of TLR4 in HSC, while chimeric mice with wild-type HSC but TLR4-mutant bone marrow showed a normal fibrogenesis in response to BDL. On a molecular level, LPS lead to an activation of NF-KB downstream of TLR4 in quiescent HSC and to a subsequent downregulation of the TGF-β pseudoreceptor Bambi. This in turn was associated with an increased activation of these cells in response to inflammatory stimuli such as LPS and finally the development of liver fibrosis. Of note, the hypothesis that gut-derived LPS is an important mediator of hepatic fibrogenesis is further supported by a recent study demonstrating a reduction in hepatic fibrogenesis in LPS-binding protein deficient mice.\textsuperscript{28} In line to these data from murine models, it was shown in humans that certain TLR4-SNPs significantly reduced risk for fibrosis progression in patients with chronic hepatitis C virus infection.\textsuperscript{29}

In summary these data suggest that the rise of portal and systemic LPS levels in the course of chronic liver diseases are associated with a TLR4/TGF-β mediated activation of HSC and contributes to the excessive secretion and deposition of collagens and other extracellular matrix proteins and leading to the development of liver cirrhosis. Given the current lack of chemopreventive agents and the strong implication of the gut microbiota in hepatofibrogenesis, there is a growing rationale to explore the therapeutic benefit of modulating the gut microbiota in patients with a high risk for the development of liver fibrosis. However, before considering such strategies further investigation are required to reveal how individual components of the intestinal microbiota are involved in the pathophysiology of this disease.

The Role of Gut Microbiota in the Development of Hepatocellular Carcinoma

Experimental data from rodent models as well as robust clinical data suggest a role of inflammation in the pathophysiology of HCC development. The liver is constantly exposed to microbial products from the enteric microflora, such as endotoxin, which activates proinflammatory signaling.
pathways and might contribute to the development of liver cancer as it has been previously demonstrated for liver cirrhosis.\textsuperscript{25} Recently, murine model have suggested a link between intestinal colonization by H. hepaticus and the induction of HCC.\textsuperscript{30,31,32} Huang et al. reported the presence of Helicobacter ssp. DNA in liver biopsies from patients with HCC while being virtually absent from control samples.\textsuperscript{33} On the other hand, Rocha et al. confirmed an association between the presence of Helicobacter ssp DNA and cirrhosis, but not a correlation between the presence of Helicobacter ssp DNA and HCC.\textsuperscript{34} In contrast, we recently failed to confirm a correlation between the presence of helicobacter DNA in patients’ stool and development of HCC in patients with viral hepatitis.\textsuperscript{35} Therefore, further studies on a possible link between intestinal microbiota and HCC in humans are warranted.

In an experimental setting, Yu et al. demonstrated that depletion of host microflora suppresses tumor formation in a toxic model of hepatocarcinogenesis. Treatment of rats with polymyxin B and neomycin, which are bactericidal for most enteric gram-negative organisms, from 4 days prior to DEN administration until 21 days after injection significantly reduced the number and size of HCC nodules.\textsuperscript{36} These data were corroborated by findings of Dapito and colleagues demonstrating in a recent publication that gut sterilization protects from development of liver cancer when mice were subjected to a combination of diethylaminoethylamine (DEN) and hepatotoxin carbon tetrachloride (CCl₄), a model that features several characteristics of the cirrhotic environment of chronically injured livers in which human HCC mostly arises, namely chronic injury, inflammation, fibrogenesis, and increased of endotoxin levels.\textsuperscript{37} The antibiotic treatment led to a reduction of tumor number and size compared with mice that did not receive antibiotics. Moreover, mice that were grown in specific germ free conditions demonstrated fewer and smaller tumors in this model compared with mice that were grown under non-germ free (SPF) conditions.\textsuperscript{37}

Patients with liver cirrhosis and liver cancer frequently develop an intestinal dysbiosis which is also found in mice after DEN-administration, which show an increase growth rate of the E. coli and Atopobium cluster, while the percentages of benign bacteria (Lactobacillus group, Bifidobacterium group, and Enterococcus group) are significantly decreased.\textsuperscript{38} Interestingly, Zhang et al. demonstrated that already the induction of such a dysbiosis is sufficient to promote hepatocarcinogenesis by enhanced portal LPS levels. This finding was supported by the fact that treatment with probiotics on the one hand dramatically mitigated enteric dysbacteriosis and hepatic inflammation and, on the other hand also decreased liver tumor growth.\textsuperscript{38} Interestingly, in the above mentioned study of Dapito and colleagues, chronic treatment of mice with a low, nontoxic dose of LPS during DEN/CCl₄-induced hepatocarcinogenesis led to a significant increase in tumor number, tumor size, and liver and/or body weight ratio compared with control animals, thus further arguing for a direct influence of gut microbiota on hepatocarcinogenesis.\textsuperscript{39} Proinflammatory TLR4 signaling has been demonstrated to be involved in sensitizing cells toward profibrotic signaling pathways and thereby leading to liver fibrosis.\textsuperscript{37} TLR4 might therefore also be directly involved in hepatocarcinogenesis. To test this hypothesis both Yu et al. as well as Dapito et al. compared tumorigenesis in TLR4 mutant and wild-type mice. Interestingly, despite both authors describing a reduction of tumor number and size in TLR4 mutant mice, contrary results were found regarding tumor incidence: Yu et al. found 25% less tumors in TLR4 mutant mice while Dapito et al. described similar tumor incidences when TLR4 mutant mice were compared with TLR4 wild-type mice.\textsuperscript{37} Even considering these differences, the available data clearly demonstrate that TLR4 is involved in LPS mediated hepatocarcinogenesis.

On a cellular level, it was demonstrated that both HSC and Kupffer cells as well as hepatocytes are sensitive to LPS by expressing TLR4. Using chimeric mice, Dapito et al. demonstrated that HSC and hepatocytes but not bone-marrow derived cells (including Kupffer cells) mediate the tumor-promoting effects of TLR4 in development of HCC models. In contrast, by using a similar approach Yu et al. found that both resident liver cells and Kupffer cells are needed for TLR4 mediated hepatocarcinogenesis. Thus, further studies in mice or rats with conditional TLR4 deletion are required to distinguish the relative contribution of TLR4 expressed in the different cell types to hepatocarcinogenesis.

Obesity and high fat diet have been identified as major risk factors for HCC (see above). Emerging evidence has indicated that alterations of intestinal microbiota are associated with obesity;\textsuperscript{39} thus it seemed likely that these alteration might represent a major pathogenetic factor HCC development. In this line, Yoshimoto and colleagues demonstrated that administration of antibiotics and gut sterilization lead to a significant decrease in HCC development in an obesity related model of hepatocarcinogenesis in mice.\textsuperscript{40} The authors demonstrated that obesity induced alterations of gut microbiota lead to elevated levels of deoxycholic acid (DCA), a gut bacterial metabolite, which induces the secretion of various inflammatory and procarcinogenic factors in the liver and thus facilitated HCC development. In this model eradication or modulation of the gut microflora blocked the DCA--inflammation--HCC axis, thus preventing obesity related liver tumors.\textsuperscript{40}

Thus, several lines of evidence suggest that the intestinal microflora is critically involved in the pathogenesis of HCC by creating an LPS dependent proinflammatory microenvironment of the liver. Considering the potential implications of these findings for prevention and therapy of HCC, Dapito et al. tested the time frame during which bacterial LPS exert the most important effects on hepatocarcinogenesis. Importantly it became apparent that gut sterilization only prevented development of HCC but did not lead to the regression of already established tumors, highlighting rather a preventive than a therapeutic potential for such a treatment. Of note, when the impact of the more selective and well tolerated gut decontamination with rifaximin\textsuperscript{41,42} on hepatocarcinogenesis was tested only a very moderate effect became apparent.\textsuperscript{37} Aside from gut decontamination, Zhang et al. provided evidence for a beneficial effect of probiotics in preventing HCC but did not provide any data on the optimal time-point of treatment. Moreover, no data on a potential effect on already existing tumors were provided in this study.\textsuperscript{38}
Overall, the new data summarized in this review highlight a profound impact of intestinal microbiota and gut light of a lack of chemotherapeutic strategies and limited chemotherapeutic options for treatment of liver cancer. Modulation of the gut microbiota may also benefit the therapeutic efficacy of antibiotics, prebiotics, and probiotics might represent novel strategies to prevent the progression from chronic hepatitis to liver cirrhosis and HCC.

**Disclosure of Potential Conflicts of Interest**
No potential conflict of interest was disclosed.
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