Translational Research in Chronic Liver Diseases

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Abstracts
Poster Abstracts
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TRANSLATIONAL RESEARCH IN
CHRONIC LIVER DISEASES

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Scientific Organization:
R. Bartenschlager, Heidelberg (Germany)
M.A. Kern, Heidelberg (Germany)
M.P. Manns, Hannover (Germany)
P. Schirmacher, Heidelberg (Germany)
D. Schuppan, Boston (USA)
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Session I

Chronic hepatitis
Novel antiviral therapy approaches to HBV infection

Ulrike Protzer
Institute of Virology, Technische Universität München/Helmholtz Zentrum München, Germany

The human hepatitis B virus (HBV) is the prototype member of the family of hepadnaviridae, small enveloped viruses, which replicate their compact and highly organized DNA genome via reverse transcription. In humans, HBV may cause inflammatory liver disease, hepatitis B. With more than 350 million chronically infected people at high risk to develop liver cirrhosis or hepatocellular carcinoma, HBV is one of the most important human pathogens. In the recent years, the viral life cycle has been characterized in considerable detail, our understanding of immunology and pathogenesis of hepatitis B has largely improved, and nucleos(t)ide analogues have been established as antivirals. However, current treatment options are still limited because they only rarely eliminate the virus, and thus long-term treatment is required. Following a general introduction, I will therefore discuss which steps in the viral life cycle may serve as targets for novel therapeutic strategies.
**Hepatitis B virus (HBV) derived lipopeptides for HBV entry inhibition and hepatocyte-specific drug delivery**

Walter Mier¹, Alexa Schieck¹, Andreas Schulze², Thomas Müller¹, Christa Kuhn², Stephan Urban²

¹Department of Nuclear Medicine, University of Heidelberg, Heidelberg, Germany
²Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany

A hallmark of Hepatitis B Virus (HBV) infection is the extraordinary efficiency by which the virion targets hepatocytes. This feature has been attributed to the specific binding of (a) viral envelope protein(s) – particularly the L – to a yet unknown host-factor.

We have previously identified acetylated peptides comprising an N-terminal segment of the preS1-domain of L that efficiently block HBV entry *in vitro* (Gripon et al., J Virol. 2005; 79: 1613–22) and *in vivo* (Petersen et al., Nat Biotechnol. 2008; 26: 335–41). These peptides have therefore been assumed to represent the ligand for this host receptor.

Using a series of synthetic peptides which have been characterized in their HBV-inhibitory activities, we performed biodistribution experiments in mice to study and identify sequence elements that mediate liver targeting *in vivo*. We found that the wild type peptide HBVpreS/2-48myr accumulated in the liver of even non HBV-susceptible mice with extraordinary selectivity, suggesting the presence of a species-independent but hepatocyte-specific peptide receptor. Removal of the N-terminal acyl moiety abrogated the accumulation of the peptide in the liver and resulted in fast renal filtration. Mutational analyses revealed that a conserved 7 a.a. sequence motif is necessary for liver-targeting. Peptides containing this sequence bind to hepatocytes after s.c. application. In contrast, peptides carrying D-amino acid exchanges within this motif showed a disperse distribution in different organs. Beside important implications regarding the determinants for HBV species specificity (host restrictions may not be related to the absence of a receptor), HBVpreS-lipopeptides open a highly selective way to deliver any kind of drug to hepatocytes or hepatoma cells. These approaches include delivery of e.g. interferons, inhibitors of HCV or HBV replication, inhibitors of the cell cycle progression for HCC treatment, the delivery of siRNAs or peptides for MHC-mediated antigen presentation.
New insights into the hepatitis C virus replication cycle: Definition of novel drug targets

Ralf Bartenschlager
Department of Molecular Virology, Hygiene Institute, University of Heidelberg, Im Neuenheimer Feld 345, D-69120 Heidelberg, Germany
E-Mail: ralf_bartenschlager@med.uni-heidelberg.de

The hepatitis C virus (HCV), a member of the flavivirus family, primarily infects the liver. Unusual for this group of viruses, HCV frequently establishes persistence and persistently infected persons have an increased risk to develop liver disease including liver cirrhosis and hepatocellular carcinoma.

With the availability of recently established highly efficient cell culture systems for HCV, studies of the complete viral replication cycle became possible. Together with earlier studies of the viral genome organization, the biochemical characterization of viral enzymes and the determination of the X-ray crystal structure of several viral proteins, prime targets for antiviral therapy have been established. These are the NS3 serine-type protease and the NS5B RNA-dependent RNA polymerase. First clinical trial with compounds targeting these enzymes have shown great promise that selective drugs will become available, but at the same time it is becoming obvious that drug resistance will develop rapidly. Therefore the identification of additional targets for antiviral therapy is urgently needed. With the advent of the new culture systems such novel targets have emerged. Most notable are inhibitors targeting the NS5A replicase factor as well as host cell proteins that HCV needs for its successful replication. The most notable example of the latter is inhibitors of cyclophilins that appear to activate the viral replicase machinery. Sequestration of cyclophilin by cyclosporine analogues potently inhibits HCV replication in cell culture and in patients arguing that targeting a cellular rather than a viral structure is a feasible therapeutic concept. This example illustrates the cellular pathways that HCV usurps to achieve efficient propagation and highlights new concepts pursued to develop efficient drugs for the treatment of chronic hepatitis C.
Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) worldwide. A protective vaccine is not available yet and therapeutic options are still limited. Current standard therapy, pegylated interferon-α (PEG-IFN-α) combined with ribavirin (RBV), is often difficult to tolerate and results in a sustained virologic response in only 50% of patients. As a consequence, the number of patients presenting with long-term sequelae of chronic hepatitis C, including HCC, is expected to further increase for the next 10–20 years. Given this scenario, there is an urgent need to develop more effective and better tolerated therapies for chronic hepatitis C. A detailed understanding of the viral life cycle underpins these efforts, and exciting progress has recently been made in this area. The development of complete cell culture systems now allows the investigation of the entire viral life cycle in vitro. HCV cell entry is a complex multistep process involving numerous cellular factors. The viral genome consists of a 9.6-kb positive-strand RNA composed of a 5’ noncoding region (NCR), a long open reading frame, and a 3’ NCR. Internal ribosome entry site (IRES)-mediated translation yields a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases. HCV structural proteins include core and the envelope glycoproteins E1 and E2. The nonstructural proteins include the p7 ion channel, the NS2-3 protease, the NS3-4A serine protease and RNA helicase, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp). HCV RNA replication takes place in a membrane-associated replication complex, composed of viral proteins, replicating RNA, altered cellular membranes (membranous web), and other host factors. Recent elegant studies have identified and characterized such host factors, including cyclophilins. In addition, complex interactions between HCV RNA replication as well as virion assembly and release and the cellular lipid metabolism have been uncovered. In this context, a role for lipid droplets and the VLDL pathway in HCV production has recently been described. In principle, each step of the HCV life cycle represents a target for antiviral intervention. Specific inhibitors of the biochemically and structurally well-characterized NS3-4A serine protease and NS5B RdRp are currently the most advanced, with the first candidates approaching phase III clinical evaluation. Not surprisingly, the development of specific inhibitors faces major issues, including modest in vivo activity of initially promising compounds, toxicity, and the rapid development of antiviral resistance. Therefore, PEG-IFN-α and RBV are likely to remain the therapeutic backbone for many years. Ultimately, however, the ongoing intense research efforts should result in innovative therapeutic and preventive strategies for chronic hepatitis C.
Session II

Protection, regeneration and cell-based therapies
Modulation of liver regeneration by cytokines – Is there an application?

Christian Trautwein
Medizinische Klinik III, Universitätsklinikum RWTH-Aachen, Germany

Liver regeneration describes the unique potential of the liver to restore liver mass. The molecular mechanisms of this process have been best studied after partial hepatectomy, but are relevant for any stimulus which leads to liver cell injury. The quiescent hepatocyte in the uninjured liver stays in G0-phase of the cell cycle. Organ loss triggers hepatocytes to enter the cell cycle. During this event different factors and pathways become activated which control cell cycle entry. In recent years these mechanism have been better characterised on a molecular level.

The different factors which are involved in the regenerative response include cytokines that are also activated during bacterial infection and sepsis, e.g. interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF). The receptors and downstream pathways of both cytokines have been defined. In order to better study the molecular events triggered by IL-6 and TNF during liver regeneration we and others have developed hepatocyte-specific knockout mice. The aim of these knockout mice is to target crucial signalling molecules and after deletion block specific intracellular pathways of the respective cytokine in hepatocytes. This approach enables to exactly define the contribution of each cascade for the process of liver regeneration in distinct animal models. Thus these studies contributed and will also in future define molecular pathways that are crucial for liver regeneration. Additionally, these studies have the potential to uncover potential therapeutic options to enhance, but also to block hepatocyte proliferation during liver regeneration, but also during carcinogenesis. In the presentation the actual status of the scientific knowledge of IL-6 and TNF-dependent pathways for hepatocyte proliferation will be summarised and potential links for a clinical application will be shown.
The rationale of stem cells and cytokines for the treatment of chronic hepatitis

M. Ott
Abteilung für Gastroenterologie, Hepatologie und Endokrinologie, Medizinische Hochschule Hannover, Germany

Patients with chronic liver failure by far outnumber patients with either hereditary liver disease or acute liver failure. If causative treatment is not available (i.e. antiviral treatment) medicine does not provide many choices for the treatment of chronic liver disease. In this context stem cells or secreted proteins of stem cells are expected to offer great potential for antifibrotic treatment in the future. Mesenchymal stem cells and subsets of hematopoietic stem cells have been isolated and tested for antifibrotic effects in animal models of chronic liver disease. Although in a few studies those cells have shown fibrinolytic and antioxidative activities after transplantation in animals with chronic liver disease, most of the available data were inconclusive or did not show any therapeutic effect. Nevertheless, clinical applications of bone marrow (stem) cell infusions in chronic liver disease have been performed in the last three years and published. The majority of these clinical trials show some improvement in clinical or laboratory scorings such as the Child-Pugh score or bilirubin/albumin levels. The small number of patients in each study, various (stem) cell preparations and application routes, no control groups except in one study, little information about additional therapies in these patients and mixed aetiologies of chronic liver disease undermine the value of these trials. One patient died a few days after arterial infusion of bone marrow cells, which raises questions about the safety of the approach in these severely ill patients. Many more animal data on safety, on which (stem) cells to use, on the best dosage, the most efficient application route as well as on therapeutic mechanisms involved, are urgently needed.
What makes liver grow, what makes it stop and how it escapes disasters

George Michalopoulos
Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261 USA, E-Mail: michalopoulosgk@upmc.edu

Regeneration of liver following tissue loss is a well orchestrated process streamlined by the coordinated action of growth factors and cytokines and promoted by proliferation of specific liver subpopulations. HGF and ligands of EGFR are the primary mitogens for hepatocytes and they are released from the adjacent biomatrix (HGF) or enter through the portal circulation (EGF) to activate their cognate receptors within 30–60 minutes after partial hepatectomy (PHx). The effects of other cytokines (norepinephrine, IL6, TNF, bile acids, serotonin, etc.) coordinate activation of specific transcription factors and optimize the time kinetics of the process. Acute elimination of the HGF receptor in normal rats results in cessation (ShRNA) or very severe decrease (morpholino-Met) of the regenerative activities after PHx. Similar effects, though of lesser intensity, are seen after ShRNA treatment against the EGFR. Extracellular Matrix (ECM) remodeling mediated by uPA at the earliest stages of regeneration is balanced by restoration of matrix biosynthesis towards the end of regeneration. ECM signaling was disrupted in mice carrying LoxP sites around Integrin-Linked Kinase (ILK). Acute elimination of ILK caused massive hepatic necrosis, suggesting a role for ECM signaling in such processes. Slower genetic elimination of ILK caused enhanced hepatocyte and biliary cell proliferation for the first 30 weeks of age and altered ECM composition. The modified and adjusted livers were 2x the size of control mice, showing that ECM signaling controls functions related to the “hepatostat” (the homeostatic mechanisms regulating liver to body weight). In addition, when the enlarged and adapted livers were subjected to PHx, they return to a weight larger by 59% over the ILK$^{loxp/loxp}$ original (already 2X over the control mice). Glypican-3, a protein over-expressed in human liver cancer, appears to be a major factor derived from pericellular biomatrix and involved in cessation of hepatocyte growth at the end of regeneration. When regeneration fails, cellular alterations occur, depending on the failing population. Many previous studies in rodents have shown that cells of the biliary compartment function as facultative stem cells for hepatocytes and may rescue the failing liver. Our recent studies also demonstrated that biliary cells in fulminant hepatitis in humans express high levels of hepatocyte associated transcription factors (e.g. HNF4a) as they transition from a biliary to a hepatocytic phenotype. Periportal hepatocytes in rodents function as facultative stem cells for biliary epithelium in situations of regenerative failure. In humans, periportal hepatocytes express biliary associated transcription factors in chronic biliary disease. Facultative (opportunistic) functions of hepatocytes and biliary cells as stem cells for each other are well documented and may be the pathway that rescues the liver when standard regenerative processes fail.
Novel protective strategies for liver surgery

Pierre-Alain Clavien
Swiss HPB Center, Department of Surgery, University Hospital Zurich, Switzerland

Over the years strategies in liver surgery have been developed to expand indications for resection and transplantation, often the only curative option for many patients. The research and understanding of liver regeneration and ischemia/reperfusion injury is essential for this progress.

The liver possesses the unique ability to regenerate its volume after major tissue loss or injury. This regenerative process involves precise initiation followed by termination after restoration of the lost liver mass. The initiation process is mediated by multiple signaling molecules and cytokines (e.g. HGF, EGF, TNF-α, IL-6, and VEGF). The action of transforming growth factor β and other members of the activin family appear to terminate the regeneration.

We could recently show the critical involvement of platelets in liver regeneration. Here serotonin, stored and secreted by platelets, is an essential co-mitogen. In a knock-out mouse with absence of serotonin in the platelets (TPH-1), a 70% hepatectomy failed to induce liver regeneration. Restoration of the serotonin levels rescued the regenerative response. This finding contributes to the understanding of factors involved in liver regeneration and might open the door to innovative strategies.

A limiting factor in liver surgery is the size and function of the remnant liver after resection. Studies in mice suggest that failure to regenerate is the most important contributing factor. A liver remnant below a certain threshold volume cannot sustain its function and results in a small-for-size syndrome. Pharmacological approaches, such as the use of pentoxifylline, a TNF-α synthesis inhibitor, reduced the likelihood of poor liver function in a murine model of partial liver transplantation. This approach is currently under clinical investigation.

Occlusion of the right or left portal branch causes atrophy of the ipsilateral liver lobe and hypertrophy of the contralateral lobe. We demonstrated superiority of portal vein ligation compared to embolization in a rat model. Entrapment of macrophages in the occluded segment due to a foreign body reaction was observed after embolization and might explain this observation. This may explain the reduced regeneration in the embolized model compared to the ligation model.

Ischemia and reperfusion injury is often an inevitable process. Marginal organs such as fatty and cirrhotic livers are even more prone to injury. Ischemic preconditioning and intermittent clamping, two treatment strategies to attenuate this injury, have already made their way to clinical practice.

The past two decades have brought an impressive number of studies and substantial progress in both liver surgery and liver transplantation. A few have reached the human and many may pave the way to novel therapies.
Presentation of selected posters
An oncogenomics-based in vivo RNAi screen identifies new tumor suppressors in liver cancer

Lars Zender\textsuperscript{1,6*}, Wen Xue\textsuperscript{1*}, Johannes Zuber\textsuperscript{1}, Stefan Kubicka\textsuperscript{4}, J.M. Luk\textsuperscript{5}, Peter Schirmacher\textsuperscript{3}, Richard W. McCombie\textsuperscript{1}, Michael Wigler\textsuperscript{1}, James Hicks\textsuperscript{1}, Gregory J. Hannon\textsuperscript{1,2}, Michael P. Manns\textsuperscript{4}, Scott Powers\textsuperscript{1}, and Scott W. Lowe\textsuperscript{1,2**}

\textsuperscript{1}Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
\textsuperscript{2}Howard Hughes Medical Institute, Cold Spring Harbor, NY 11724, USA
\textsuperscript{3}Institute of Pathology, University Hospital Heidelberg, D-69120 Heidelberg, Germany
\textsuperscript{4}Department of Gastroenterology and Hepatology, Medical School Hannover, D-30625 Hannover, Germany
\textsuperscript{5}Department of Surgery, University of Hong Kong, Hong Kong, China
\textsuperscript{6}Current address: Helmholtz Centre for Infection Research (HZI), D-38124 Braunschweig, Germany, and Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, D-30625 Hannover, Germany

Here we combine microRNA based short hairpin RNA (shRNA) technology with a progenitor cell derived mouse model of hepatocellular carcinoma to perform an in vivo RNA interference screen for new tumor suppressor genes. We generated a series of low complexity pools of shRNAs targeting genes found in focal deletions identified by comparative genomic hybridization of \(\sim\) 100 human hepatocellular carcinomas. These pools were introduced into liver progenitor cells expressing the Myc oncogene and tested for their ability to promote tumorigenesis in vivo; remarkably, pools containing shRNAs targeting deleted genes gave rise to tumors whereas those containing randomly selected shRNAs did not. Through further analyses, we identified and validated 13 new tumor suppressor genes, most of which have not been linked to cancer before. One gene, \textit{EXPORTIN 4 (XPO4)}, encodes a nuclear export protein whose substrates include SMAD3 and a putative translational regulator, EIF5A. Interestingly, we show that \textit{EIF5A2} is amplified in human tumors, is required for efficient proliferation of tumor cells lacking XPO4, and can promote hepatocellular carcinoma development in mice. Our result establishes the feasibility of in vivo RNAi screens for genes that modulate epithelial cancer phenotypes, and illustrate how combining next generation mouse models, RNAi and genomic information from human cancer may facilitate the function annotation of the cancer genome.
Growth of hepadnavirus infected hepatocytes strongly reduces virion productivity and cccDNA loads in uPA mice

Tassilo Volz¹, Marc Lütgohetmann¹, Tim Broja¹, Ansgar W. Lohse¹, Jörg Petersen¹, Maura Dandri¹
¹Department of Medicine, University Hospital Hamburg-Eppendorf, Germany

**Introduction:** Chronic hepatitis B virus (HBV) infection is assured by maintenance of the covalently closed circular DNA (cccDNA), the template of viral transcription. In quiescent hepatocytes cccDNA is a stable molecule which can persist throughout the life span of the hepatocytes. However, immune mediated cell division may favour cccDNA dilution in the course of chronic HBV infection. Aim of this study was to investigate stability and activity of the cccDNA in infected hepatocytes undergoing cell division in vivo.

**Methods:** Primary tupaia hepatocytes (PTH) chronically infected with woolly monkey (WM-) HBV were isolated from high viremic (10E⁹ WM-HBV DNA/ml) immuno-deficient uPA mice. Cell suspension consisted of 50% PTH and was re-transplanted into 20 uPA recipients. PTH engraftment and proliferation were monitored by immunohistochemistry and real-time PCR.

**Results:** Intrahepatic viral loads (rcDNA and cccDNA/PTH) were determined by RT-PCR at different time points and loads were compared to levels estimated in cell suspension, where all PTH appeared HBcAg-positive and harboured 1000rcDNA and 2.5 cccDNA per PTH. Strong expansion of engrafted PTH was demonstrated by PCNA staining 5 days after transplantation and continued up to 40 days. Cell growth induced strong reduction (median 2-log) of intrahepatic viral loads in all mice analysed, which was accompanied by strong reduction of pgRNA. Notably, a rapid and significant reduction (Δ1-log) of cccDNA levels occurred while PTH repopulated diseased mouse livers.

**Discussion/Conclusion:** This study reveals that cell division in the setting of liver regeneration causes rapid reduction of viral productivity and cccDNA dilution in chronically infected hepatocytes.
Interferon-α therapy does not modulate hepatic expression of classical type I interferon inducible genes

Volker Meier¹, M.D., Sabine Mihm¹, Ph.D., Giuliano Ramadori¹, M.D.
¹Division of Gastroenterology and Endocrinology, Department of Internal Medicine, Georg-August-University Goettingen, Germany

Introduction: Hepatitis C virus (HCV) infection is a major cause of chronic liver disease. Treatment with interferon-alpha² (IFN-α²) can induce viral clearance and marked biochemical and histological improvement. IFN-α² treatment has been shown to stimulate the expression of type I IFN regulated genes in peripheral blood mononuclear cells (PBMCs) of hepatitis C patients; however, whether it affects hepatic expression remains unknown. This study thus aimed comparing hepatic gene expression with particular emphasis on type I IFN inducible genes in patients with chronic hepatitis C before and during an IFN-α² monotherapy.

Methods: Responsiveness to IFN-α² therapy was monitored by determining serum and hepatic viral load. Differential gene expression analysis was performed by two different techniques, namely suppression subtractive hybridization (SSH) and differential display (DD). Expression of two prototype type I IFN regulated genes was quantified in further PBMC and liver samples.

Results: Among different genes found to be up-regulated during an effective, i.e. virus clearing, IFN-α treatment, only a single one was identified which can be accounted to type I IFN responsive genes. Parallel quantitative real time PCR analyses demonstrated significant induction of the type I IFN regulated genes MxA and PKR in PBMC, but not in the liver.

Conclusion: Taken together, while IFN-α treatment leads to the induction of type I IFN regulated genes in PBMC, such an induction appears not to occur in the liver of hepatitis C patients. The mechanism by which IFN-α treatment causes viral clearance thus might be independent on hepatic activation of type I IFN regulated genes.
Session III

Fibrosis/Fibrogenesis
Non-invasive monitoring of liver fibrosis

Detlef Schuppan
Beth Israel Deaconess Medical Center, Division of Gastroenterology and Hepatology, Harvard Medical School, Boston, MA, USA

We have made striking progress in our understanding of the biochemistry and cell biology of liver fibrosis and cirrhosis. This includes numerous agents and emerging strategies that prevent fibrosis progression or even induce reversal of established fibrosis and cirrhosis in rodent models of liver fibrosis. However, translation into the clinic requires improved noninvasive markers or techniques that permit the exact measurement of hepatic fibrosis, and in particular of fibrogenesis and fibrolysis. This is necessary since liver biopsy has an inherent sampling error and progression from stage 0 to stage 4 (cirrhosis) usually takes 20–30 years, even in patients classified as fast progressors.

The current serum markers and serum marker algorithms allow only a crude differentiation between no to mild and moderate to severe fibrosis (Metavir stages 0–1 vs. 2–4, resp.), making them unsuitable for assessing progression or the effect of antifibrotic therapies. Transient elastography or MR elastography/diffusion weighted MRI, though sampling larger volumes of the liver or the whole liver, resp., lack the necessary sensitivity and specificity for exact fibrosis quantification.

Major efforts are invested in finding better serum markers that reflect fibrogenesis or fibrolysis rather than fibrosis. Methods employed are proteomic techniques such as 2-D differential gel electrophoresis (DIGE) or isobaric tagging (iTRAQ) followed by identification of differentially expressed proteins/peptides by mass spectrometry. Comparison is made between normal and fibrotic livers with various stages of fibrosis and rates of progression. Preferentially this is done in well defined rodent fibrosis models, followed by validation in the human system.

Alternative strategies are based on targeted imaging of hepatic fibrogenesis or fibrosis. These exploit the upregulation of certain cell surface markers on fibrogenic cells or free binding sites on collagen. High affinity ligands are created that are linked to imaging agents for SPECT or MRI. This approach is not trivial, since imaging agents have to be small to penetrate into the fibrotic matrix, must be nontoxic with elimination half lives of less than one hour, and must display very high affinity, e.g., via oligomerization. In addition, the high unspecific background uptake of liver has to be reduced. Proof of concept was provided by successful imaging of fibrogenesis in rodent models of liver fibrosis using an integrin alphaVbeta6 targeted construct.

The availability of such noninvasive methodologies to quantify hepatic fibrosis and fibrogenesis/fibrolysis will serve as pacemaker for the clinical development and validation of potent antifibrotic agents.
Interfering with profibrogenic cytokines in hepatic fibrosis

Steven Dooley
II. Medizinische Klinik, Universitätsmedizin Mannheim der Universität Heidelberg, Germany

Liver transplantation is currently the only available treatment for terminal liver failure. Since donor organs are highly limited, there is a strong interest in new therapies. Current efforts aim at discarding the source of damage, which represents the most efficient strategy of healing. In alcoholic liver disease (ALD), this can be achieved by avoiding further alcohol consume, which is complicated by addiction behaviour of the patients. Regarding HBV or HCV infections, which represent about 30% of CLDs, virostatic treatments are currently in use to decrease virus load, thus improving patient conditions to some extent. However, the fact that virus infection cannot be completely abrogated narrows efficiency of this therapy.

Intense basic and clinical research in the last decades has led to deep knowledge in the molecular pathophysiology of hepatic fibrosis. Identified fibrogenic stimuli include, among others, activation of cytokine signaling pathways, which trigger inflammatory and immune responses, extracellular matrix deposition and scarr formation as well as morphological plasticity and apoptosis in the different cell types of the liver. Intracellular signaling pathways for these liver fibrosis related cytokines are widely delineated and inhibitors to signaling molecules are being explored in vivo or in cultured cells. Add on approaches based on these inhibitors that target these proinflammatory and profibrotic cytokine signaling pathways required for disease progression are promising candidates to provide medical treatment.

Proliferative cytokines including PDGF, FGF and TGF-α signal through tyrosine kinase receptors, inhibitors of which are already undergoing clinical trials in other tissues. Anti TGF-β approaches were established and successfully utilized for the treatment of experimental fibrogenesis. Dominant negative TGF-β receptors were applied to suppress fibrosis. Similarly TGF-α binding proteins like decorin, antagonistic cytokines such as bone morphogenetic protein-7, hepatocyte growth factor, IL-10, or IFN-γ were as efficient as camostat mesilate, a protease inhibitor that possibly abrogated proteolytic activation of TGF-β. Further, our group recently overexpressed Smad7 in bile duct ligation induced liver fibrosis and achieved efficient inhibition of intracellular TGF-β signaling, thereby counteracting profibrogenic effects in cultured HSCs and in vivo.

Although the above results strongly indicate that targeting fibrogenic signaling pathways can prevent fibrotic responses, and despite substantial progress in uncovering the cellular and molecular mechanisms of fibrosis, there are no approved therapies available.

Major reasons are the complexity of the disease, represented by functional multiplicity of involved cytokines, crosstalk of disease related signaling pathways and cell cell communication, leaving a black box of unwished side effects. This may be solved by comprehensive modeling of the complex regulatory network based on functional genome wide data of chronic liver diseases, which is now accessible with the field of Systems Biology. It is expected that downstream branch points of multi-
functional cytokines like TGF-β will be identified, which act as regulators of disease related synexpression groups. Targeting these will increase specificity and reduce side effects. In addition, cell type specificity and time dependent changes need to be considered and will finally lead to strongly improved drugs.

On the other hand, more robust biomarkers and clinical trial endpoints, e.g. the use of non invasive assays with labeled compounds to identify fibrotic stages and monitor treatment efficacy must be developed and validated to allow the assessment of such drugs in clinical trials.
Reversibility of fibrosis: Therapeutic implications

John P. Iredale
Professor of Medicine, University of Edinburgh, Edinburgh, UK

Liver fibrosis represents the end stage of the wound healing response of the liver and results from the excessive secretion of matrix proteins by activated Myofibroblast-like hepatic stellate cells (HSC) and myofibroblasts recruited from mesenchymal stem cells. Additionally hepatic, particularly periportal, myofibroblasts may contribute to the wound healing response in the liver. In this activated phenotype HSC proliferate and are the major source of collagens I and III that characterise fibrosis. In addition, activated HSC express both matrix degrading metalloproteinases (MMPs) and their inhibitors the tissue inhibitors of metalloproteinases (TIMPs) – leading to the hypothesis that progressive fibrosis is in part the result of a failure of matrix degradation.Whilst previously viewed as irreversible, we have used models of biliary and parenchymal liver injury, to demonstrate that established fibrosis is reversible and have studied these models to determine the critical roles of individual cell lineages and the changes in cell behaviour and matrix turnover that mediate resolution of fibrotic change.

Recovery from fibrosis induced by both CCl4 and bile duct ligation will occur over 4–6 weeks following withdrawal of the insult (cessation of dosing with CCl4 and bilio-jejunal reanastamosis respectively). Recovery is associated with histological resolution and a return of the normal architecture and histological and biochemical evidence of matrix degradation. Resolution is accompanied by apoptosis of the activated HSC. In association with HSC apoptosis the hepatic levels of TIMPs 1 and 2 decrease to levels comparable with normal untreated liver and collagenase activity within liver homogenates increases in parallel, coinciding with evidence of matrix remodelling. Therefore, HSC apoptosis appears to serve the dual function of removing the cells responsible for both producing the neomatrix and ensuring its protection from collagenase digestion through expression of the TIMPs. Apoptosis may represent a default pathway for stellate cells; this pathway is forestalled during progressive injury because HSC are provided with survival signals. Increasing evidence indicates that contact with a collagen-I rich fibrotic matrix may promote survival of activated HSC. In addition, TIMPs 1 and 2 act as autocrine survival signals for HSC by reducing MMP mediated matrix turnover. Additionally MMP activity may be critical to the cleavage and inactivation of survival factors including n-cadherin and Pro-NGF.

The development of a more advanced cirrhosis is, however not entirely reversible. Remodelling of this lesion results in the conversion of a micronodular to attenuated macronodular cirrhosis. The features of the irreversible components of fibrosis include a relative hypocellularity of the persistent scar and the cross-linking of matrix within the scar. Most recently, by showing that macrophage depletion retards resolution of fibrosis, we have demonstrated a key role for these cells in liver remodelling. These observations will be discussed in greater depth during the presentation.

Our increasing understanding of the process of spontaneous recovery from liver fibrosis is likely to be invaluable to the design of future therapeutic strategies targeted at this and other fibrotic disorders. Therapeutic approaches to enhance matrix degradation, myofibroblast apoptosis and the potential for macrophage and stem cell based therapies will be discussed in the presentation.
New mechanistic treatment concepts in liver fibrosis

Massimo Pinzani, M.D., Ph.D.
Dipartimento di Medicina Interna – Centro di Ricerca, Alta Formazione e Trasferimento “DENOTHe”, Università di Firenze, Florence, Italy

The last two decades have witnessed a considerable progress in the understanding of the mechanisms responsible for the fibrogenic progression of chronic liver diseases. However, no drugs are approved as antifibrotic agents in humans. Considering the central role attributed to hepatic stellate cells (HSC) in liver fibrosis, this cell type has represented and still represents is a major focus of antifibrotic research. Indeed, the well-described pathway of HSC activation, subsequent fibrogenesis, with the potential for apoptosis and reversibility, provides a logical framework to define sites of intervention. Consequently the search for effective anti-fibrogenic strategies is based on the knowledge gained in the area of HSC biology, including the biology of the factors (growth factors, cytokines, etc.) conditioning their pro-fibrogenic attitude. Regardless, it is absolutely clear that the best anti-fibrogenic treatment would be represented by any strategy able to eliminate the primary cause of parenchymal damage, metabolic overload or excessive oxidative stress. Since the fibrogenic process is in its essence a compensatory phenomenon aimed at maintaining a sufficient tissue continuity and cohesion in the presence of a continuous microscopic parenchymal collapse, it would be erroneous to attempt to cure fibrogenic chronic liver diseases (CLDs) only with antifibrogenic drugs, once some effective compounds will become available for clinical use. Once this primary requirement is fulfilled, the association with an anti-fibrogenic drug would be relevant for stabilizing the cure and favor an optimal remodeling. Putative anti-fibrogenic drugs include: 1. agents able to reduce inflammation and immune response, 2. agents able to reduce the activation of ECM-producing cells and their pro-fibrogenic properties (proliferation, motility, ECM deposition, contraction), 3. agents with pro-apoptotic potential for ECM-producing cells, 4. agents able to increase fibrillar ECM degradation. It should be stressed that most of the evidence indicating a beneficial effect of these drugs derives from studies performed in vitro or in animal models of fibrogenesis. Therefore it is still debatable whether or not these agents could be truly effective. Considering the above limitations, it is about time to select some of the most promising agents emerging from preclinical studies and start testing their clinical efficacy. However, testing for clinical efficacy may prove difficult and expensive. Appropriate end-points for studies need to be defined and agreed as the time frame for regression of fibrosis is likely to be adequately measurable only in years. Consequently, the relative need to set long-term prospective studies represents a major limitation for the enthusiasm of researchers embarking in this task. Since all CLDs are in general characterized by a very slow course to cirrhosis, the above mentioned limitations contrast with the possibility that any suitable antifibrogenic treatment could effectively render the fibrogenic evolution even slower and eventually reduce the number of patients reaching end-stage disease within a reasonable life time-frame.
Session IV

Steatohepatitis and oxidative stress
Experimental therapeutic concepts for alcoholic steatohepatitis: From pathology to therapy

Helmut K. Seitz
Department of Medicine and Center of Alcohol Research, Salem Medical Center and University of Heidelberg, Heidelberg, Germany

Established treatment targets in alcoholic steatohepatitis (ASH) are endotoxins and cytokines as well as oxidative stress. Various treatment strategies against endotoxins and cytokines have been used including steroids, TNF-α antibodies, pentoxifylline, enteral nutrition as well as the application of the molecular absorbent redistribution system (MARS). With respect to oxidative stress, studies using antioxidants have shown to be ineffective. Since the treatment strategies mentioned are still not convincing with respect to short and long-time survival, new therapeutic strategies are needed.

Experimental therapies may include 1) the administration of antibiotics to decrease gut endotoxins deriving from bacteria; 2) clearance of endotoxins from the blood by using detoxification procedures including antibodies against endotoxins or extracorporeal detoxification using certain types of detoxification columns; 3) the use of thalidomide which counteracts the effect of tumor necrosis factor α; 4) the administration of antibodies against interleukin-1, since interleukin-1 levels have been found to be extremely high in ASH, and 5) the use of cytochrome P4502E1 inhibitors eliminating alcohol induced hepatic cytochrome P4502E1 which leads to the generation of reactive oxygen species. Animal experiments have shown that chlorothiazole which is a selective CYP2E1 inhibitor, improves alcoholic liver disease and also improves ethanol associated DNA damage. 6) Also, inhibitors of caspase-12 which is activated via endoplasmatic reticulum stress and TRAF2 translocation have been experimentally used. 7) The administration of betain which may interfere with an altered methionine-homocystein metabolism resulting in a decrease of homocystein, an important factor for endoplasmic reticulum stress and for the activation of ERK 1/2 may be another therapeutic approach. Betain may also lead to an increase in phospholipids, an important factor in membrane structure. 8) The administration of polyenylphosphatidylcholine, a membrane stabilizing factor, was found to be ineffective in a US multicenter trial. However, selective patients may benefit from this compound.

Protone decoupled 31P magnetic resonance spectroscopy could eventually identify these patients. 9) Finally, adiponectine could be another compound of interest.

Some of these approaches have been used in animal experiments resulting in an improvement of ASH, however, human data are urgently needed to determine their effectiveness.
Mechanistic approaches to NASH therapy

C.P. Day
University of Newcastle, The Medical School, Center for Liver Research, Newcastle upon Tyne, UK

The original ‘two hit’ model of NASH pathogenesis – now ten years old suggested that steatosis – the first ‘hit’ – sensitised the liver to the injurious effects of the second ‘hits’ – oxidative stress and cytokine-mediated stress. Ten years later endoplasmic reticulum (ER) stress has been added to the list of second hits; non-inflammatory mediators of fibrosis have been identified and steatosis may now be considered a ‘bystander’ rather than being intimately involved in the pathogenesis of progressive disease. Oxidative stress is thought to arise principally as a result of increased free fatty acid (FFA) oxidation in hepatocytes. Pro-inflammatory cytokines are produced both by hepatocytes, in response to an increased supply of FFAs, and by Kupffer cells stimulated by gut-derived endotoxin arising as a result of small intestinal bacterial overgrowth and increased gut permeability; there may also be a contribution from adipose tissue derived macrophages. ER stress also arises as a result of lipid overload and results in oxidative stress, apoptosis and insulin resistance. Non-inflammatory mediators of fibrosis include insulin and glucose, oxidative stress, hepatocyte apoptosis, endotoxin and adipokines including angiotensin, norepinephrine and leptin along with low levels of the anti-inflammatory and anti-fibrotic adipokine adiponectin. Recent evidence that blocking triglyceride synthesis in animal models of NASH reduces steatosis but increases liver cell injury and fibrosis, suggests that the conversion of potentially toxic FFA to triacylglycerol may actually be a protective mechanism rather than part of the pathogenesis of progressive disease, with the correlation between steatosis severity and progressive disease almost certainly due to the mediators of progressive disease also causing steatosis. Based on these mechanisms current therapies are based on reducing the supply of FFA to the liver (weight loss and insulin sensitizers), reducing oxidative stress (with antioxidants), reducing endoplasmic reticulum stress (with molecular ‘chaperones’), anti-inflammatory agents, hepatoprotectants (ursodeoxycholic acid), anti-fibrotic agents, drugs aimed at reducing levels of adipokines (ACE inhibitors and ARBs) and drugs aimed at reducing gut-derived endotoxin. At present the best evidence from human studies has been provided for the glitazone class of insulin sensitizers and weight loss induced by obesity surgery, with angiotensin receptor blockade, cannabainoid receptor blockers and IKK blockers, perhaps the most promising agents being trialled at present.
Modulation of oxidative stress and CD95-mediated apoptosis

R. Reinehr, D. Häussinger
Klinik für Gastroenterologie, Hepatologie und Infektiologie, Universitätsklinikum Düsseldorf, Düsseldorf, Germany

CD95 ligand (CD95L) and other pro-apoptotic stimuli, like pro-apoptotic bile acids (e.g. glycochenodeoxycholate, GCDC) or hyperosmolarity, were recently reported to trigger a rapid NADPH oxidase-driven oxidative stress response in rat hepatocytes (1, 2) which does finally lead to CD95 oligomerization (3), CD95 translocation to the plasma membrane, DISC formation, caspase 8-activation (1–6), Bid cleavage and thereby mitochondrial amplification of the apoptotic machinery (7), which involves disruption of mitochondrial electron transport chain (8), mitochondrial hydroperoxide generation (9) and mitochondrial permeability transition (10). Thus earlier reported CD95L- or pro-apoptotic bile acid-induced ROS formation by mitochondria (8–11) may rather represent a downstream consequence than the cause of CD95 activation. Despite NADPH oxidase and mitochondria, also an endoplasmic reticulum (ER) stress response induced by hydrophobic bile acids has been reported recently (11, 12). Thus ROS generation in general might be seen as a crucial step in the initiation (i.e. NADPH oxidase) and propagation (i.e. mitochondria) of hepatocyte apoptosis and thereby represents a promising target in order to prevent liver cell damage.

Within the last years many different approaches have been undertaken in order to attenuate hepatocellular ROS formation using N-acetylcysteine (NAC), S-adenosyl-L-methionine (SAMe), betaine or tauroursodeoxycholate (TUDC) but unfortunately, it is still a matter of debate whether these approaches really effect the development of ROS-mediated chronic liver diseases, such as alcoholic (ASH) or non-alcoholic fatty liver disease (NAFLD/NASH), in humans. While the antioxidant SAMe has been shown to inhibit liver cell damage in vivo induced by a variety of conditions in previous studies (13, 14), SAMe failed to exhibit a sustained hepatoprotective effect in recent work (15, 16). Betaine, an organic osmolyte, can protect against bile acid-induced apoptosis in vitro and in vivo by inhibiting otherwise observed bile acid-induced mitochondrial cytochrome c release (17). Also TUDC potently inhibits bile acid-induced apoptosis, in vivo (18) and in vitro (19, 20) by reducing the mitochondrial membrane permeability transition (MMPT) and subsequent cytochrome c release (18). In addition, recent studies in isolated rat hepatocytes revealed that despite of TUDC-induced Erk activation (21), TUDC inhibits bile acid-induced apoptosis at a level upstream of caspase-8 activation and in an Erk-independent manner (19).

In summary, generation of reactive oxygen species is a crucial step in death receptor-mediated hepatocyte apoptosis and therefore, prevention of ROS formation might represent a promising therapeutic strategy in order to prevent liver cell damage induced by a variety of agents, such as CD95L, hydrophobic bile acids or hyperosmolarity.
References:


Session V

**Hepatocellular carcinoma**
Application of comparative functional genomics for molecular classification of liver cancer

Snorri S. Thorgeirsson
Laboratory of Experimental Carcinogenesis, CCR, National Cancer Institute, NIH, Bethesda, MD 20892-4262, USA

The variability in the prognosis of individuals with hepatocellular carcinoma (HCC) suggests that HCC may comprise several distinct biological phenotypes. These phenotypes may result from activation of different oncogenic pathways during tumorigenesis and/or from a different cell of origin. Comparative functional genomics approach has been used to address whether the transcriptional characteristics of HCC can provide insight into the cellular origin of the tumor. We integrated gene expression data from rat fetal hepatoblasts and adult hepatocytes with HCC from human and mouse models. Individuals with HCC who shared a gene expression pattern with fetal hepatoblasts had a poor prognosis. The gene expression program that distinguished this subtype from other types of HCC included markers of hepatic oval cells, suggesting that HCC of this subtype may arise from hepatic progenitor cells. Analyses of gene networks showed that activation of AP-1 transcription factors in this newly identified HCC subtype might have key roles in tumor development. Transforming growth factor β (TGF-β) is known to exhibit tumor stage dependent suppressive (that is, growth inhibition) and oncogenic (that is, invasiveness) properties. We have asked if a TGF-β specific gene expression signature could refine the classification and prognostic predictions for HCC patients. Applying a comparative functional genomics approach we demonstrated that a temporal TGF-β gene expression signature established in mouse primary hepatocytes successfully discriminated distinct subgroups of HCC. The TGF-β positive cluster included two novel homogeneous groups of HCC associated with early and late TGF-β signatures. Kaplan-Meier plots and log-rank statistics indicated that the patients with a late TGF-β signature showed significantly shortened mean survival time compared to the patients with an early TGF-β signature. Also, tumors expressing late TGF-β-responsive genes displayed invasive phenotype and increased tumor recurrence. We also showed that the late TGF-β signature accurately predicted liver metastasis and discriminated HCC cell lines by degree of invasiveness. Finally, we established that the TGF-β gene expression signature possessed a predictive value for tumors other than HCC. These data demonstrate the clinical significance of the genes embedded in TGF-β expression signature for the molecular classification of HCC.
Telomere shortening in chronic liver disease, cirrhosis and liver cancer

K. Lenhard Rudolph
Institute of Molecular Medicine and Max-Planck-Research-Group on Stem Cell Aging, University of Ulm, Ulm, Germany. E-Mail: lenhard.rudolph@uni-ulm.de

Telomere shortening limits the proliferative capacity of primary human cells to 50–70 cell divisions by induction of cellular senescence. There is growing evidence that telomere shortening limits organ maintenance in chronic diseases and in aging tissues. Telomerase knockout mice (TERC−/−) with dysfunctional telomeres show premature aging of high turnover organs and an impairment in liver regeneration in response to chronic or acute liver damage. In humans, telomere shortening occurs in most organs during aging. Moreover, telomere shortening is accelerated in chronic diseases. Critical telomere shortening and hepatocyte senescence is a hallmark of human cirrhosis indicating that telomere shortening promotes the impairment of liver regeneration and the evolution of cirrhosis in response to chronic liver disease in humans. In agreement with this hypothesis, biomarkers of DNA damage and telomere dysfunction increase during human aging and in cirrhosis patients. In addition to its role in regeneration and aging, telomere shortening influences cancer formation. Telomere shortening increases tumor initiation by induction of chromosomal instability. In contrast, telomere dysfunction and increasing levels of chromosomal instability impair tumor progression and most human HCC overexpress telomerase – the enzyme that can synthesize telomeres de novo.
Innovative targeted therapeutic approaches in HCC

K. Breuhahn
Institut für Pathologie, Universitätssklinikum Heidelberg, Germany

Over the last years, several key molecular changes have been identified and characterized which are responsible for hepatocellular carcinoma (HCC) growth and tumor cell dissemination. These include alterations in the growth factor-dependent auto- and paracrine stimulation of tumor cells as well as non-malignant cells which affect cell division, apoptosis, differentiation, and migration. As was shown for Sorafenib in the treatment of non-resectable HCC, especially cellular tyrosine kinases are proper drugable targets. We have demonstrated that high level expression of insulin-like growth factor (IGF)-II in approximately 40% of human HCC promotes the full program of malignant competence. IGF-II overexpression and autocrine activation of the tyrosine kinase IGF-IR denominates a group of HCCs with lower apoptosis rate as well as elevated tumor cell proliferation and mobility. Therefore, a full range of highly selective small molecule inhibitors has been developed to specifically inhibit IGF-IR. Indeed, substances such as the cyclolignan picropodophyllin efficiently inhibit IGF-II-induced protumorigenic effects in vivo and in vitro. In addition, analyses of novel growth factor-dependent downstream effectors aberrantly expressed in HCC lead to the identification of cyclooxygenases and transcription factors that mediate tumor growth and invasiveness. Generally HCCs express cyclooxygenases (COX)-2 mostly at higher levels compared with surrounding liver parenchyma which is associated with HCC cell resistance to apoptosis. Inhibition of COX-2 activity using nonsteroidal anti-inflammatory drugs (e.g., meloxicam) induced anti-neoplastic effects, which were attributable to the induction of apoptosis and reduction of mitogenesis. In addition, the far upstream sequence element (FUSE) binding protein (FBP) transcription factor family mediates proliferation and tumor cell motility; however, with different functional specificity. Thus, the concerted high level overexpression of FBP family members in more >70% of HCCs represents a novel therapeutic target structure for which first results have demonstrated that small chemical compounds efficiently reduces FBP bioactivity. In summary, growing knowledge about the molecular interplay of different tumor-relevant pathways will enable cancer scientists and physicians to develop more efficient and specific therapies that prevent HCC growth and tumor cell dissemination.
Immunotherapy of hepatocellular carcinoma

Tim F. Greten
Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Germany

No systemic cytotoxic chemotherapy has proven to be safe and efficient for the treatment of patients with hepatocellular carcinoma (HCC) (1). Therefore, new treatment options are urgently needed. Recently, the multi-tyrosine kinase inhibitor sorafenib was shown to enhance overall survival in patients with advanced disease (2). However, the median survival for patients with HCC remains below 12 months. In addition, there is also accumulating evidence that immunotherapy might become a potent therapeutic option for patients with HCC (3) for several reasons: First, it has been shown that patient’s survival directly depends on the type and number of tumor infiltrating immune cells (4) (5) (6), indicating that immune responses have a direct effect on the clinical course of the disease. Second, HCC has been shown to be the first cancer, against which a potent vaccine is available. Vaccination against hepatitis B infection has been shown to effectively prevent the development of HCC (7). Third, local-ablative treatment options, which cause physical destruction of tumors, are associated with the release of high antigen load, which could induce anti-tumor immune responses. In addition, more than 80% of all patients with HCC have significant liver cirrhosis. Cohort studies indicate that HCC has become the major cause of liver-related death in patients with compensated cirrhosis (8). Therefore, this patient population might represent an ideal group for a “preventive cancer vaccine”. Interestingly we have been able to demonstrate that HCC patients mount spontaneous tumor-specific immune responses (9) indicating that immune-suppressor mechanisms might counter-balance these immune responses. Indeed, we have been able to detect different types of immune suppressor mechanisms in HCC patients such as regulatory T cells and myeloid derived suppressor cells (10) (11). Recently we have performed a clinical trial with the aim to specifically deplete immune suppressor cells such as regulatory T cells which could potentially augment the effect of either spontaneous or vaccination induced T cell responses in HCC patients. The results from these studies will be presented.

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List of Speakers, Moderators and Scientific Organizers

Prof. Dr. R. Bartenschlager
Molekulare Virologie
Universitätsklinikum Heidelberg
Im Neuenheimer Feld 345
D-69120 Heidelberg
Germany

Dr. K. Breuhahn
Universität Heidelberg
Pathologisches Institut
Im Neuenheimer Feld 220-221
D-69120 Heidelberg
Germany

Prof. Dr. Dr. h.c. mult. M.W. Büchler
Allgemein-/Viszeralchirurgie
Universitätsklinikum Heidelberg
Im Neuenheimer Feld 110
D-69120 Heidelberg

Prof. Dr. P.-A. Clavien
Universitätsspital Zürich
Visceralchirurgie
Rämistrasse 100
CH-8091 Zürich
Switzerland

Prof. Dr. C.P. Day
University of Newcastle
The Medical School
Center for Liver Research
William Leech Building
Framlington Place
Newcastle-upon-Tyne NE2 4HH
Great Britain

Prof. Dr. H.-P. Dienes
Pathologie
Klinikum der Universität zu Köln
Kerpener Str. 62
D-50924 Köln
Germany

Prof. Dr. S. Dooley
Innere Medizin II
Klinikum Mannheim
Theodor-Kutzer-Ufer 1–3
D-68167 Mannheim
Germany

Prof. Dr. T.F. Greten
Abteilung für Gastroenterologie,
Hepatologie und Endokrinologie
Medizinische Hochschule Hannover
Carl-Neuberg-Str. 1
D-30625 Hannover
Germany

Prof. Dr. D. Häussinger
Gastroenterologie/Hepatologie
Universitätsklinikum Düsseldorf
Moorenstr. 5
D-40225 Düsseldorf
Germany

Prof. Dr. J. Iredale
Queen's Medical Research Inst.
Centre for Inflammation Research
47 Little France Crescent
Edinburgh EH16 4TJ
Great Britain

Dr. M.A. Kern
Institut für Pathologie
Universitätsklinikum Heidelberg
Im Neuenheimer Feld 220/221
D-69120 Heidelberg
Germany

Prof. Dr. A.W. Lohse
Medizinische Klinik I
Universitätsklinikum Eppendorf
Martinistr. 52
D-20251 Hamburg
Germany
Prof. Dr. M.P. Manns  
Klinik für Gastroenterologie,  
Hepatologie und Endokrinologie  
Medizinische Hochschule Hannover  
Carl-Neuberg-Str. 1  
D-30625 Hannover  
Germany

Dr. V. Meier  
Gastroenterologie  
Universitätskliniken Göttingen  
Robert-Koch-Str. 40  
D-37075 Göttingen  
Germany

G.K. Michalopoulos, M.D.  
Professor of Pathology  
University of Pittsburgh  
School of Medicine  
Department of Pathology  
S-410 Biomedical Science Tower  
Pittsburgh, PA 15261  
USA

Prof. Dr. D. Moradpour  
C.H.U.V.  
Div. of Gastroenterology  
& Hepatology BB07-2400  
Rue du Bougnon 44  
CH-1011 Lausanne  
Switzerland

PD Dr. M. Müller-Schilling  
Innere Medizin IV  
Universitätsklinikum Heidelberg  
Im Neuenheimer Feld 410  
D-69120 Heidelberg  
Germany

Prof. Dr. M. Ott  
Abteilung für Gastroenterologie,  
Hepatologie und Endokrinologie  
Medizinische Hochschule Hannover  
Carl-Neuberg-Str. 1  
D-30625 Hannover  
Germany

Prof. Dr. M. Pinzani  
Università di Firenze  
Ospedale Careggi  
Fisiopatologia Epatica  
Clinica Medica II  
Viale G.B. Morgagni 85  
I-50134 Firenze  
Italy

Prof. Dr. U. Protzer  
Institut für Virologie  
TU München  
Trogerstr. 30  
D-81675 München  
Germany

PD Dr. R. Reinehr  
Gastroenterologie/Hepatologie  
Universitätsklinikum Düsseldorf  
Moorenstr. 5  
D-40225 Düsseldorf  
Germany

Prof. Dr. K.L. Rudolph  
Klinik für Innere Medizin  
Universitätsklinikum Ulm  
Robert-Koch-Straße 8  
D-89081 Ulm  
Germany

Prof. Dr. P. Schirmacher  
Pathologie  
Universitätsklinikum Heidelberg  
Im Neuenheimer Feld 220/221  
D-69120 Heidelberg  
Germany

D. Schuppan, M.D., Ph.D.  
Professor of Medicine  
Beth Israel Deaconess  
Medical Center  
Harvard Med. School, Dana 501  
330 Brookline Ave.  
Boston, MA 02215  
USA
Prof. Dr. H.-K. Seitz
Innere Medizin
Krankenhaus Salem
und Alkoholforschungszentrum
Universität Heidelberg
Zeppelinstr. 11–33
D-69121 Heidelberg
Germany

Prof. Dr. W. Stremmel
Innere Medizin IV
Universitätsklinikum Heidelberg
Im Neuenheimer Feld 410
D-69120 Heidelberg
Germany

S.S. Thorgeirsson, M.D., Ph.D.
National Institutes of Health
NCI, NIH, MSC 4262
Laboratory of Experimental Carcinogenesis
37 Convent Drive
Bethesda, MD 20892
USA

Prof. Dr. C. Trautwein
Medizinische Klinik III
Universitätsklinikum Aachen
Pauwelsstr. 30
D-52074 Aachen
Germany

PD Dr. S. Urban
Abteilung für molekulare Virologie
Hygiene-Institut
Im Neuenheimer Feld 324
D-69120 Heidelberg
Germany

T. Volz
Universitätsklinikum Eppendorf
Medizinische Klinik I
Martinistr. 52
D-20246 Hamburg
Germany

Dr. L. Zender
Helmholtz-Zentrum für Infektionsforschung
Inhoffenstraße 7
38114 Braunschweig
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Genetic dissection of liver fibrogenesis in BXD recombinant inbred lines (RIL) as a genetic reference population

Rabea Hall, Katrin Hochrath, Susanne Weber, Roman Müllenbach, Frank Grünhage, and Frank Lammert
Department of Medicine II, Saarland University Hospital, Saarland University, Homburg, Germany

Background: Recombinant inbred lines are a viable and a common mouse resource for the investigation of complex diseases. They represent an everlasting genetic reference population that allows repeated phenotyping of genetically identical individuals. Our aim is the analysis of liver fibrosis in BXD RIL-lines as a model that allows integration of our results into a phenotypic and transcription data network.

Methods: Liver fibrosis was induced by challenging BXD RILs twice weekly with CCl₄ (12 injections i.p., 0.7 mg/kg). We determined (i) histological fibrosis stages, (ii) hepatic collagen contents, (iii) gender disparities in fibrosis progression, and (iv) profibrogenic QTLs by genome-wide scans and pair-scans.

Results: The 27 BXD lines display marked differences in fibrosis susceptibility. Fibrosis stages vary from F0 to F3, and strain means of collagen contents range from 181 to 506 µg/g liver. We identified potential QTLs that determine fibrosis susceptibility on chromosomes (Chr) 3 and 12. QTLs that determine line-specific gender differences in fibrosis map to Chr 4 and 14 and epistatic interactions were observed between loci on Chr 1 and 15 as well as Chr 4 and 18.

Conclusions: Hereby we demonstrate that BXD lines provide a reference population for the analysis of profibrogenic susceptibility genes. We conclude that the use of this panel, coupled with transcriptome profiling, will lead to novel insights into the complexity of the genetic control of liver fibrosis and allow modelling of QTL networks during liver injury.
The role of the receptor for advanced glycation end products (RAGE) in hepatic fibrosis

Daniel Neureiter¹, Christina Lohwasser², Yury Popov²,³, Michael Bauer², Detlef Schuppan²,³
¹Institute of Pathology, Paracelsus Private Medicine University, Salzburg, Austria
²Department of Medicine I, University of Erlangen-Nuernberg, Germany
³Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

Introduction: The role of advanced glycation end products (AGE) and their specific receptor (RAGE) in the pathogenesis of liver fibrogenesis is largely unexplored.

Methods: In vitro RAGE and matrix related gene expression in rat and human hepatic stellate cells (HSC) was measured after stimulation with the RAGE ligands AGE-BSA and Nε-(carboxymethyl) lysine (CML)-BSA, or with TNF-α. In vivo RAGE expression was examined in models of hepatic fibrosis due to bile duct ligation and thioacetamide. The effect of AGE-BSA and CML-BSA on HSC proliferation, signal transduction and a panel of profibrogenic and fibrolytic transcripts was studied in rat and human HSC in vitro.

Results: In hepatic fibrosis RAGE expression was enhanced in activated HSC, but also in endothelial, inflammatory and activated bile duct epithelial cells. HSC expressed RAGE which was upregulated after stimulation with AGE-BSA, CML-BSA, and TNF-α. RAGE stimulation with AGE-BSA and CML-BSA did not alter HSC proliferation, apoptosis, fibrogenic signal transduction and fibrosis-related gene expression, except for marginal up-regulation of procollagen-α1(I) mRNA by AGE-BSA.

Discussion/Conclusion: Activation of HSC leads to upregulation of RAGE in vitro and in vivo. However, since RAGE stimulation by AGEs in HSC does not lead to significant changes in parameters related to fibrogenesis or fibrolysis, the AGE-RAGE axis does not contribute directly to hepatic fibrogenesis.
Transcriptomic analysis of TGF-β1 profibrotic impact on primary mouse hepatocytes

Iryna Ilkavets¹, Patricio Godoy², Anastasia Bachmann¹ and Steven Dooley¹
¹Medical Faculty Mannheim, Heidelberg University Medicine II, Gastroenterology and Hepatology, Mannheim, Germany
²Leibniz Research Center for Working Environment and Human Factors – IFADO, Dortmund, Germany

Introduction: Transforming growth factor beta 1 (TGF-β1) is a key effector cytokine in liver fibrosis. Although the intense investigations, the mechanisms of liver fibrosis remain elusive. In this study, we aimed to identify signaling pathways that are differentially regulated upon TGF-β1 stimulation and might serve as markers for enhanced pathophysiological level of TGF-β1 in liver.

Methods: To access TGF-β1 time course response of mouse hepatocytes as well as ALK5-dependent genes in genome wide resolution, we utilized Affymetrix oligonucleotide microarrays containing 45,101 probe sets that correspond to about 25,000 mouse genes. Probe set intensities were normalized with quantile algorithm, and significantly regulated genes were discriminated with one-way ANOVA in Bioconductor, with false discovery rate selected lower than 0.05. Using KEGG and David tools, the significantly changed gene targets were mapped into annotated pathways.

Results: We identified 2,196 differentially regulated genes upon 1, 6 and 24 h treatment of primary mouse hepatocytes with 5 ng/ml TGF-β1. TGF-β1 dependent gene targets were mapped into TGF-β, Wnt, MAPK and Notch signaling as well as ErbB, PPAR and insulin signaling pathways revealing relationships broadly altered in primary mouse hepatocytes. Further, by blocking the TGF-β type I receptor (ALK-5) with SB431542, ALK-5 dependent and independent TGF-β1-responsive genes were discriminated.

Discussion/Conclusion: We conclude that robust genome wide transcriptome analyses of TGF-β1 treated mouse hepatocytes provide an overall signature of the impact of this cytokine, allowing previously unexpected functional predictions. Furthermore, disturbing the system by shutting off specific branches of the pathway may help to identify function/disease related components/steps with drugable potential.
NeoHepatocytes from patients with chronic liver disease can be used for autologous cell therapy

S. Ehnert¹, A. Lehmann², U. Böcker³, S. Dooley³, A. Nüssler¹
¹Department of Traumatology, Klinikum Rechts der Isar, TUM, Munich Germany
²Department of Surgery, University Medicine Berlin, Campus Virchow, Berlin, Germany
³II Medical Clinic, University Medicine Mannheim, Ruprecht-Karls-University Heidelberg, Germany

Introduction: Recently, we have shown that injection of hepatocyte-like cells can increase the survival in rats after extended liver resection. In order to apply this technology in humans with chronic liver diseases in an autologous approach, it is mandatory to verify whether NeoHepatocytes derived from these patients show similar metabolic patterns as NeoHepatocytes generated from healthy controls.

Methods: NeoHepatocytes were generated from monocytes of alcoholic patients and healthy controls and compared to human hepatocytes. We measured glucose and urea production as well as hepatocyte markers and the TGF-beta signaling pathway using PCR, Western blot and reporter assays.

Results: The yield of monocytes from patients (2.2 ± 0.8*10⁷ cells/ml blood) and controls (2.5 ± 0.9*10⁷ cells/ml blood) was comparable. There were no obvious morphological differences between the 2 sets of NeoHepatocytes after 20 days of differentiation. Although albumin expression remained very low, other hepatocyte markers, e.g. cytokeratin-18, transferrin and ADH1, increased significantly. Glucose and urea production was comparable with primary hepatocytes. We could show fat accumulation by insulin, TGF-beta and ethanol in NeoHepatocytes and hepatocytes, but not in monocytes and PCMOs. TGF-beta signaling was comparable among the investigated cell types. However, Smad1 and 3 expression was reduced in NeoHepatocytes (~ 30 and ~ 60%). Subsequently, expression of TGF-beta regulated genes, e.g. CTGF, fibronectin and collagen was lower.

Discussion/Conclusion: We could successfully generate NeoHepatocytes from patients with chronic liver disease. Cell quality and function was comparable with healthy controls, suggesting that these cells may be useful to bridge time with an autologous setting, till a suitable organ for transplantation is available.
Exploring the role of hepatocyte damage in liver fibrosis

Katrin Hochrath¹, Roman Müllenbach¹, Steven Dooley², and Frank Lammert¹
¹Klinik für Innere Medizin II, Universität des Saarlandes, D-66421 Homburg/Saar, Germany
²Medizinische Fakultät Mannheim, Universität Heidelberg, D-68167 Mannheim, Germany

Introduction: Genetic variation leads to individual differences in the fibrotic response to various environmental impacts (alcohol, virus, fat, toxins), resulting in different levels of predisposition.

The aim of our work is to identify predisposing genetic factors. Hepatocyte-specific gene knockouts of genes involved in pro-fibrogenic signalling (Dooley et al. 2008) and apoptosis have been shown to modify fibrogenesis in various mouse models.

The hepatocytes’ demise not only initiates fibrogenesis by activation of Kupffer cells and hepatic stellate cells, but may contribute to the pool of fibrogenic cells by undergoing epithelial-mesenchymal transition (EMT).

We have previously identified genetic loci modifying sensitivity to CCl₄-induced liver fibrosis by QTL mapping in experimental mouse crosses (Hillebrandt et al. 2002) and subsequently humans (Hillebrandt et al. 2005).

Hypothesis: Differential susceptibility of hepatocytes to damage induced by chemicals, viruses or cytokines is a contributing factor to fibrosis susceptibility.

Methods: Cultured primary hepatocytes from inbred mouse strains are challenged with CCl₄ or TGF-beta, hepatocytes from Abcb4 knockout mice with taurocholate, and the resulting cell damage is quantified by measuring levels of released lactate dehydrogenase (LDH) in relation to total LDH.

Results from CCl₄ challenge are correlated with fibrosis scores from the same inbred strains after CCl₄ i.p. injection (Hillebrandt et al. 2002).

Results: Damage assessment in cultured hepatocytes after challenge with TGF-beta reveals a highly variable response, indicating sufficient variation between inbred mouse strains to make QTL mapping of predisposing factors in various damage models a feasible strategy.
Electron microscopic study of the liver stem cells in children with chronic hepatitis B with a focus on liver fibrosis. The first pediatric analysis

Joanna M. Lotowska¹, Maria E. Sobaniec-Lotowska¹, Dariusz M. Lebensztejn²
¹Department of Clinical Pathomorphology, ²IIIrd Department of Pediatrics, Medical University of Białystok, Poland

Introduction: It is assumed, that liver stem cells (syn. liver progenitor/oval cells) constitute a heterogenic cell population accounting for 1–3% of the normal liver cell pool, which is located mainly in periportal areas. They exhibit a two-directional differentiating ability, which in the course of regeneration and hepatocarcinogenesis is a major source of precursor cells both for hepatocytes and for epithelial cells of bile ductules. The most common pathological process involving the liver stem cells occurs in chronic hepatitis in the areas of regeneration/proliferation, as well as during the phase of fibrosis and structural reorganization of hepatic parenchyma. The main aim of the study is ultrastructural assessment of oval cells in children with chronic hepatitis B, with special emphasis on their location in areas of fibrosis. It is the first report on pediatric material.

Methods: Morphological investigations were conducted on the liver biopsy material obtained from 40 children aged 3–16 years with chronic hepatitis B. The stage of fibrosis was earlier assessed histologically using the arbitrary semiquantitative numerical scoring system according to Ishak et al., currently used in hepatology. The material for ultrastructural investigations was fixed in glutaraldehyde and paraformaldehyde, routinely processed for transmission-electron microscopic analysis and examined using an Opton 900 PC microscope.

Results: Ultrastructural examination of biopsy specimens obtained from children with chronic hepatitis B showed the presence of two types of oval cells, i.e. hepatic progenitor cells (HPCs) and intermediate hepatocyte-like cells (IHCs) in some fragments of the parenchyma, especially in areas of intensive peripoortal fibrosis (at least stage 2 according to Ishak). These cells were most commonly found in the vicinity of the limiting plate of the lobule. IHCs were bigger than HPCs, even twice. Their cell nuclei were less abundant in heterochromatin than progenitor cell nuclei. They often resembled cell nuclei of mature hepatocytes. The IHC cytoplasm showed lower electron density than that of HPCs and contained more better developed cell organelles, mainly mitochondria and elements of the endoplasmic reticulum, with prevalence of the granular endoplasmic reticulum channels. Among the intracellular organelles, small structures could be seen that might correspond to newly formed peroxisomes. Sometimes the cells showed apical alterations in the form of newly formed capillary bile canaliculi. Worthy of notice is that activated nonparenchymal hepatic cells, i.e. transformed hepatic stellate cells and Kupffer cells, were seen very close to the IHCs.

Discussion/Conclusion: We found a distinct relationship between the prevalence of oval cells (HPCs and IHCs) and fibrosis stage in pediatric patients with chronic hepatitis B.
Small hepatocytes in cell therapy as treatment for acute liver diseases

Daniel Knobeloch\textsuperscript{1}, Patrick Kupczyk\textsuperscript{1}, Antje Lehmann\textsuperscript{1}, Matthias Glanemann\textsuperscript{1}, Nian Liu\textsuperscript{2}, Sabrina Ehnert\textsuperscript{3}, Andreas Nüssler\textsuperscript{3}
\textsuperscript{1}Department of General Surgery, Charité Campus Virchow, Berlin, Germany
\textsuperscript{2}Department of Environmental Medicine, Tongji Medical University, Wuhan, China
\textsuperscript{3}Department of Traumatology, Technical University Munich, Germany

\textbf{Introduction}: Intoxication and other causes which lead to acute liver damage is associated with a high mortality unless liver transplantation is feasible. Cell based therapies is a possible alternative to stabilize patients. However, one limitation of cell-based therapies is the availability of functional human hepatocytes. Several methods to overcome this cell limitation were investigated, but only the use of hepatic precursor cells may be a real alternative. In the adult human liver, two candidates of progenitor cells have been identified: oval cells and small hepatocytes (SH cells). We describe here, for the first time, the isolation and characterization of human SH cells.

\textbf{Methods}: SH cells are isolated by a Percoll based multistep centrifugation technique and cultured in a serum free medium. First characterizations were done by immunofluorescence staining and polymerase chain reaction.

\textbf{Results}: The cells can be maintained for up to two months without losing their ability to proliferate. First results have shown that a better proliferation is achieved when human foreskin fibroblasts are used as a feeder layer rather than collagen coated surfaces. These cells are positive for albumin, transferrin and cytokeratin 18. Preliminary data suggest a week expression of cytochrome P450 1A2 and 3A4. Additionally, they do not express alpha fetoprotein. Also CD 90 is not expressed at detectable levels.

\textbf{Discussion/Conclusion}: Several markers show that SH cells possess a close relationship to adult hepatocytes. The investigated markers of these cells present also that they are not tumor- or stem cell like. This SH cells are due to the proliferation capacity with access at any time. Further detailed characterization of SH cells may help to determine the relationship between oval cells and adult hepatocytes. This will, consequently, reveal the future potential use of SH cells in the regeneration of acute damaged livers.
Improvement of NeoHepatocyte differentiation and function by using autologous serum

Sara Saidy-Rad¹, Helen Vester¹, Sabrina Ehnert¹, Jürgen Burkhart², Wolfgang Thasler³, Ulrich Stöckle¹, Andreas Nüssler¹
¹Department of Traumatology, MRI, TU Munich, Munich, Germany
²Blood Donation Service of the Bavarian Red Cross, Munich, Germany
³Department of General Surgery, LMU Munich, Munich, Germany

Introduction: In Germany over 3 Million patients are suffering from chronic liver disease, with clinical manifestations from fibrosis to cirrhosis. Liver transplantation is the only definitive treatment for end stage liver disease. Donor organ scarcity raises a growing interest in new therapeutic options, e.g. transplantation of NeoHepatocytes. Aim of this study was to optimize the yield and the function of NeoHepatocytes for autologous cell therapy.

Methods: PBMCs, isolated of whole blood from the blood bank by density gradient centrifugation, were dedifferentiated to "programmable cells of monocytic origin" (PCMOs) and after that differentiated to NeoHepatocytes in the presence of different AB sera and autologous serum. The amount of PCMOs was quantified with sulforhodamin B, while their activation, which probably has an effect on the differentiation, was measured with PCR and zymogram of the supernatant. Phase I and II enzyme activities of NeoHepatocytes were measured with fluorescent based assays. NeoHepatocytes were further stained for fat after insulin treatment and glucose-6-phosphatase.

Results: Isolating the PBMCs 24 hours after the blood donation had no effect on differentiation potential. However, the yield was lower. The amount of PCMOs, their activation and the enzyme activity of NeoHepatocytes varied considerably depending on the different sera. With the autologous approach, constantly the highest amount of cells and peak activity of phase I and II enzymes were detected. Only NeoHepatocytes in the autologous setting were entirely stained for glucose-6-phosphatase like human hepatocytes. Fat droplets were visible only in cells cultured with autologous serum and in human hepatocytes.

Discussion/Conclusion: Our data demonstrate that use of autologous serum improves both the yield and the function of NeoHepatocytes. This is probably due to a lower activation of PCMOs. The possibility of creating NeoHepatocytes from whole blood is a first step towards a possible clinical application especially using the autologous approach.
Modulation of miRNAs by bile acids in the context of liver regeneration

R.E. Castro¹,², Y. Zeng³, C.J. Steer²,⁴, B.T. Kren², and C.M.P. Rodrigues¹
¹Med. UL, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal; Departments of ²Medicine, ³Pharmacology and ⁴Genetics, Cell Biology, and Development, University of Minnesota Medical School, Minneapolis, Minnesota, USA

Introduction: Bile acids (BA) are strong modulators of apoptosis and proliferation in hepatocytes. In addition, BA modulate mRNA levels during liver regeneration. Here, we used microRNA (miRNA) analysis to gain insight into the mechanisms by which BA modulate cell fate and how this impacts on liver regeneration.

Methods: RNA was isolated from livers harvested at 3–72 h, following 70% partial hepatectomy (PH) or sham operations, from either 0.4% UDCA or control diet-fed rats. miRNA expression profiles were determined using a custom microarray platform; real time RT-PCR was used for validation. miRNA-21 and target protein Programmed Cell Death 4 (PDCD-4) were evaluated in isolated primary rat hepatocytes, following BA exposure for 4–48 h. In functional studies, miRNA-21 was overexpressed by transfecting cells with a specific miRNA-21 precursor.

Results: Our results showed that miRNAs involved in regulating cell fate were significantly modulated during LR. Real time RT-PCR analysis confirmed the specific increase of miRNA-21 after PH in rats fed either UDCA or control diets. In rat hepatocytes, miRNA-21 expression was slightly increased in control and UDCA exposed cells. Interestingly, deoxycholic acid (DCA) was a stronger modulator of miRNA-21, decreasing expression up to 50% at 48 h. Consistently, PDCD-4 was significantly increased in cells incubated with DCA. Finally, miRNA-21 overexpression inhibited PDCD-4 protein expression, increased cell viability, and prevented DCA-induced PDCD-4 expression and cell death.

Discussion/Conclusion: In conclusion, BA modulate miRNA expression, in particular miRNA-21, in both regenerating rat liver and primary hepatocytes. In addition, repression of miRNA-21 by DCA may contribute for its pro-apoptotic role through PDCD-4. Ultimately, a better knowledge of the mechanisms by which BA modulate cell fate, as well as the switches that control liver regeneration, may have broad implications for developing novel therapeutic options.

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Fluvastatin can reduce the hepatitis C virus-RNA and the pro-inflammatory cytokines

R. Mihăilă1, E.C. Rezi2, C. Domnariu3, R. Ciucă4, A. Zaharie2, L. Bera1, M. Deac1, R. Mihăilă5
1”Lucian Blaga University”, Sibiu, Romania
2County Clinical Emergency Hospital, Sibiu, Romania
3Public Health Centre, Sibiu, Romania
4C.S. Casa Paltinul L.R.S., Sibiu, Romania
5Public Health Authority Sibiu, Romania

Introduction: Studies made in vitro showed that there are statins that inhibit the geranylation and farnesylation of certain proteins, which are responsible for the dissolution of the hepatitis C virus (HCV) replicative complex. We aimed at studying the effect of fluvastatin on the HCV viremia and of the hepatic pro- and anti-inflammatory serum cytokines.

Methods: We took into our study 22 consecutive patients with chronic viral hepatitis C, who were treated with fluvastatin 40 mg/day, for 28 days. The following hepatic biochemical tests were performed before and after the treatment: IL-6, IL-8, TNF-alpha, IL-10, HCV RNA (through real time PCR), cholesterolemia, triglyceridemia, creatininemia, glycemia and hemogram.

Results: The average age of the patients was 49.95 ± 11.35 years. Gender distribution counted 13 women and 9 men. Average viremia decreased from 3024177.27 ± 4861526.3 UI/ml to 956477.27 ± 1102873.40 UI/ml (p = 0.028). The average values of the IL-6, IL-8, TNF-alpha decreased after the treatment significantly from the statistics point of view (p < 0.001, p < 0.000001 and respectively p < 0.0001). IL-10 did not significantly vary between the two determinations (5.63 as against 5.85). ALAT in only one patient and ASLT in 2 patients reached values three times over the upper limit of the normality. The rest of the biochemical tests did not vary significantly between the two determinations.

Discussion/Conclusion: Due to the fact that fluvastatin significantly reduced the value of the HCV viremia, as well as the serum levels of the hepatic pro-inflammatory cytokines, without influencing the IL-10 level (anti-inflammatory cytokine), it might represent a therapeutic option in the patients with chronic viral hepatitis C, who have no indication for interferon, and who relapsed after the classic antiviral treatment.

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Study on the correlation between APRI score and Forns index to viremia, pro- and anti-inflammatory cytokines and the hepatic biochemical tests in the patients with chronic viral hepatitis C

R. Mihăilă¹, R. Mihăilă², E.C. Rezi³, C. Domnariu⁴, R. Ciucă⁵, A. Zaharie³, L. Bera², M. Deac²
¹Public Health Authority Sibiu, Romania
²“Lucian Blaga University”, Sibiu, Romania
³County Clinical Emergency Hospital, Sibiu, Romania
⁴Public Health Centre, Sibiu, Romania
⁵C.S. Casa Paltinul L.R.S., Sibiu, Romania

Introduction: We aimed at studying, whether fibrosis, non-invasively estimated through the APRI score and Forns index, may be correlated to viremia, pro and anti-inflammatory cytokines and the hepatic biochemical tests in the patients with chronic viral hepatitis C.

Methods: We took into our study 25 consecutive patients with chronic viral hepatitis C, who after randomization, were treated with lovastatin 20 mg/day or with fluvastatin 40 mg/day, for 28 days. The following hepatic biochemical tests were made before and after the treatment: IL-6, IL-8, TNF-alpha, IL-10, hepatitis C virus RNA (through real time PCR), cholesterolemia, triglyceridemia, creatininemia, glycemia and hemogram and the patients were initially examined ultrasoundographically from the hepatic point of view. We studied the correlation between them and the APRI score and Forns index through the Pearson test.

Results: The average life of the patients was 55.64 ± 11.36 years. Gender distribution counted 17 women and 8 men. APRI score average was 1.22 ± 1.12 and 28% of the patients recorded significant hepatic fibrosis (score > 1.2). The average of the Forns index was 8.74 ± 2.04, and 84% of the patients registered important hepatic fibrosis (p > 6.9). APRI score was correlated directly in decreasing order to ASATi (r = 0.913), the degree of the posterior hepatic attenuation of ultrasounds, ALATi, long axis of the spleen, IL-10f, IL-6f, age, IL-6i, TNF-alpha i, FASI, IL-10i, hepatic echogenity, and inversely with viremia i and the post-therapeutic decrease of viremia. Forns index was directly correlated in decreasing order to: the degree of posterior hepatic attenuation of ultrasounds (r = 0.684), age, TNF-alpha i, ASATi, TNF-alpha f, ALATi, total bilirubin i, hepatic echogenity, IL-6f and inversely with the post-therapeutic decrease of viremia, viremia i and the portal vein diameter.

Discussion/Conclusion: Although the two methods for estimating fibrosis found a different proportion of significant hepatic fibrosis, both of them are directly correlated to some of the echographic markers of the portal hypertension and some pro- and antiinflammatory hepatic cytokines and inversely to the initial viremia and its post-therapeutic decrease.

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Cytokine expression profiles in the liver of individuals chronically infected with hepatitis B virus do not correlate with virological and biochemical parameters

Marc Lütgehetmann¹, Jan-H. Bockmann¹, Tassilo Volz¹, Ansgar W. Lohse¹, Jörg Petersen¹ and Maura Dandri¹
¹Department of Medicine, University Hospital Hamburg-Eppendorf, Germany

Introduction: Experiments in chimpanzees and transgenic mice have shown that inflammatory cytokines can suppress viral replication. Aim of this study was to analyze expression profiles of intrahepatic cytokines in HBV-chronically infected patients and correlate values with virological and biochemical parameters.

Methods: Nucleic acids were extracted from 17 treatment naïve CH-B liver biopsies (9 HBeAg-positive and 8 HBeAg-negative) and 4 healthy controls. Viral activity (rcDNA/cccDNA) and cytokine expression levels were measured by real-time PCR.

Results: All patients had active liver disease with ALT ranging from 0.2–3.6 ULN, viremia ranged from 100 to 4 x 10⁹ copies/ml and intrahepatic activity ranged between 3.2 and 42 rcDNA/cccDNA. Results: We found upregulation of TNFα, lymphiotxin-α and IFNγ in CH-B livers compared to healthy controls (p = 0.039, p = 0.025 and p = 0.016 respectively), while IL10 expression was significantly downregulated (p = 0.016), indicating predominance of TH1 cytokine profile in liver of CH-B individuals. However, expression levels of TNFα, TGFβ and IFNγ did not correlate with HBV replicative activity and levels of IFNα, IFNβ, TGFβ, IL1, IL2, IL8, IL12, CCL2, CXCL10 and CSF2 did not differ from healthy controls, supporting the notion that HBV is a “stealth” virus. Furthermore there was no correlation between ALT and levels of TH1 cytokines. Surprisingly, expression of IL6, which mediate activation of adaptive immunity, was significantly lower (p = 0.039) in infected livers compared to controls.

Discussion/Conclusion: The lack of correlation between viral activity and cytokines indicate that the antiviral effect achieved by these cytokines within the chronic phase of infection is not sufficient to significantly suppress HBV productivity.
Growth of hepadnavirus infected hepatocytes strongly reduces virion productivity and cccDNA loads in uPA mice

Tassilo Volz¹, Marc Lütgehetmann¹, Tim Broja¹, Ansgar W. Lohse¹, Jörg Petersen¹, Maura Dandri¹
¹Department of Medicine, University Hospital Hamburg-Eppendorf, Germany

Introduction: Chronic hepatitis B virus (HBV) infection is assured by maintenance of the covalently closed circular DNA (cccDNA), the template of viral transcription. In quiescent hepatocytes cccDNA is a stable molecule which can persist throughout the life span of the hepatocytes. However, immune mediated cell division may favour cccDNA dilution in the course of chronic HBV infection. Aim of this study was to investigate stability and activity of the cccDNA in infected hepatocytes undergoing cell division in vivo.

Methods: Primary tupaia hepatocytes (PTH) chronically infected with woolly monkey (WM-) HBV were isolated from high viremic (10^{9} WM-HBV DNA/ml) immuno-deficient uPA mice. Cell suspension consisted of 50% PTH and was re-transplanted into 20 uPA recipients. PTH engraftment and proliferation were monitored by immunohistochemistry and real-time PCR.

Results: Intrahepatic viral loads (rcDNA and cccDNA/PTH) were determined by RT-PCR at different time points and loads were compared to levels estimated in cell suspension, where all PTH appeared HBCAg-positive and harboured 1000rcDNA and 2.5 cccDNA per PTH. Strong expansion of engrafted PTH was demonstrated by PCNA staining 5 days after transplantation and continued up to 40 days. Cell growth induced strong reduction (median 2-log) of intrahepatic viral loads in all mice analysed, which was accompanied by strong reduction of pgRNA. Notably, a rapid and significant reduction (Δ1-log) of cccDNA levels occurred while PTH repopulated diseased mouse livers.

Discussion/Conclusion: This study reveals that cell division in the setting of liver regeneration causes rapid reduction of viral productivity and cccDNA dilution in chronically infected hepatocytes.
MicroRNA involved in chronic liver disease of hepatitis C infected patients

Melanie Scheffler, Ali Manav, Ingo Strack, Ulrich Töx*, Uta Drebber, Inga Wedemeyer, Hans Peter Dienes and Margarete Odenthal Institute for Pathology and *Department of Gastroenterology and Hepatology University Hospital Cologne, Cologne, Germany

Introduction: MicroRNAs (miRNAs) are short regulatory RNA that result in posttranscriptional gene repression and are involved in cell differentiation and carcinogenesis. In the present study we analysed miRNA, which we have previously identified by an altered expression profile during myofibroblastic transition of HSC, in chronic hepatitis.

Methods: For induction of experimental fibrosis, bile duct ligation rat model was performed. In addition, 84 liver biopsies representing different stages of fibrosis (S1/n = 21, S2/n = 24, S3/n = 17, S4/n = 9) were analysed. miR-122, miR-125b, miR-22, miR-143 were analysed by reverse transcription real-time PCR of total polyadenylated RNA, which was extracted by the Trizol method from native murine liver samples and from formalin-fixed, paraffin-embedded (FFPE) human liver biopsies.

Results: During fibrogenesis in rat and in man, the liver-specific miRNA 122 was repressed. However, miR-143, which was differentially expressed during myofibroblastic differentiation, did not alter in chronic hepatitis C or experimental fibrosis induced by cholangitis. In contrast, miR-22 and miR-125b levels were significantly reduced during progression of fibrosis (p < 0.05), in both, in experimental fibrosis and in chronic hepatitis C.

Discussion/Conclusion: miRNAs will be subject of further studies because they are suggested to be involved in the mechanisms of liver fibrosis and might serve as new diagnostic and therapeutic targets.
**MicroRNA expression in patients with chronic hepatitis C biopsies and therapy with peginterferon**

M. Kölz¹, A. Manav¹*, M. Scheffler¹*, M. Odenthal¹, F. Wrba², H. Hofer³, P. Ferenci³, H. Holzmann⁴ and Hans-Peter Dienes¹

¹Institute for Pathology, University of Cologne, Kerpenerstr. 62, D-50924 Cologne, Germany
²Clinical Institute for Pathology, Medical University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna, Austria
³Internal Medicine 3, Department of Gastroenterology and Hepatology, Medical University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna, Austria
⁴Institute of Virology, Medical University of Vienna, Kinderspitalgasse 15, A-1090 Vienna, Austria

*¹Authors are equally distributed.

**Introduction**: MicroRNAs are a class of small noncoding RNA molecules that control gene expression on a posttranscriptional level. Previous data has shown that in response to HCV infection interferon can induce certain cellular microRNAs (1, 30, 448) in human hepatoma cells, Huh 7. Additionally microRNA 122 has been identified as a highly liver specific microRNA, facilitating HCV replication. In order to study the role of these interferon induced microRNAs in predicting a trend to clinical response to treatment with peginterferon we analysed the expression of 2 of these microRNAs (30 and 448) and the liver specific microRNA 122 in liver biopsies of HCV-patients before treatment.

**Methods**: The expression of microRNAs 122, 30 and 448 were analysed in 94 formalin fixed and paraffin embedded (FFPE) liver biopsies of HCV patients by real-time-PCR. The response status of the patients as well as the HCV serum level and histologic parameters (fibrosis according to Ludwig and activity by Metavir) were compared with the expression level of these microRNAs.

**Results**: Nonresponder showed a slightly reduced level of microRNA 122 as well as patients with a higher Metavir score but that was not statistically significant. However, microRNA 122 was significantly reduced in biopsies with a higher grade of fibrosis. MicroRNA 30 and 448 did not alter in the different response groups. Furthermore, all 3 microRNAs did not show a correlation with the HCV serum level.

**Discussion/Conclusion**: In conclusion our data show that the microRNA level in biopsies before treatment do not significantly alter in the different clinical response groups.
Lack of an association between TLR3 gene polymorphisms and response to IFN-α therapy in chronic HCV infection

Eva Askar, Giuliano Ramadori, Sabine Mihm
Georg-August-Universität, Department of Internal Medicine, Division of Gastroenterology and Endocrinology, Robert-Koch-Str. 40, D-37075 Göttingen, Germany

Introduction: The natural outcome of hepatitis C virus (HCV) infection is highly variable and ranges from asymptomatic to mild disease to cirrhosis and hepatocellular carcinoma. There is a marked inter-individual variability regarding disease progression, the host’s immune response, and the outcome of an interferon-α (IFN-α) therapy. Toll-like receptor 3 (TLR3) senses double-stranded RNA, which is both a secondary structure of the HCV genome and an intermediate of the viral replication cycle. This study aimed at analyzing biochemical parameters of liver disease and responsiveness to an IFN-α based therapy with regard to two single nucleotide polymorphisms (SNPs) within the TLR3 gene, namely rs5743305 (T/A) within the promoter region and rs3775291 (C/T) within exon 4.

Methods: Biochemical parameters and the initial virological responsiveness to an IFN-α therapy were recorded for a total of 142 patients with chronic hepatitis C. Genomic DNA was purified from peripheral blood mononuclear cell or serum samples. Genotyping of the respective SNPs was applied by allelic discrimination using commercially available TaqMan genotyping assays.

Results: The TLR3 promoter and exon 4 SNP genotypes (TT:TA:AA) and (CC:CT:TT) followed the distribution of 54:62:26 and 60:68:14, respectively. Thus, genotype distributions and minor allele frequencies were similar to that given for Caucasians in public database and no deviation from Hardy-Weinberg equilibrium was found (p = 0.2955 and 0.4581), respectively. We found no significant association of TLR3 genotypes with demographic parameters, biochemical parameters of liver disease, or the outcome of an IFN-α based therapy.

Discussion/Conclusion: Data thus argue against a predictive impact of the TLR3 SNPs under study for liver disease parameters or therapy responsiveness in chronic HCV infection.
Antihypertensives reduce ethanol and TGF-beta induced cellular damage of mouse hepatocytes

*Department of Medicine II, University Hospital at Mannheim, University of Heidelberg; Germany
**Department of Traumatology, Technical University Munich, Germany
***Department of Medicine, Salem Medical Center, University of Heidelberg, Germany

Introduction: Alcohol dependent liver damage is continuously increasing in developed countries and is a leading cause of death worldwide. Chronic alcohol uptake is reported to induce synthesis and activation of TGF-beta. The resulting signal transduction in liver cells is critically required for progression of liver disease.

Methods: In mouse hepatocytes, we determined cellular damage by LDH release, reactive oxygen species (ROS) production, glutathione (GSH) reduction and Annexin V staining. Neutral lipids were visualized by Oil Red O staining. TGF-beta signaling was analyzed by PCR, Western blot and adenovirus based Smad3 reporter assay.

Results: Ethanol, as well as TGF-beta, rapidly induces oxidative stress in primary hepatocytes. Along with an increase in ROS and reduction of cellular GSH, about 45% of the total LDH is released into the culture supernatant after 72 h. Annexin V staining identified the majority of the cells affected to be apoptotic. The antihypertensives Amlodipine, Captopril, Furosemide, Metoprolol, Propanolol and Spironolactone reduced oxidative stress and LDH release in a dose dependent manner.

These antihypertensives seem to induce expression of heme oxygenase-1 (HO-1), which buffers oxidative stress. Consequently blocking of HO-1 activity by ZnPP9 reduced the protective effect of the antihypertensives on ethanol/ TGF-beta-dependent cellular damage.

Furthermore, all substances altered TGF-beta signaling, shown by Western blot and Smad3/4 reporter assay.

Discussion/Conclusion: Our results suggest that medication with Amlodipine, Captopril, Furosemide, Metoprolol, Propanolol and Spironolactone might have strong influence on the progression of fibrotic liver disease and thus these patients could benefit from an adjusted antihypertensive therapy.
Up-regulation of hepatic heme-oxygenase-1 mRNA expression in IL-6 knock out mice during acute phase inflammation

Ghayyor Ahmad, Pierluigi Ramadori, Giuliano Ramadori
Georg-August-Universität, Department of Internal Medicine, Division of Gastro-enterology and Endocrinology, Robert-Koch-Str. 40, D-37075 Göttingen, Germany

Introduction: In a previous study, we have shown a time- and dose-dependent IL-6 induced up-regulation of heme-oxygenase-1 (HO-1) mRNA expression during acute phase inflammation. Accordingly, Yamaji et al. (2008) described a significant reduction of HO-1 mRNA and protein expression in animals receiving CCl₄ intraperitoneal injection after a pre-treatment with anti IL-6 antibodies. In the current work, we aimed to investigate the HO-1 expression during acute phase conditions in IL-6 knock out mice.

Methods: Male IL-6 knock-out mice (B6.129S2-IL6tm1Kopf/J) and wild-type C57 Black6 mice 6–8 weeks old were injected intramuscularly 10 ml/kg turpentine oil. Alternatively, mice were intraperitoneally injected 50 µg of LPS. Animals were sacrificed at different time points after treatment, using pentobarbital sodium anaesthesia. Livers of the animals were excised, and processed for further analysis. Sera were analyzed for IL-6 concentration with ELISA.

Results: Hepatic HO-1 mRNA levels were found to be increased 40- to 45-fold in IL-6 knock out mice and 15- to 65-fold in wild type mice, respectively. The serum IL-6 levels of wild type mice were also elevated up to 5,000 pg/ml and were found to be closely correlated to HO-1 expression in these animals.

Discussion/Conclusion: Though IL-6 plays an important role in the development of acute phase response and is also considered the main regulator of HO-1 expression, the results obtained from the current work suggest also an alternative mechanism for HO-1 up-regulation during acute phase inflammation.
Prevalence of anti-hepatitis E virus antibodies in blood donors from Saxony, Germany

Chwan-Heng Wang¹²*, Anna Wang², Hong-Sheng Wang¹, Shu-Yuan Tschen²
¹Department of Research and Diagnosis, Dr. Wang GmbH, Tübingen, Germany
²Department of Validation and Surveillance, Dr. Wang GmbH, Zurich, Switzerland

Introduction: Hepatitis E virus (HEV) is a non-enveloped, single stranded RNA virus associated with enterically transmitted epidemic of acute, self-limiting hepatitis. Recently, HEV was shown to be an etiologic agent of chronic hepatitis in organ-transplant recipients. A number of chronic cases in immunocompromised patients have been described where the traditional risk factors are not defined (Kamar N et al., N Engl J Med. 2008; 358; 811–7). Due to prolonged viremic phase after infection, HEV has emerged as a member of transfusion-transmitted infection (Colson P et al., Emerg Infect Dis. 2007; 13: 648–9). In this study, we took an attempt to evaluate the seroprevalence of HEV in healthy adult population of blood donors in Saxony, Germany.

Methods: A total of 300 serum samples collected in the years 2006 and 2007 from healthy blood donors were tested for HEV antibodies (IgM and IgG) and antigen by enzyme-linked immunosorbent assay (ELISA). IgG titre was measured according to different reference standards (100 IU/ml, 200 IU/ml, 300 IU/ml, and 400 IU/ml). According to our protocols, the sensitivity of our HEV antigen assay is equivalent to non-nested polymerase chain reaction (PCR) assay.

Results: Overall 15% were found to be positive for hepatitis E virus IgG antibodies. 25% of these samples had high (> 300 IU/ml) and 75% low avidity (< 200 IU/ml). In addition, 2% of the plasma was shown to be positive IgM as well as antigen.

Discussion/Conclusion: Our study showed a high prevalence of HEV antibodies in healthy blood population living in Saxony, Germany. Subclinical infected donors carrying infectious HEV may be present in our regions. Transfusion by bloods derived from those persons could associate with serious diseases. To prevent any possible transmission, therefore, it may be appropriate to include screening for HEV also in the pretransfusion blood testing schedule.
Appearance of anti-mitochondrial antibodies in hepatitis E infected patients: Initiation of chronic mechanism in genetically predisposed individuals with supporting immune environment

Chwan-Heng Wang¹, Hong-Sheng Wang², Anna Wang³, Shu-Yuan Tschen¹,³
¹Department of Research and Diagnosis, Dr. Wang GmbH, Tübingen, Germany
²Department of Emergency, Nanjing Tong-Ren Hospital, Nanjing, China
³Department of Validation and Surveillance, Dr. Wang GmbH, Zurich, Switzerland

Introduction: Anti-mitochondrial antibodies (AMAs) are the serological hallmark for chronic autoimmune hepatitis (AIH) and/or primary biliary cirrhosis (PBC) (Hepatology. 2008; 48: 550). Hepatitis E virus (HEV) is an emerging disease in industrialized countries. Recently, scientists conclude that HEV infection cannot only evolve to chronic hepatitis, but also be responsible for rapidly progressing cirrhosis in immune-suppressed patients (Am J Transplant. 2008; 8: 1744). Till now, however, no literature regarding association between AMAs and HEV can be cited. In this report, we described for the first time detection of AMAs in the course of HEV infection.

Methods: The presence of freeze-stored sera allowed for a retrospective trace-back for HEV infection story. Serological diagnosis of HEV was done by the assaying for the presence of anti-HEV IgM/IgG and RNA. Anti-SMA (smooth muscle antibodies), ANAs (anti-nuclear antibodies), and AMAs were determined via indirect immunofluorescence and ELISA.

Results: Appearance of AMAs in four cases of HEV patients has been observed. Investigations for other viral infections (HAV, HBV, HCV, HCMV, EBV) and alcoholic fatty liver disease were negative. The median alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were 219 U/L and 125 U/L, respectively. The median AMA titer was 68 IU/ml.

Discussion/Conclusion: This result supports the hypothesis that HEV infection could be a potential inducer of AMAs and therefore may pay a role in the chronicity of liver diseases and/or pathogenesis of PBC in genetically predisposed individuals with supporting immune environments. If AMAs detected in patients with HEV infection have pathological significance under changing immune conditions in the long-term should be assessed. Clarification of this causal relationship may have important relevance to both treatment and management.
Hepatic HIF-1 alpha gene expression in an aseptic model of acute phase response: A role for IL-6

Pierluigi Ramadori, Ghayyor Ahmad, Giuliano Ramadori
Georg-August-University, Department of Medicine, Division of Gastroenterology and Endocrinology, Göttingen, Germany

Introduction: The liver is considered to be the main source of acute phase proteins. It has recently become apparent that hypoxia inducible factor (HIF), as well as being responsive to hypoxia, can also be activated in response to a number of non-hypoxic stimuli including bacterial LPS, TNF-alpha, HGF, and reactive oxygen species (ROS).

Methods: In the present work, we used a rat and murine model of intra-muscular Turpentine Oil (TO)-injection to induce an acute phase response. The same model was performed with the use of IL-6 knockout mice. HIF-1 and HIF-2 alpha expression was investigated at mRNA and protein level by RT-PCR, western blot and EMSA analysis in the non-injured livers.

Results: In rat livers HIF-1 gene expression started to increase increased 2 h after the injection with a peak of expression between 4 h and 6 h (4.7 ± 1.7 and 4.4 ± 0.8 respectively). The protein levels also showed a clear increase from the 4 h until 24 h with a successive decline. No changes in HIF-2 mRNA or protein levels were detected. In murine livers, the number of transcripts observed in IL-6 KO animals was strongly reduced compared to the wild type strain (only 2.7-fold increase versus 6.4-fold increase at the peak of expression respectively) as well as the protein expression.

Discussion/Conclusion: These results indicate that during aseptic acute phase response HIF-1 expression is induced at mRNA and protein level in the liver and that this induction might depend mainly on IL-6 pathway.
Association of CD14 gene single nucleotide polymorphisms rs2569190 (C→T) and rs5744455 (C→T) with transcriptional activity and sCD14 serum concentration in healthy subjects and hepatitis C patients

R. Bregadze, A. Mansur, G. Ramadori, S. Mihm
Georg-August-University, Internal Medicine, Gastroenterology and Endocrinology, Göttingen, Germany

Introduction: The single nucleotide polymorphism (SNP) rs2569190 within the CD14 endotoxin receptor gene was found to be associated with advanced liver fibrosis in patients with alcoholic liver disease. We aimed to investigate the association of the T allele gene variant with transcriptional activity in vivo and with serum concentration of soluble CD14 (sCD14), which is believed to modulate the inflammatory response. We included also the SNP rs5744455 located in promoter region and being in linkage disequilibrium with the above mentioned one.

Methods: Genomic DNA and total cellular RNA were extracted from freshly isolated peripheral blood mononuclear cells (PBMC) from 42 healthy subjects and 42 chronic HCV-infected patients. Genotyping was performed by allelic discrimination in 5'-nuclease reactions. CD14 transcripts were quantified by real time RT-PCR and the sCD14 serum concentrations were determined by ELISA.

Results: Genotype distribution (CC:CT:TT) for both SNPs was in agreement with Hardy-Weinberg equilibrium. Analysis of the amount of CD14 transcripts with regard to genotypes revealed no significant association. Rs2569190 TT-homozygotes showed higher sCD14 serum concentration than heterozygotes or CC-homozygotes (5.3, 4.4, and 4.9 µg/ml for healthy subjects and 5.8, 5.3, and 5.1 µg/ml for patients, respectively). However, this difference did not reach statistical significance. Instead we found a significant difference in sCD14 serum concentrations with regard to rs5744455 genotypes in healthy subjects (CC: 5.3, CT+TT: 3.9 µg/ml; p = 0.01).

Discussion/Conclusion: According to our data, we found no evidence for a significant association of rs2569190 genotypes with CD14 gene transcription rate in PBMC or with sCD14 serum concentration. Possibly, promoter SNP rs5744455 is more important for gene expression and disease susceptibility.
Effect of gamma irradiation on gene expression of MIP-2 and its receptor in rat liver

I. Malik¹, F. Moriconi¹, N. Sheikh¹, G. Ahmed¹, J. Dudas¹, C.F. Hess², M. Rave-Fränk², H. Christiansen², G. Ramadori¹

¹Department of Internal Medicine, Division of Gastroenterology and Endocrinology, ²Department of Radiation Oncology, University Hospital, Georg-August-University, Robert-Koch-Str. 40, D-37075 Göttingen, Germany

Background: Liver is considered to be radiation sensitive when exposed to irradiation. Macrophage inflammatory protein-2 (MIP-2) is known to be one of the major inducible chemokines with the ability to attract neutrophils to the site of inflammation. Aim of this study was to investigate the gene expression of MIP-2 after liver gamma-irradiation in rat.

Material and methods: Rats were exposed to a single gamma-irradiation (25 Gy). Livers of treated animals and of controls sham-irradiated were investigated at 1, 3, 6, 12, 24, and 48 h after liver irradiation. Cryostat sections were used for immunohistological studies. MIP-2 concentration in serum was measured by ELISA. Total RNA was extracted for real-time PCR and Northern blot analysis. Hepatocytes primary cultures were also irradiated (8 Gy).

Results: The most striking finding of this study is to find the early (3–6 h) recruitment of inflammatory cells attached to endothelium of the portal vessels after single dose of gamma-irradiation in rat liver.

MIP-2 serum concentration was significantly increased up to (up to 774 ± 80 pg/ml or 15-fold) after irradiation as compared to sham-irradiated controls. A statistically significant induction of the chemokines at early time points: recruitments (MIP-2) (61.4 ± 5.3-fold) (1–3 h), CXCL-1 (KC), LIX and monocyte chemoattractant protein-3 (MCP-3) were found by RT-PCR. Results of MIP-2 and KC were confirmed by Northern blot analysis. CXCR-2 (MIP-2, KC and LIX receptor) was also early up-regulated (1–6 h) in irradiated rat liver tissue.

We could observe significant up-regulation of MIP-2 and LIX in irradiated hepatocytes primary cultures. Surprisingly, anti-inflammatory and repair cytokines were also early induced in irradiated rat liver (1–3 h).

Conclusion: MIP-2 is the main mediator of inflammatory cells recruitment process into the portal area in irradiated rat liver.
Protection of apoptotic liver cells by cellular prion protein \textit{in vivo} – Evidence by $^{18}$F-FDG-MicroPET

Chwan-Heng Wang$^1$, Ming-Hsieh Lin$^{2,3}$, Jyh-Cheng Chen$^3$, Ren-Shyan Liu$^3$

$^1$Department of Research and Diagnosis, Dr. Wang GmbH, Tübingen, Germany
$^2$Department of Nuclear Medicine, Zhongxiao Branch, Taipei City Hospital, Taipei, Taiwan
$^3$Department of Biomedical Imaging and Radiological Sciences, School of Biomedical Science and Engineering, National Yang-Ming University, Taipei, Taiwan

\textbf{Introduction}: The biological function of the cellular prion protein (PrPc) is unclear. PrPc associates with lipid rafts, highly glycolipid-rich membrane domains containing a large variety of signaling molecules. PrPc was shown to serve as an anti-apoptotic factor \textit{in vivo} and may function as a guardian of neuronal integrity. Chronic lymphocytic inflammation seems to specify the organ tropism of prion (Science. 2005; 307: 1107). Typically, prions not only accumulate in nervous and lymphoid tissues but also in spleen, heart and liver. Study regarding the possible role of prion in liver cells is, however, rare. Recently, we described an \textit{in vivo} system for molecular imaging of apoptotic process and tracing functional molecules in liver by $^{18}$F-FDG-MicroPET. To obtain an insight into the biological function of PrPc, interaction of PrPc specific monoclonal antibodies with the Fas-induced apoptotic liver cells was monitored by $^{18}$F-FDG-MicroPET.

\textbf{Methods}: Mice were injected with Fas antibody for induction of apoptosis and $^{18}$F-FDG-MicroPET was then performed. For comparative study, mice were challenged with anti-PrPc together with anti-Fas. $^{18}$F-FDG-MicroPET was acquired to image serially the sites, extent, and severity of apoptosis induced by control, anti-Fas, and anti-Fas/anti-PrPc. Dynamic images of 60 min post injections were obtained. Serial SUVs (standard uptake values) of liver were recorded as time activity curve (TAC). The $^{18}$F-FDG clear half time of TAC and SUV at immediate and 60 min post injection were obtained.

\textbf{Results}: The static images showed increased radioactivity in the liver of mice p.i. than controls. Intravenously administrated $^{18}$F-FDG localized preferentially in the liver, kidney, heart and spleen; increased uptake in these organs was easily visualized after injection of anti-Fas in animals. Higher dosages increased $^{18}$F-DFG uptake in the liver and kidney and delayed recovery of these organs as seen compared with lower doses. The F18 clear half time in anti-Fas induced cases with anti-PrPc are more rapid than that with anti-Fas alone.

\textbf{Discussion/Conclusion}: Transitional introduction of anti-PrPc into mouse apoptotic liver cells may result in the rearrangement of interactive proteins involved in protection again Fas-induced apoptosis. We report for the first time that PrPc influence the regulation of apoptotic liver cells \textit{in vivo}. This evidence strongly suggested that PrPc in liver cells undergoing apoptosis exhibited antiapoptotic effects. Revealing the correlation between presence/absence and/or different proteins might further contribute to our understanding of certain interactive cellular proteins of the complex role of PrPc in cell biology.
Interferon-α therapy does not modulate hepatic expression of classical type I interferon inducible genes

Volker Meier¹, M.D., Sabine Mihm¹, Ph.D., Giuliano Ramadori¹, M.D.
¹Division of Gastroenterology and Endocrinology, Department of Internal Medicine, Georg-August-University Goettingen, Germany

Introduction: Hepatitis C virus (HCV) infection is a major cause of chronic liver disease. Treatment with interferon-alpha₂ (IFN-α₂) can induce viral clearance and marked biochemical and histological improvement. IFN-α₂ treatment has been shown to stimulate the expression of type I IFN regulated genes in peripheral blood mononuclear cells (PBMCs) of hepatitis C patients; however, whether it affects hepatic expression remains unknown. This study thus aimed comparing hepatic gene expression with particular emphasis on type I IFN inducible genes in patients with chronic hepatitis C before and during an IFN-α₂ monotherapy.

Methods: Responsiveness to IFN-α₂ therapy was monitored by determining serum and hepatic viral load. Differential gene expression analysis was performed by two different techniques, namely suppression subtractive hybridization (SSH) and differential display (DD). Expression of two prototype type I IFN regulated genes was quantified in further PBMC and liver samples.

Results: Among different genes found to be up-regulated during an effective, i.e. virus clearing, IFN-α treatment, only a single one was identified which can be accounted to type I IFN responsive genes. Parallel quantitative real time PCR analyses demonstrated significant induction of the type I IFN regulated genes MxA and PKR in PBMC, but not in the liver.

Conclusion: Taken together, while IFN-α treatment leads to the induction of type I IFN regulated genes in PBMC, such an induction appears not to occur in the liver of hepatitis C patients. The mechanism by which IFN-α treatment causes viral clearance thus might be independent on hepatic activation of type I IFN regulated genes.
Plasma level of nitric oxide in hepatitis B patients: Difference between acute and chronic state

Chwan-Heng Wang1*, Ming-Hsieh Lin2, Anna Wang3, Shu-Yuan Tschen3
1Department of Research and Diagnosis, Dr. Wang GmbH, Tübingen, Germany
2Department of Nuclear Medicine, ZhongXiao Branch, Taipei City Hospital, Taipei, Taiwan
3Department of Validation and Surveillance, Dr. Wang GmbH, Zurich, Switzerland

Introduction: The intrahepatic expression of iNOS is elevated in chronic hepatitis B patients and correlated well with the severity of the disease, which indicated that inducible nitric oxide synthase (iNOS) may have a critical role in the pathogenesis of chronic viral hepatitis B. Nitric oxide (NO) synthesized by iNOS can function as an antimicrobial agent able to kill or reduce replication of microorganisms, and plays an important role in immune regulation (Int J Infect Dis. 2008; 12: 12). The objective is to investigate if the increased iNOS activity can be represented by plasma level of nitric oxide in acute as well as chronic HBV patients.

Methods: Eight cases with acute HBV infections (group I; anti-HBc IgM+/HBsAg+; mean age = 42), 69 patients with chronic active hepatitis B virus infection (group II; HBsAg+/HBeAg+; mean age = 52), and 100 healthy subjects (group III; HBsAg-/HBeAg-; mean age = 48) were enrolled. Nitric oxide was measured in classified groups (patients and controls) as the serum metabolic products of nitrates and nitrites using a modification of the Griess reaction. HBsAg, HBeAg, anti-HBc IgM and HBV-DNA were analysed by commercial kits.

Results: Total peroxide level in plasma was significantly higher in subjects with acute HBV infection (112 ± 68.17 µM vs. 78.43 ± 28.77 µM; p < 0.001) but significant lower in those chronic HBV subjects than that of controls (51 ± 32.17 µM vs. 78.43 ± 28.77 µM; p < 0.001). In our hand, no significant relationship was found between plasma NO level and numbers of HBV DNA (average copy number = 10^5-6 copies/ml).

Discussion/Conclusion: Our result indicates that low level of NO in plasma of chronic HBV hepatitis is not positively correlated with increased tissue expression of iNOS. The iNOS may be differentially expressed at intracellular or local tissue level and is not quantitatively reflected by the plasma level of NO. Whether other intracellular effectors induced by IFN-gamma mediated immune response and cellular oxidative stress during HBV chronic development are interactively involved remains to be further investigated.
Differences of bile acid composition in liver biopsies of non-alcoholic and alcoholic steatohepatitis


BSMU, Chernivtsi, Ukraine

Introduction: The two most common forms of steatohepatitis are alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH). While mechanisms of steatohepatitis pathogenesis are actively studied, exact bile acid composition of liver tissue in steatohepatitis remains largely unclear; however, bile acids may play a role as potential mediators of liver damage. The aim of current study was to assess if the histological findings in steatohepatitis can be correlated with bile acid composition in biopsies.

Methods: Bile acid composition in liver biopsies was studied by gas-fluid chromatography in liver tissue of patients with non-alcoholic (n = 11), and alcoholic steatohepatitis (n = 13). Liver biopsies were investigated and graded for steatosis, inflammation, and fibrosis.

Results: All bile acids were statistically significantly increased in liver biopsies of steatohepatitis patients compared with control group (p < 0.05). Deoxycholic, chenodeoxycholic and cholic acids were elevated by 90.1, 63.6 and 45.5%, respectively in patients with steatohepatitis (p < 0.05). Cholic acid was the prevailing bile acid in NASH and controls, while more hydrophobic bile acid species were elevated in ASH compared with controls (p < 0.05). Significant correlations were found in NASH patients between hepatic chenodeoxycholic acid and fibrosis, and between cholic acid and tri-hydroxy/di-hydroxy bile acids and signs of inflammation (p < 0.05). In ASH, cholic acid and tri-hydroxy/di-hydroxy bile acids strongly correlated with steatosis (p < 0.01).

Discussion/Conclusion: Distinct pattern of bile acids in the liver of patients with steatohepatitis is shown. Association between bile acids profiles and histological picture of liver suggests possible role of specific bile acids in disease progression, either as a cause or consequence of the disease.
Differential expression pattern of the lipid droplet-associated proteins perilipin, adipophilin, and TIP47 in chronic steatotic liver diseases

Beate Katharina Straub¹,², Pamela Stoeffel¹, Hans Heid², Ralf Zimbelmann², and Peter Schirmacher¹

¹Institute of Pathology, Im Neuenheimer Feld 220/221, D-69120 Heidelberg, Germany
²German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

Introduction: Fatty change is the most frequent liver pathology in western countries and is caused by a broad range of disorders such as alcohol abuse and metabolic syndrome. The surface layer of lipid droplets (LDs) contains certain amphiphilic proteins, so-called PAT proteins, named after their constituents, perilipin, adipophilin, and TIP47, which are fundamental for the stabilization of LDs in cells. Whereas adipophilin and TIP47 are ubiquitously expressed, perilipin was thought to be restricted for LDs of adipocytes and steroidogenic cells.

Methods: We characterized the expression pattern of the LD-associated proteins perilipin, adipophilin, and TIP47 in chronic steatotic liver diseases with protein biochemical and molecular biological methods as well as with immunofluorescence microscopy and immunohistochemistry.

Results: Adipophilin and TIP47 surround LDs of vitamin A storing hepatic stellate cells and of normal and steatotic hepatocytes. Perilipin is found to be de novo expressed in hepatocytes during steatogenesis; its association with LDs is different from TIP47 and adipophilin, with respect to size and localization of LDs, suggesting that the different PAT proteins play specific roles during LD-maturation. In immunoblot, using different cryoconserved liver specimens, and immunohistochemistry of paraffin-embedded liver cases, both, the amounts and staining intensities of adipophilin and perilipin correlated significantly with the amount of hepatocytic fat (degree of steatosis). Yet, no correlation was observed between staining intensity of adipophilin or perilipin and the underlying etiology (alcohol, metabolic syndrome, HCV) or presence or absence of steatohepatitis.

Discussion/Conclusion: Antibodies against PAT-proteins may be used as markers for hepatic steatosis. As perilipin is restricted to adipocytes and steatotic hepatocytes, perilipin may represent a potential target for the suppression of hepatic steatosis.
The development of model of non-alcoholic fatty liver disease in Wistar and Sprague-Dawley rats

Charles University in Prague, Faculty of Medicine in Hradec Kralove, Department of Physiology, Hradec Kralove, Czech Republic

Introduction: Nonalcoholic fatty liver disease (NAFLD) is an important cause of liver-related morbidity and mortality. NAFLD is often the hepatic manifestation of metabolic syndrome and represents a wide spectrum of conditions ranging from non-progressive hepatic steatosis, to nonalcoholic steatohepatitis that may progress to cirrhosis and end-stage liver disease. The aim of this project was to induce and characterize the nutritional models of NAFLD in rats, with respect to duration of diet feeding and the use of rat strain.

Methods: Wistar or Sprague-Dawley male rats were fed ad libitum a standard pelleted diet (ST-1, 10% of energy from fat); medium-fat gelled diet (MFGD, 35% of energy from fat) and high-fat gelled diet (HFGD, 71% of energy from fat) for 3 and 6 weeks. Then serum ALT, AST, glycaemia, levels of triacylglycerols and cholesterol were measured. Respiration of isolated liver mitochondria was assessed using high-resolution respirometry. Malondialdehyde content in the liver and tissue cytokines (IL-6, TGF-β1) were measured and histopathological samples were prepared.

Results: Feeding with HFGD (less with MFGD) induced periportal small-droplet steatosis with mild focal inflammation without necrosis in comparison with control group (ST-1); changes were more pronounced in 6-week model and in Sprague-Dawley strain. There were no significant differences among groups in serum biochemical parameters. We found an increase in tissue IL-6 in HFGD in comparison with ST-1 in all groups. There were no significant differences in TGF-β1.

Discussion/Conclusion: Sprague-Dawley rats fed high-fat liquid diet for 6 weeks seems to be the proper model for NAFLD in experiment.

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The effect of gamma radiation on the expression of genes involved in fat metabolism in rat liver

Gesa Martius¹, Ihtzaz Malik¹, Josef Dudas¹, Nadeem Sheikh¹, Margret Rave-Fränk², Hans Christiansen², Giuliano Ramadori¹, Clemens F. Hess²
¹Department of Internal Medicine, Division of Gastroenterology and Endocrinology, ²Department of Radiology, Division of Radiotherapy and Radiooncology University Hospital, Georg-August-University, Robert-Koch-Str. 40, D-37075 Göttingen, Germany

Introduction: The liver is a radio-sensitive organ and radiation-induced fat liver disease is increasingly recognized as a frequent cause of liver dysfunction. Fat was found to accumulate in form of vacuoles in hepatocytes 12 h after gamma irradiation. This study aimed at investigating the expression of several fat metabolism genes in the rat after liver irradiation.

Methods: Rat livers were irradiated selectively with 25 Gy. Livers were taken 1 h, 3 h, 6 h, 12 h, 24 h and 48 h after irradiation. Total RNA and proteins were isolated and genes of interest were investigated by real time-PCR and by Northern and Western blot analysis. Frozen liver sections were used to stain fat with Nile red dye. Additionally, hepatocytes were isolated from rat liver, cultured in the presence or absence of pro-inflammatory cytokines and irradiated (8 Gy).

Results: Significant down-regulation of all investigated fat metabolism genes was observed. Surprisingly, the gene expression of peroxisome proliferators-activated receptor gamma coactivator 1alpha (PGC-1alpha) and carnitine palmitoyl transferase 1alpha (CPT-1alpha) was significantly down-regulated at early points (1–12 h) after irradiation. PGC-1alpha was the highest down-regulated gene reached its maximum at 12 h. Sterol regulatory element binding protein 1 (SREBP-1) was significantly down-regulated at 3 h but prolonged its down-regulated expression until 48 h standing along with PGC-1alpha and CPT-alpha. Regulation of SREBP-1 and estrogen related receptor alpha (ERR-alpha) were also confirmed by western blot analysis. Carnitine palmitoyl transferase beta (CPT-beta) was the only up-regulated gene after liver irradiation in rat.

Another finding was to observe the intracellular accumulation of fat which increased with time after rat liver irradiation.

In vitro, we observed that irradiation could alone induce the gene expression of CPT-alpha, acetyl-CoA-carboxylase 2 (ACC-2) and PGC-1alpha in isolated hepatocytes at late time points (12–48 h). Additionally, CPT-alpha expression was also significantly increased by pro-inflammatory cytokines treated hepatocytes.

Discussion/Conclusion: Our study identified genes that might be involved in pathogenesis of fat liver disease. Studies regarding regulation and kinetics are reasonable.
Identification of hepatitis C virus (HCV) non-structural protein 2 as a modulator of cyclosporin A-mediated inhibition of HCV RNA replication

Sandra Ciesek1,2, Eike Steinmann2, Michael P. Manns1, Christiane Brohm2, Norbert Tautz3, Johan Neyts4, Heiner Wedemeyer1, and Thomas Pietschmann2

1Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Germany
2Department for Experimental Virology, Twincore, and Hannover Medical School, Hannover, Germany, and Helmholtz Centre for Infection Research, Braunschweig, Germany
3Department of Medical Molecular Biology, University of Lübeck, Germany
4Rega Institute for Medical Research, KU Leuven, Leuven, Belgium

Worldwide more than 130 million people are chronically infected with the hepatitis C virus. Employing subgenomic HCV replicons it has recently been shown that Cyclosporin A (CsA) inhibits HCV RNA replication. The HCV-specific antiviral effect of CsA was linked to its ability to interact with cyclophilins, which in turn were recognized as essential cellular co-factors. Since various resistance mutations map to NS5B and as Cyclophilin B was found to associate with NS5B and to stimulate its RNA binding activity it is believed that NS5B is a primary target of CsA-dependent inhibition. However, standard subgenomic replicons do not express HCV proteins core, E1, E2, p7 and NS2, and therefore it is unclear if the function of any of these proteins may also be affected by CsA or if these proteins may modulate the antiviral effect of CsA.

In this study, we noted that CsA inhibits replication of a JFH1-derived full length genome much more efficiently than a subgenomic replicon. This increased sensitivity is apparently independent of core, E1, E2 and p7, since replicons with deletions of these proteins individually or in combination also were inhibited with a ca. 5-fold lower IC50 as compared to a replicon encoding NS3-5B only. Therefore, these data imply that NS2 is an additional target for CsA-dependent inhibition or could modulate the antiviral activity against NS3 to NS5B proteins. Additional experiments suggest that this effect is independent of the NS2/NS3 protease function and CsA calcineurin inhibition. If this effect is also linked to the ability of CsA to interact with cyclophilins is currently under investigation.
Differential effects of multikinase inhibitors sorafenib (Nexavar®) and U0126 on phosphorylation patterns of hepatocellular carcinomas cells

Christian Quack¹, Eva Rieser¹, Monika Braun¹, Stefan Maßen¹, Andreas Koch², Martina Müller², Christine S. Falk¹
¹NCT National Center for Tumor Diseases, DKFZ & Institute for Immunology, Heidelberg, im Neuenheimer Feld 305, 69120 Heidelberg, Germany
²Department of Internal Medicine IV, Hepatology and Gastroenterology, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany

Introduction: Hepatocellular carcinoma (HCC) represents one of the most prevalent cancer entities worldwide. A variety of molecular mechanisms and signalling pathways are involved in hepatocarcinogenesis. Among these, the MAP kinase (RAS/RAF/MEK/ERK) signalling pathway plays an essential role in the regulation of hepatocyte proliferation and metabolic function. Dysregulation of this signalling pathway following mutation or constitutive activation is critical in HCC pathogenesis and highlights it as an attractive target for chemotherapeutic agents. Sorafenib (Nexavar®), an oral drug developed as B-RAF inhibitor, is suggested to be a promising agent for HCC therapy. However, the molecular basis for differential effects of various MAPK inhibitors on other signal cascades is poorly understood.

Methods: To analyze the consequences of MAP kinase inhibition with the B-Raf inhibitor sorafenib (Nexavar®) or the MEK inhibitor U0126 on phosphorylation and stability of kinases beyond the MAP kinase pathway, the HCC lines HepG2, Hep3B and Huh7 were treated with these inhibitors or the solvent DMSO. Phosphorylation status and stability of several kinases within and outside the MEK/ERK pathway were quantified by the multiplex protein array technology.

Results: Stability and phosphorylation status of MAP kinases as well as other kinases and transcription factors differed substantially between sorafenib and U0126 treatment. While ERK1/2 phosphorylation was immediately decreased by both inhibitors, differences were seen in the phosphorylation of some components of the PI3 kinase pathway and some transcription factors. In addition, substantial protein degradation was observed in sorafenib- but not in U0126-treated HCC cells. These effects were partially dependent from p53 and the degradation of kinases and transcription factors was virtually independent of caspase activity.

Discussion/Conclusion: Although B-RAF and MEK belong to the same MAPK pathway, blocking of one of these components by inhibitors collaterally affects other signal cascades and transcription factors. The relevance of these observations for cell proliferation, apoptosis induction and surface molecule expression is currently under intensive investigation. Nevertheless, these initial observations may already help to understand the highly variable response rates in different clinical studies using multikinase inhibitors.
An oncogenomics-based in vivo RNAi screen identifies new tumor suppressors in liver cancer

Lars Zender1,6*, Wen Xue1*, Johannes Zuber1, Stefan Kubicka4, J.M. Luk5, Peter Schirmacher3, Richard W. McCombie1, Michael Wigler1, James Hicks1, Gregory J. Hannon1,2, Michael P. Manns4, Scott Powers1, and Scott W. Lowe1,2**

1Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
2Howard Hughes Medical Institute, Cold Spring Harbor, NY 11724, USA
3Institute of Pathology, University Hospital Heidelberg, D-69120 Heidelberg, Germany
4Department of Gastroenterology and Hepatology, Medical School Hannover, D-30625 Hannover, Germany
5Department of Surgery, University of Hong Kong, Hong Kong, China
6Current address: Helmholtz Centre for Infection Research (HZI), D-38124 Braunschweig, Germany, and Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, D-30625 Hannover, Germany

Here we combine microRNA based short hairpin RNA (shRNA) technology with a progenitor cell derived mouse model of hepatocellular carcinoma to perform an in vivo RNA interference screen for new tumor suppressor genes. We generated a series of low complexity pools of shRNAs targeting genes found in focal deletions identified by comparative genomic hybridization of ~100 human hepatocellular carcinomas. These pools were introduced into liver progenitor cells expressing the Myc oncogene and tested for their ability to promote tumorigenesis in vivo; remarkably, pools containing shRNAs targeting deleted genes gave rise to tumors whereas those containing randomly selected shRNAs did not. Through further analyses, we identified and validated 13 new tumor suppressor genes, most of which have not been linked to cancer before. One gene, EXPORTIN 4 (XPO4), encodes a nuclear export protein whose substrates include SMAD3 and a putative translational regulator, EIF5A. Interestingly, we show that EIF5A2 is amplified in human tumors, is required for efficient proliferation of tumor cells lacking XPO4, and can promote hepatocellular carcinoma development in mice. Our result establishes the feasibility of in vivo RNAi screens for genes that modulate epithelial cancer phenotypes, and illustrate how combining next generation mouse models, RNAi and genomic information from human cancer may facilitate the function annotation of the cancer genome.
Hepatic fibrosis and hepatocellular carcinoma are determined by independent gene sets in a murine reference population

Susanne Weber, Rabea Hall and Frank Lammert
Department of Medicine II, Saarland University Hospital, Homburg, Germany

Introduction: Hepatic fibrosis is a non-specific reaction in response to chronic liver injury. It is commonly caused by exogenous factors such as viral hepatitis or alcohol abuse but recent studies also point to a polygenic predisposition. The fibrogenic response may lead to cirrhosis and predisposes to hepatocellular carcinoma (HCC), which is also determined by the interaction of multiple genetic and environmental factors. Our specific aim now is to model both conditions in the same murine reference population.

Methods: As reference population, we availed of phenotypic and genotypic data from recombinant inbred mouse strains. The strain set was generated by intercrossing C57BL/6 and DBA/2 mice and comprises up to 80 BXD lines. We determined hepatic collagen contents after CCl₄ challenge for 6 weeks and correlated the data to all published BXD phenotypes (including tumor sizes of diethylnitrosamine [DEN]-induced HCC), liver mRNA profiles at baseline, and > 13.300 genetic markers that discriminate the two parental strains, employing WebQTL (www.genenetwork.org).

Results: Comparing the data sets, fibrosis and HCC phenotypes showed a correlation score of only 39% (all p > 0.05), and both traits correlated significantly with distinct sets of genes. Furthermore, interval mapping showed no concordant linkage peaks for both traits. Cluster tree analysis displayed moderate associations with genetic markers but no overlap between the two phenotypes.

Discussion/Conclusion: Employing in silico analysis of BXD recombinant inbred mice, we could not identify a genetic association between both experimental conditions. Our findings indicate that independent gene sets determine fibrogenesis and carcinogenesis in the liver.
PGE2-synthase (mPGES)-2 as a potential therapeutic target in hepatocellular carcinoma (HCC)

Marco Breinig, Benjamin Goeppert, Volker Ehemann, Peter Schirmacher and Michael André Kern
Department of General Pathology, University Hospital Heidelberg, Germany

Introduction: To date, no effective therapeutic options are available for HCC-management. A growing body of evidence demonstrates that the inhibition of Cyclooxygenase (COX)-2, the rate-limiting enzyme in prostaglandin-biosynthesis, exerts antineoplastic activity in in vitro and in vivo HCC models. In this regard, prostaglandin E2 (PGE2) is discussed as the major protumorigenic factor.\(^{(1,2,3,4)}\) PGE2-synthesis is catalyzed by differentially-regulated PGE2-synthases (mPGES-1 and -2), with the COX-derived PGH2 used as substrate. Since continuing intake of COX-2-inhibitors has been found to provoke cardiovascular complications, we tested if PGE2-synthases may represent candidates for a more specific therapeutic approach.

Methods: Expression of COX-2 and mPGES-2 in HCCs was analyzed using tissue micro-arrays (TMAs). mPGES-2 expression in HCC-cell lines (HuH7, PLC, HepG2, Hep3B) was investigated using Western immunoblotting. RNA-interference (RNAi) was employed to specifically reduce the expression of mPGES-2. Functional consequences of mPGES-2-knockdown were tested using MTT-assays (viability) and FACS-analyses (cell cycle, apoptosis).

Results: TMA-analyses revealed an increase in mPGES-2-expression with decreasing differentiation of HCCs, which was in diametrical opposition to the expression-profile of COX-2. Additionally, mPGES-2 was overrepresented in cirrhotic liver. mPGES-2-knockdown reduced the cell viability of all HCC cell lines tested, which was associated with alterations in cell cycle distribution and apoptosis-induction.

Discussion/Conclusion: We show that mPGES-2 represents a potential new therapeutic target in HCC. Since HCCs frequently arise from a cirrhotic background our data showing an increased expression of mPGES-2 in cirrhotic liver insinuate that targeting mPGES-2 could constitute a potential preventive strategy. Collectively, the specific inhibition of mPGES-2, which exclusively reduces PGE2 availability, could represent a therapeutic approach advantageous over COX-2 inhibition which leads to a general blockade of prostaglandin synthesis, deemed accountable for the adverse side-effects of COX-2 inhibitors.

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Selective inhibition of casein kinase-2 in comparison to other dedicated Wnt/beta-catenin pathway-associated kinases by natural and chemical modified polyphenoles

K.S. Lerche¹, R. Günther², H. Hofmann², R. Gebhardt¹
¹Institute of Biochemistry, Medical Faculty, University of Leipzig, Johannisallee 30, D-04103 Leipzig, Germany
²Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Brüderstr. 34, D-04103 Leipzig, Germany

Introduction:
In former times, *Rheum officinale*, commonly known as Chinese rhubarb, was used as an anticancer drug in the Orient. Main ingredients are anthraquinones with the basic moiety of emodin. We investigated several modifications of the emodinic core as targets for tumor therapy and looked for the potency of natural flavonoids that may act in a preventive manner.

The Wnt/β-catenin signal transduction pathway is a complex network of proteins, which is involved in normal physiological processes as well as in the pathophysiology of the HCC. In this work we have investigated CK-1, GSK-3beta and CK-2, which are quantitatively elevated in most proliferating tissues such as tumor cells.

Methods: With the intention to find inhibitors of CK-2 with high potency in association with high selectivity, we were virtually docking various polyphenoles into their X-ray crystallographic structures and analyzed their binding affinities and possible reasons of them. Secondly, we collected *in-vitro* data of selected hit-compounds, measured by an *in-vitro* kinase phosphorylation assay. Further on, we tested some of the compounds on primary hepatocytes in comparison to HepG2 with a cytotoxic assay.

Results: The virtual screening showed a selective inhibition of CK-2 in the upper nanomolar range. Some of the tested compounds had interesting biological properties. Acetylated compounds, for example, seemed to act as prodrugs and ethylester variants showed a higher cytotoxic potential to HepG2 cells than to normal hepatocytes.

Discussion/Conclusion: The elucidation of molecular details of protein-inhibitor interactions discloses further possibilities for compound modifications and target-orientated signalling pathway interference.
Transient telomere dysfunction promotes hepatocarcinogenesis

Yvonne Begus-Nahrmann¹, André Lechel¹, P. Schirmacher², Lenhard Rudolph¹
¹Institute of Molecular Medicine, University of Ulm, Germany
²Institute of Pathology, University of Heidelberg, Germany

We propose that telomere dysfunction is followed by telomerase activation during human carcinogenesis. Our aim is to test this hypothesis by transient induction of telomere dysfunction at an early time point of liver damage/hepatocarcinogenesis in telomerase (mTERC+/+) competent mice.

Introduction: The hallmarks of human hepatocellular carcinoma include chromosomal instability, short telomeres, and telomerase reactivation. Previous work in mouse models has shown that telomere shortening increases tumor initiation (microscopic tumor lesions) but impairs tumor progression. Our current model indicates that telomere dysfunction induces chromosomal instability leading to tumor initiation. However, initiated tumor cells need to reactivate telomerase in order to stabilize telomeres and genomic instability to prevent genetic chaos and tumor cell death. Since telomerase knockout mice lack the telomerase gene tumor initiation is increased but tumor progression is impaired.

Methods: Hepatocarcinogenesis was induced by DEN treatment in 15 days old mice. At an early time point (2–4 months after DEN injection) transient telomere dysfunction was induced by TRF2 inhibition. TRF2 is a telomere binding protein, which is essential for telomere capping function. To inhibit TRF2 we used inducible transgenic mice expressing a dominant negative mutant from of TRF2 in the liver (LAP-rtTA, TRF2ΔBΔM double transgenic mice). Expression of the mutant form of TRF2 was induced by 3 repeated intrasplenic injections of doxycycline in 2 week intervals. Control animals (TRF2ΔBΔM) were treated in the exact same way but did not express mutant TRF2 in response to doxycycline injection due to the lack of LaprtTA.

Results: Intrasplenic injection of doxycycline induced expression of TRF2ΔBΔM in the liver of double-transgenic mice correlating with transient induction of telomere dysfunction in 2–4 month old mice as measured by induction of anaphase bridges (morphological correlate of chromosomal fusions). After 13 months both the LAPrtTA-TRF2ΔBΔM (n = 23) and the control group (n = 29) developed hepatocellular carcinomas. However, transient telomere dysfunction in LAPrtTA-TRF2ΔBΔM double transgenic mice strongly increased tumor formation. The tumor size was significantly increased by more than two-fold in male LAPrtTA-TRF2ΔBΔM mice compared to controls (p < 0.0001). In addition, the number of tumors was significantly increased female LAPrtTA-TRF2ΔBΔM mice compared to controls (p = 0.019). Increased tumor formation in double transgenic mice exposed to telomere dysfunction was associated with increased levels of tumor cell proliferation measured by PCNA staining. Experiments on chromosomal instability and gene expression changes induced by transient telomere dysfunction are currently conducted.
**Discussion/Conclusion:** The results provide first experimental evidence that transient induction of telomere dysfunction during early stages of hepatocarcinogenesis leads to a significant acceleration of tumor formation. These results appear to be important for human hepatocarcinogenesis, which is characterized by telomere shortening and chromosomal instability.
Smad7 and negative regulation of TGF-β signaling in liver tumorigenesis

J. Dzieran¹, B. Bauche¹, A. Tannapfel², S. Dooley¹, N.M. Meindl-Beinker¹
¹Molecular Alcohol Research in Gastroenterology, II. Medical Clinic, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany
²Institute of Pathology, Ruhr-University Bochum, Bochum, Germany

Introduction: Hepatocellular carcinoma (HCC) is one of the deadliest and most common cancers worldwide. In liver cells, cytokines of the TGF-β family participate in a number of different processes ranging from cell damage to growth control and tumorigenesis. TGF-β has characteristics of a tumor suppressor in early stages of liver damage and regeneration. During cancer progression it changes to a tumor promoter supporting invasion and metastasis. R-Smad proteins are the main intracellular mediators of the TGF-β signaling cascade, whereas Smad7 is a very efficient inhibitor. Here, we analyze the input of Smad7 in aberrant TGF-β signaling in HCC.

Methods: To quantify Smad7 expression in HCC samples we performed real-time PCR and Northern blot analysis. Western Blot and immunochemical stainings were used to determine protein levels. Additionally, mutation analyses have been carried out for the most conserved promoter region of Smad7.

Results: In 16 of 20 samples from HCC patients, including 6 with HBV infection, Smad7 expression is increased when compared to healthy tissue from the same patients. From the limited number of samples currently investigated, an impact of HBV infection or cirrhosis was not visible. Analysis of the most conserved promoter region of Smad7 did not reveal any mutations in these samples. Comparing analysis of 8 different human HCC cell lines showed an overexpression of Smad7 in HuH-7 and FLC-4 cells. In line with this, FLC-4 cells lack TGF-β dependent activation of Smad2 as measured with a phospho-Smad specific antibody. Furthermore, staining revealed that nuclear translocation of Smad2 was abolished in FLC-4 and PLC/PRF/5 cells. A link of these observations with intrinsic Smad7 overexpression remains to be shown.

Discussion/Conclusion: In summary, we show that deregulated Smad7 expression may contribute to loss of antiproliferative TGF-β effects, making it a potential oncogene and promising target for drug development.
Gene expression profiling: A comparison of epithelial liver tumours

Reena Buurman\textsuperscript{1}, Doris Steinemann\textsuperscript{1}, Anja Weigmann\textsuperscript{1}, Peer Flemming\textsuperscript{4}, Thomas Becker\textsuperscript{2}, Jakobus Flik\textsuperscript{3}, Hans Kreipe\textsuperscript{4}, Brigitte Schlegelberger\textsuperscript{1}, Ludwig Wilkens\textsuperscript{1,4}, Britta Skawran\textsuperscript{1}

\textsuperscript{1}Institute of Cell and Molecular Pathology, \textsuperscript{2}Department of Visceral and Transplantation Surgery, \textsuperscript{3}Institute of Virology, \textsuperscript{4}Institute of Pathology, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

**Introduction:** Differential diagnosis of epithelial liver tumors using small biopsies is a difficult diagnostic field. More information on different gene expression profiles of epithelial liver tumors would help to delimit histopathologically malignant hepatocellular carcinoma (HCC) from benign hepatocellular adenoma (HCA) and focal nodular hyperplasia (FNH), respectively. Therefore gene expression profiling was performed on tumours cytogenetically well characterized by array based comparative genomic hybridization.

**Methods:** Global expression profiles of 24 HCC, 8 HCA and 7 FNH were measured by microarray analysis using a genome-wide microarray containing 43000 spots. Taqman assays were applied to validate the expression pattern of significant genes.

**Results:** Based on these microarray results, hierarchical cluster analysis branched all HCC from HCA and FNH. The most differentially expressed genes represent the family of metallothioneins, which are drastically decreased in HCC compared to FNH and HCA. The most significant gene is MT1F, expressed at high levels in HCA and FNH in comparison to HCC, followed by MT1G, MT1X and MT2A, showing the same pattern. Taqman Assays of MT1F and MT1G validated these significant microarray results.

**Conclusions:** We have shown that the expression of metallothionein is significantly decreased during development of malignant epithelial liver tumors. These data suggest that the different expression of metallothionein is a putative biomarker, differentiating between malignant HCC on the one hand and benign HCA and FNH on the other and therefore should be a focus of further research.
Analysis of C-kit expression in human hepatocellular carcinoma and in the corresponding peritumoral tissue

T. Mansuroglu¹, D. Baumhoer¹,*, J. Dudas¹, F. Haller², S. Cameron¹, T. Lorf³, L. Füzesi², G. Ramadori¹
¹Department of Gastroenterology and Endocrinology, ²Department of Pathology, ³Department of Surgery, University of Göttingen, Germany
*Present address: Institute of Pathology, University of Basel, Switzerland

Introduction: The aim of the current study was to examine and compare the expression pattern of the stem cell factor receptor c-kit in human hepatocellular carcinoma (HCC) with the corresponding peritumoral tissue. In order to prove the immunohistochemical results, HCC cell lines were investigated for c-kit expression.

Methods: Expression of c-kit (SCF-receptor) has been evaluated in 72 HCC and in the corresponding surrounding non-tumorous tissue. Additionally to immunohistochemical analysis reverse transcription polymerase chain reaction (RT-PCR) was employed to examine the mRNA expression of c-kit protooncogene. Moreover, three HCC cell lines (HUH-7, HepG2 and SK-Hep1) were used for gene-expression analysis of c-kit mRNA.

Results: C-kit expression was detected immunohistochemically in 70% of HCC with different degrees of intensity and could also be found in about 90% of the corresponding peritumoral liver tissues. C-kit mRNA was detectable in 83% of HCC and in 75% of the corresponding peritumoral non-cirrhotic as well as in 100% of corresponding peritumoral cirrhotic samples. In addition, two of the three HCC cell lines (HUH-7 and SK-Hep1) showed a well detectable PCR-product for c-kit.

Conclusions: Hepatocytes express the c-kit receptor under altered pathological conditions. The presence of c-kit in HCC cell lines supports the assumption that SCF might play a role in the regulation of proliferative activity of tumorous and non-tumorous hepatic cells.

Keywords: c-kit, hepatocellular carcinoma, liver cirrhosis, hepatocytes

Correspondence to:
Prof. Dr. Dr. h.c. G. Ramadori
Center of Internal Medicine
Department of Gastroenterology and Endocrinology
University of Göttingen
Robert-Koch-Str. 40
D-37099 Göttingen / Germany
Tel.: +49 551 396301, Fax: +49 551 398279
E-Mail: gramado@med.uni-goettingen.de
Overexpression of the FUSE-binding protein (FBP) family members stimulates proliferation and migration of HCC cells

Mona Malz¹, Vera Riehmer¹, Achim Weber², Michaela Bissinger¹, Marc-Oliver Riener², Christopher Soll³, Arndt Vogel⁴, Peter Angel⁵, Peter Schirmacher¹, Kai Breuhahn¹
¹Institute of Pathology, University Hospital Heidelberg, Germany
²Department of Pathology, University Hospital Zurich, Switzerland
³Visceral and Transplantation Surgery, University Hospital Zurich, Switzerland
⁴Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Germany
⁵German Cancer Research Center (DKFZ), Heidelberg, Germany

Introduction: The single-strand nucleic acid-binding factors FBP (far upstream sequence element (FUSE)-binding protein)-1, -2 and -3 are multifunctional factors which are involved in RNA processing including transcript splicing and stabilization. FBP family members have also been ascribed to bind torsionally stressed genomic regions and subsequently regulate the expression of tumor-relevant genes such as c-MYC.

Methods: Expression of FBP family members in healthy liver (n = 3), and hepatocellular carcinoma (HCC) (n = 27) was analyzed on transcript (semiquantitative real-time PCR) and protein levels (Western immunoblotting). Nuclear FBP-1, -2, and -3 expression in the course of hepatocarcinogenesis was examined using tissue-microarrays (TMAs) containing healthy liver, dysplastic nodules, and HCCs (n = 272). In vitro, functional effects after siRNA-mediated inhibition of all FBP family members were analyzed in HCC cell lines (HuH-7, Hep3B) using MTT-assays (viability), BrdU-ELISAs (proliferation), caspase-assays/PARP-cleavage (apoptosis), and scratch-assays (motility).

Results: At the transcript and protein levels a strong overexpression and positive correlation among all FBP family members was observed in HCC tissues compared to healthy liver tissue (FBP-1: 81.5%, FBP-2: 67%, FBP-3: 74%). Nuclear accumulation of FBPs significantly correlated with the process of malignant transformation (Spearman correlation: r > 0.3, p < 0.0001), tumor cell proliferation (Ki-67: r > 0.5, p < 0.0001), and poor survival of HCC patients (p < 0.05). No correlation between FBP and c-MYC expression was detected. Selective inhibition of FBP-1 and FBP-3 significantly reduced tumor cell viability in HCC cells which was based on reduced proliferation but not apoptosis. In addition, FBP-1 and FBP-2 but not FBP-3 stimulated hepatocyte growth factor (HGF)-induced migration in HCC cells. No changes of c-MYC expression were detected after siRNA-mediated inhibition of all FBP family members.

Discussion/Conclusion: Concerted activation of FBP family members represents a novel and frequent pro-tumorigenic mechanism promoting tumor growth and motility of human liver cancer cells. Although FBP family members do not modulate the expression of previously described target genes, they stimulate partly redundant effects in tumor cells. Thus, the FBP-system represents a potential target structure in the treatment of human cancer.
Facilitating translational research on human liver tissue specimens – Report on tissue banking experience over the last 10 years

M. Ilowski¹, T.S. Weiss², A. Reichert¹, S. Gashi¹, F. Stadler¹, S. Kirchner², H.-J. Schlitt², K.-W. Jauch¹, W.E. Thasler¹
¹Department of Surgery, Ludwig-Maximilians-University of Munich Hospital Grosshadern, Munich, Germany
²Centre for Liver Cell Research, Department of Surgery, University of Regensburg Hospital, Regensburg, Germany

Introduction: Research with human tissue is constantly extending. As part of translational research tissue banks are linking scientists and clinicians. This leads to a variety of related questions and problems concerning legal aspects and the technical procedure of collection, processing, storage and transfer of tissue specimens. Therefore a tissue bank with specific guidelines and a prospective data collection is of vital importance.

Methods: Over the last ten years a surgical tissue-bank focussing on liver pathology was developed with a regulatory framework to cover the necessity of ethical, legal and quality guidelines. In consequence the Human Tissue and Cell Research (www.htcr.de) foundation was established and is acting as an „honest broker“, monitoring tissue collection, processing and transfer. To apply appropriate procedures of tissue collection pre-, intra- and postoperative parameters (e.g. medication, ischemia time, storage conditions) were observed. Therefore tissue quality assessments (e.g. RNA degradation) of liver samples were performed to evaluate critical factors of tissue processing.

Results: From over 1200 donors who underwent liver surgery, tissue specimens (278 donors with primary and 973 donors with secondary liver tumours) were collected together with the corresponding blood samples. The analysis of the liver specimens showed no significant change in tissue quality after three hours ischemia and the usage of RNAlater had a protective effect on RNA stability. So far over 50 scientific projects used samples of the HTCR tissue bank.

Discussion/Conclusion: The liver tissue bank, certified according to ISO 9001: 2000 in 2007 by TÜV Süd, has built up a research platform which offers multiple approaches for translational research regarding chronic liver diseases in future.
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