Falk Workshop

Inflammation & Cancer

January 26 – 27, 2012
Medical Center
Hamburg-Eppendorf
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Abstracts
Poster Abstracts

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Abstracts of Invited Lectures
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INFLAMMATION & CANCER

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Scientific Organization:
A.W. Lohse, Hamburg
R. Thimme, Freiburg
G. Tiegs, Hamburg
C. Trautwein, Aachen

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Session I

Viral hepatitis

Inflammation and cancer: Hepatitis B, hepatitis C and HCC

Massimo Levrero, Cecilia Scisciani, Francesca Guerrieri, Barbara Testoni, Laura Belloni, Valeria Schinzari, Natalia Pediconi
Department of Internal Medicine (DMISM) and the Life Nano-Science Laboratory – Sapienza University of Rome, Rome, Italy

Hepatocellular carcinoma (HCC) ranks among the commonest cancers in many countries. A recent estimate indicates that HCC represents the fifth most common cancer of males, and the eighth most common cancer in females, with a total of 560,000 new cases each year (1). However, because of its very poor prognosis, HCC is the third leading cause of cancer death worldwide. (2).

Hepatocellular carcinoma (HCC) represent an intriguing “model human solid tumor” in that multiple viruses (hepatitis B virus [HBV] and hepatitis C virus [HCV] infection), toxins (alcohol and aflatoxin B1 ingestion) and chronic metabolic alterations (non-alcoholic fatty liver diseases and diabetes) lead, often in combination, to chronic inflammation, epigenetic and genetic changes that result in cancer development through a combination of both “common” and distinct “etiology specific” pathways. Increasing experimental evidence suggests that both HBV and HCV contributes to HCC by directly modulating pathways that promote the malignant transformation of hepatocytes and, in the case of HBV encoded HBx protein, to affect the epigenetic control of chromatin dynamics (3).

Alterations of the p53 family of tumor suppressors (p53 mutations and DNp73 isoforms deregulation) are found in more than 50% of human HCCs (4). In addition, the activation of several signaling pathways, both in cirrhotic tissue and in overt HCC, has been implicated in human hepatocarcinogenesis (5), including proliferation signaling pathways (e.g. epidermal growth factor [EGF] and transforming growth factor alpha [TGF-α], insulin-like growth factor [IGF], hepatocyte growth factor [HGF]/MET, and the downstream AKT/mTOR and Ras/RAF/MAPK transduction pathways), pathways involved in liver development and cell differentiation (WNT/ β-catenin and Hedgehog signaling pathways), pathways involved in liver inflammation (IL-6, transforming growth factor beta [TGF-β] and the lymphotoxin/NFkB cascades) and neoangiogenesis (VEGF and FGF) (5).

The integration of genetic and transcriptomic analysis has allowed to define specific subsets of human HCCs (6, 7), to identify both genetic alteration and genes and subsets of genes that are up- or downregulated in specific HCC subtypes or define an hepatic lesions or transitions (i.e. regenerative nodule vs. dysplastic nodule vs. early cancer) (8) or are associated with alterations that are common to all HCCs irrespective to their etiology (7). In addition to genetic and epigenetic abnormalities modifying oncogene and tumor suppressor genes, deregulation of microRNAs (miRNAs) may also contribute to liver carcinogenesis (9, 10) and a number of specific targets of miRNAs modulated in HCC have been identified.

Inflammatory cytokines, and IL-6 in particular, play a significant role is HCC development (11) and increased circulating levels of IL-6 are the best predictors of rapid progression from viral hepatitis to HCC in humans (12). HBV and HCV replication induce IL-6 synthesis both *in vitro* and *in vivo* (13, 14, 15). Upon HBV infection, IL-6 is induced in both hepatocytes (15) and non-infected Kupffer cells (14).

IL-6 released by Kupffer cells inhibits HBV replication and hepatocytes differentiation through the down-regulation of the liver enriched transcription factors HNF4a and HNF1a (14) that are recruited *in vivo* on the HBV cccDNA intermediate to boost replication (16). Extracellular HCV core protein activates STAT3 in human monocytes/macrophages/dendritic cells and modulates APC functions through an IL-6 autocrine pathway (13) but IL-6 activity on HCV replication is not established. IL-6-deficient mice are resistant to HCC induction upon challenge with DEN (diethylnitrosamine) (17). The early source of IL-6 in DEN-treated mice are Kupffer cells and inflammatory monocytes and the inducible ablation of the IκB kinase IKKβ in these cells blunts IL-6 production and HCC development (18). Obesity-promoted HCC is also dependent on enhanced IL-6 and TNF production, thus linking liver steatosis, a phenotype shared by virus- and non-virus-related HCCs, with liver inflammation and activation of the oncogenic transcription factor STAT3 (19).

Several miRNAs are differentially expressed in human HCCs as compared to paired peritumoral cirrhotic tissues or cirrhotic livers without HCC (9, 10). Other miRNAs have been shown to selectively modulate HCV (e.g. miR-122) (20) and HBV (miR-1) (21). In a genome wide search of HBx cellular targets by ChIP-Seq we have found that that HBx protein binds in vivo the regulatory region and modulates the expression of several miRNAs, including miR21 (a bona fide “onco-miR” elevated in HCC and other cancer types) and miR224 (apparently more HCC specific, frequently up-regulated in human HCCs, peri-tumor cirrhotic tissues and cirrhotic livers without HCCs). In the liver miR224 expression is induced by the NFκB-dependent inflammatory pathways (i.e. lymphotoxins and TNF-α, that are activated in a large proportion of chronic viral hepatitis, cirrhosis and HCC patients) (22) and miR21 by the wnt/β-catenin pathway.

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Hepatitis C and hepatocellular carcinoma

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

Persistent infection with the hepatitis C virus (HCV) is a major global health problem. Around 2–3% of the world population are chronically infected worldwide and infected individuals are at high risk of developing steatosis, fibrosis and liver cirrhosis. The latter is a major predisposing factor for development of hepatocellular carcinoma (HCC).

It is generally accepted that an inflammatory response triggered by chronic hepatitis C and affecting infected liver cells leads to increased cell proliferation and fibrogenesis, thus enhancing cirrhosis and HCC development. This indirect mechanism of tumor induction would explain why HCC most often develops only 10–30 years after primary HCV infection. It would also explain why additional cofactors such as toxins or drugs (most notably alcohol), metabolic liver diseases, steatosis, non-alcoholic liver disease or diabetes accelerate HCV-associated HCC.

With the advent of adequate cell culture systems for HCV, it is becoming increasingly clear that the virus also contributes directly to HCC formation. Examples are the viral replicase (especially non-structural protein 5B) and the core protein that bind to and sequester the tumor-suppressors retinoblastoma protein (Rb) and the RNA helicase DDX3, respectively. Another example is the massive perturbation of intracellular lipid homeostasis that is linked to HCV-associated steatosis. Finally, the viral serine protease cleaves the cellular T-cell protein tyrosine phosphatase, thus enhancing epithelial growth factor-dependent signal transduction. These examples illustrate the profound alterations of host cell homeostasis induced by HCV that likely promote development of HCC.

Distinct roles of CCR2 signaling in tumor cell extravasation

Monika Julia Wolf[1*], Alexandra Hoos[2*], Judith Bauer[3], Steffen Boettcher[4], Markus Knust[5,6], Achim Weber[7], Nicole Simonavicius[3], Holger Moch[7], Markus Manz[4], Michael Stürzl[8], Roland S. Croner[8], Andreas Konrad[8], Christoph Schneider[9], Adriano Aguzzi[1], Gert van Loo[10], Manolis Pasparakis[11], Marco Prinz[5], Lubor Borsig[2*] and Mathias Heikenwälder[1,3*]

[1]Institute of Neuropathology, University Hospital Zurich, 8091 Zurich, Switzerland
[2]Institute of Physiology, Zürich Center for Integrative Human Physiology, University of Zurich, 8057 Zurich, Switzerland
[3]Institute of Virology, Technische Universität München/Helmholtz Zentrum München, Munich, Germany
[4]Division of Hematology, University Hospital Zurich, 8091 Zurich, Switzerland
[5]Department of Neuropathology, University of Freiburg, 79106 Freiburg, Germany
[6]Faculty of Biology, University of Freiburg, 79104 Freiburg, Germany
[7]Institute of Surgical Pathology, University Hospital Zurich, 8091 Zurich, Switzerland
[8]Division of Molecular and Experimental Surgery, Department of Surgery, University Hospital Erlangen, 91054 Erlangen, Germany
[9]Institute of Integrative Biology, ETH Zurich, 8952 Schlieren, Switzerland
[10]Department for Molecular Biomedical Research, VIB Gent, 9052 Gent-Zwijnaarde, Belgium
[11]Institute of Genetics, Centre for Molecular Medicine (CMMC) and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, 50674 Cologne, Germany
[*]These authors contributed equally.

Metastasis is a multistage process that requires tumor cells to leave the primary tumor, survive in the circulation, seed at distant sites and grow. Recently it has been recognized that the tumor cell microenvironment significantly influences both tumor growth and metastatic dissemination. A variety of stromal cells have been associated with cancer progression, but tumor-associated macrophages are the major population detected at primary tumor sites while myeloid-derived and/or inflammatory monocytic cells make up the major part of stromal cells in metastatic lesions. Host-derived as well as tumor cell derived cytokines and chemokines actively shape the tumor microevironment by recruitment of leukocytes and activation of pro-inflammatory mediators. The sources of chemokine expression were postulated to be endothelial cells, infiltrating leukocytes and tumor cells themselves. Indeed, local activation of endothelium through metastasizing tumor cells led to local activation of endothelium associated with enhanced production of CCL5 chemokine. Inhibition of CCL5-dependent monocyte recruitment during the initial phase of metastasis strongly attenuated metastasis. Recently, another inflammatory chemokine, CCL2, has been identified as the major factor affecting breast metastasis to the lung. Elevated levels of CCL2 have been detected at primary as well as metastatic sites in various cancers including breast, colon, prostate and cervix and correlated with poor prognosis due to metastatic progression. Monocytes recruited to tumors through CCL2-CCR2 chemokine axis polarized to an alternatively activated phenotype of macrophages that contributes to: immunosuppression, enhanced tumor cell survival and vascularization. CCL2 has been shown to induce angiogenic activation of endothelial cells along with an inflammatory response. Accordingly, treatment of mice carrying

tumors with CCL2-neutralizing antibody significantly increased survival due to inhibition of metastasis. However, the exact mechanism how CCL2 signaling facilitates metastasis remained elusive. Here we describe how tumor cells engage distinct roles of CCR2 in extravasation.

Session II

Immunology

p50 NF-κB orchestrates colorectal cancer-related inflammation

Antonio Sica[1,2]
[1]DISCAFF, Università del Piemonte Orientale Amedeo Avogadro, Novara, Italy; [2]Istituto Clinico Humanita IRCCS, Rozzano, Milan, Italy

Colorectal cancer (CRC) is a major health problem in developed countries, which provides one of the best example of pathological association between chronic inflammation and cancer development. Tumor Associated Macrophages (TAM) represent the major leukocyte population present in tumors. Despite macrophages are potentially able to express M1 polarized anti-tumor functions, evidence indicate that TAM undergo to phenotypic switch promoting an M2 polarized phenotype with tumor promotion properties. We have previously demonstrated that nuclear accumulation of p50 NF-κB, as occurring in TAM, promotes a tolerant pro-tumoral phenotype. More recently, we have shown that, endotoxin tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50. This may be relevant in the gut, where innate immune cells are central regulators of intestinal homeostasis and orchestrate the balance between immune response and tolerance. Based on this, we investigated the role of p50-driven polarized inflammation in CRC development and progression, by using two distinct models of genetic- (Apc^{Min} mice) and colitis-associated cancer (CAC). Analysis of mice survival, tumor incidence, size and histopathological stage, in Apc^{Min} versus Apc^{Min}-$p50^{-/-}$ mice, demonstrated that the p50 NF-κB subunit is required to support cancer growth at different stages of the neoplastic process, including early (tumor initiation) and late stages of tumor progression. Strikingly, using the CAC model we observed that $p50^{-/-}$ mice exhibit a dramatic intestinal inflammation (as scored by weight loss, intestinal bleeding and histological analysis of colon tissues) paralleled by reduced incidence of tumor development. Overall our results suggest that, irrespective of the etiological events triggering CRC development, the p50 NF-κB-driven inflammation is required to promote cancer development.

Inflammatory bowel disease, T cells and cancer

Markus F. Neurath, M.D.
Medizinische Klinik 1, Klinikum der Universität Erlangen, Germany

Inflammatory bowel diseases (IBD: Crohn's disease, ulcerative colitis) are associated with an increased risk for colitis associated colon cancer. This is particularly well known for patients with ulcerative colitis. In these patients, established risk factors for disease development include the extent and duration of colitis as well as the presence of primary sclerosing cholangitis and backwash ileitis. These risk factors suggest that inflammation is a direct trigger of tumor growth in IBD. Indeed, recent studies in experimental models of colitis associated colon cancer such as the AOM/DSS model have clearly shown that inflammation is triggered by T lymphocytes and antigen presenting cells and that such inflammation provides direct growth factors for tumors. One example consists of the IL-6 system whereby IL-6 released by immune cells induces tumor growth via sIL-6R and the transcription factor STAT3 in intestinal epithelial cells. Recent progress in this field is shown and discussed.

Address for correspondence:

Prof. Dr. Markus F. Neurath
Medizinische Klinik 1
Klinikum der Universität Erlangen
Ulmenweg 18
91054 Erlangen
Germany
Telephone: (0 91 31) 85-3 52 04
Telefax: (0 91 31) 85-3 52 09
E-Mail: markus.neurath@uk-erlangen.de

Immune suppressor mechanisms in patients with hepato-cellular carcinoma

Prof. Dr. Tim F. Greten
Abteilung Gastrointestinale Onkologie, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda MD 20892, USA

Several studies have shown that development of hepatocellular carcinoma (HCC) initiates a number of immune suppressive mechanisms (Korangy et al., 2010a). We have been investigating different cellular mechanisms of cell mediated immune suppression in patients with HCC. We have been able to demonstrate an increase in the frequency of $CD4^+CD25^+Foxp3^+$ Tregs in HCC (Ormandy et al., 2005). Depletion of these cells in vitro and in vivo supports tumor-specific T cell responses in patients with HCC (Greten et al., 2010). Myeloid derived suppressor cells (MDSC) represent a different suppressor cell. We have identified a new population of human MDSC, namely $CD14^+HLA\text{-}DR^{-/low}$ cells (Greten et al., 2011). The frequency of $CD14^+HLA\text{-}DR^{-/low}$ cells is increased in peripheral blood and tumors of HCC (Hoechst et al., 2008) and these cells are potent suppressors not only of T but also NK cell responses (Hoechst et al., 2009). Differential analysis of $CD14^+HLA\text{-}DR^{-/low}$ MDSC and $CD14^+HLA\text{-}DR^+$ monocytes revealed that these cells modulate TGF-beta dependent $CD4^+$ T cell developmental program *ex vivo*. While $CD14^+HLA\text{-}DR^{-/low}$ MDSC induce $Foxp3^+$ regulatory T cells, we could demonstrate that $CD14^+HLA\text{-}DR^+$ monocytes promote generation of IL-17 secreting, $RORc^+$ Th17 cells when co-cultured with naïve $CD4^+$ T cells. Interestingly MDSC were also able to catalyze the trans-differentiation of $Foxp3^+$ regulatory T cells from monocyte-induced Th17 cells (Hoechst et al., 2011). Based on these results we decided to analyze Th17 cells in patients with HCC, since controversial results have been reported on the role of Th17 cells in cancer. First we analyzed the frequency of $CD4^+IL\text{-}17^+$ cells in peripheral blood from patients with HCC, which was increased in comparison to healthy controls. In order to further analyze the function of these cells, we generated Th17 cell lines *in vitro* in the presence of IL-1beta, IL-6 and IL-23 cytokines, anti-IL-4 and anti-IFN-gamma antibodies. Th17 cells were than stained using an IL-17 capture antibody and purified by FACS sorting. *In vitro* primed Th17 cells were further purified into $CCR4^+CCR6^+$ and $CCR4^{neg}CCR6^+$ Th17 cells. When cultured with autologous $CD8^+$ T cells, $CCR4^+CCR6^+$Th17 cells suppressed the lytic function, proliferation and cytokine secretion of both antigen specific and CD3/CD28/CD2 stimulated autologous $CD8^+$ T cells. In contrast, $CCR4^{neg}CCR6^+$ $CD4^+$ T cells, which also secrete IL-17, did not affect the $CD8^+$ T cells. Suppression of $CD8^+$ T cells by $CCR4^+CCR6^+$Th17 cells was partially dependent on TGF-beta, since neutralization of TGF-beta in cocultures reversed their suppressor function. Based on these findings we evaluated the frequency $CCR4^+CCR6^+$Th17 and $CCR4^{neg}CCR6^+$Th17 cells in patients with HCC separately. Our analysis revealed also an increase in the frequency of $CCR4^+CCR6^+$Th17 but not $CCR4^{neg}CCR6^+$ Th17 cells in peripheral blood of HCC patients. Finally we demonstrated the suppressor activity of freshly isolated $CCR4^+CCR6^+$T cells on autologous $CD8^+$ T cell proliferation. This study not only underlies the importance of analysis of subsets within Th17 cells to understand their function, but also suggests Th17 cells as yet another immune evasion mechanism in HCC. In summary we propose that understanding and targeting immune suppressor function in cancer including HCC is a pivotal requirement when

immune based treatment are developed for the treatment of HCC (Korangy et al., 2010b).

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Address for correspondence:

Prof. Dr. Tim F. Greten
Direktor der Abteilung Gastrointestinale Onkologie
Medical Oncology Branch
Center for Cancer Research, National Cancer Institute, NIH
Bldg. 10 Rm 12N226
9000 Rockville Pike
Bethesda, MD 20892, USA
Telephone: (301) 451-4723
Telefax: (301) 480-8780
E-Mail: tim.greten@nih.gov
Website: http://ccr.nci.nih.gov/staff/staff.asp?profileid=17192

Adaptive immune responses against hepatocellular carcinoma

Robert Thimme
Department of Medicine II, University Hospital Freiburg

Hepatocellular carcinoma (HCC) is one of the most common tumors in the world, with a global incidence of 500,000 new cases per year. HCC is up to four times more common in men than in women and 60–90% of these tumors develop in a cirrhotic liver. If untreated, most patients die within 3–6 months after diagnosis and, even if treated, the 5-year survival rate is low. Therefore, new strategies to treat patients with HCC have a high clinical priority. Immunotherapy aimed at inducing or activating HCC-specific immune responses is one such concept; its rationale is based on the presence of high numbers of tumor-infiltrating T cells in HCC tissue, the correlation between the density of lymphocytic infiltrates in HCC lesions and prognosis, and the finding that adoptive immunotherapy with anti-CD3 and interleukin-2 stimulated autologous lymphocytes lowers postsurgical HCC recurrence rates in humans. Different HCC targets have been identified in recent years, such as α-fetoprotein (AFP), MAGE-A family genes and NY-ESO. Importantly, several studies have demonstrated the existence of HCC-specific responses in blood and tumor of HCC patients although the relative immunodominance of different antigen-specific immune responses and the peripheral versus intratumoral distribution has not been determined. In addition, no correlation with the course of tumor progression could be observed. These results suggest a failure of these adaptive HCC specific immune responses in controlling tumor progression. Several mechanisms have been suggested to contribute to HCC specific T cell failure, such as the action of regulatory T cells, inhibition by myeloid-derived suppressor cells, an impairment of tumor specific antigen processing and presentation, the action of inhibitory receptors or a lack of $CD4^+$ T cell help. Thus, the development of novel immunotherapeutic strategies requires the identification of immunodominant and protective HCC-specific epitopes and a better understanding of the mechanisms contributing to T cell failure. These important aspects will be discussed.

Session III

Cytokines and intracellular signalling

NF-κB signaling in intestinal and hepatic inflammation and cancer

Rudi Beyaert[1,2], Lars Vereecke[1,2], Brecht Rogiers[1,2], Geert van Loo[1,2]
[1]Department for Molecular Biomedical Research, Unit of Molecular Signal Transduction in Inflammation, VIB, Ghent, Belgium; [2]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium, E-Mail: rudi.beyaert@dmbr.vib-ugent.be

It is generally accepted that chronic inflammation contributes to different steps of tumorigenesis. Increased or sustained activity of the transcription factor NF-κB in immune and non-immune cells plays a key role in inflammation and inflammation-associated cancer by driving the expression of inflammatory cytokines, growth and survival factors, angiogenic factors, proteases and other proteins in immune and non-immune cells. Understanding the underlying molecular mechanisms that normally ensure the proper termination of NF-κB signaling is therefore of therapeutic interest. *A20* (also known as *TNFAIP3*) is an NF-κB responsive gene that plays a key role in the negative feedback regulation of NF-κB signaling in response to pro-inflammatory cytokines and infection. In addition, A20 inhibits TNF-induced apoptosis. A20 exerts its inhibitory functions by acting as a deubiquitinating enzyme that targets specific signaling proteins. Human *A20* is a susceptibility locus for common inflammatory diseases such as Crohn's disease, rheumatoid arthritis, and lupus. To investigate the importance of A20 expression in preventing inflammation and inflammation-associated cancer in the intestine and the liver, we have generated several cell type-specific A20 knockout mice. The characterization of these mice will be presented.

Microenvironment in pancreatic oncogenesis – How myeloid cells are linked to pancreatic cancer development

H. Algül
II. Medizinische Klinik, Klinikum rechts der Isar der TU München, Munich, Germany

Physiological levels of *Kras*^{G12D} are sufficient to induce pancreatic intraepithelial neoplasias (PanINs); the mechanisms that drive PanIN progression are unknown. We establish that in addition to oncogenic *Kras*^{G12D}, the microenvironment determines pancreatic oncogenesis. Specifically, we detailed that IL-6 transsignaling-dependent activation of Stat3/Socs3 is required to promote PanIN progression and pancreatic ductal adenocarcinoma (PDAC). Myeloid compartment induces Stat3 activation by secreting IL-6; consequently, IL-6 transsignaling activates Stat3 in the pancreas. Using genetic tools, we show that inactivation of IL-6 transsignaling or Stat3 inhibits PanIN progression and reduces the development of PDAC. Aberrant activation of Stat3 through homozygous deletion of *Socs3* in the pancreas accelerates PanIN progression and PDAC development. Our data describe for the first time the involvement of microenvironment in PanIN progression and PDAC development.

Interleukin-6 trans-signalling and colonic cancer associated with inflammatory bowel disease

Stefan Rose-John, Dirk Schmidt-Arras and Athena Chalaris
Department of Biochemistry, Christian-Albrechts-Universität, 24098 Kiel, Germany

Cytokine receptors exist in membrane bound and soluble form. While most soluble receptors are antagonists, the soluble Interleukin-6 receptor (sIL-6R) is an agonist. *In vivo,* the IL-6/soluble IL-6R complex stimulates many types of target cells not responsive to IL-6 alone, since they do not express the membrane bound IL-6R. This process has been named trans-signaling (1, 2).

We have shown that soluble gp130 is the natural inhibitor of IL-6 trans-signaling responses (1, 2). Thus, soluble gp130 can be considered a molecular tool to discriminate between gp130 responses via membrane bound and sIL-6R. We have constructed a fusion of soluble gp130 and the Fc portion of human IgG1. This dimerized sgp130Fc protein proved to be efficient in blocking responses via the IL-6/soluble IL-6R complex without affecting IL-6 responses mediated via the membrane bound IL-6R (2–8). Using sgp130Fc we demonstrate that in chronic inflammatory diseases and cancer including inflammatory bowel disease, peritonitis, rheumatoid arthritis, atherosclerosis and colon cancer, trans-signaling via the soluble IL-6R complexed to IL-6 is a crucial step for the development and the progression of the disease. We therefore hypothesize that the pro-inflammatory activities of IL-6 are mediated via the soluble IL-6R whereas the regenerative or anti-inflammatory activities of this cytokine are rather mediated via the membrane-bound IL-6R (9).

ADAM17 is responsible for the proteolytic release of TNFα, IL-6R, L-selectin and ligands of the EGF-R. We used a novel gene targeting strategy to generate mice with undetectable ADAM17 levels in all tissues. The resulting mice showed dramatically increased susceptibility to inflammation in experimental colitis. This was due to a failure to phosphorylate STAT3 via the EGF-R and, consequently, in defective regeneration of epithelial cells and breakdown of the intestinal barrier. Our results demonstrate that shedding of membrane proteins via ADAM17 orchestrates inflammatory processes (10, 11).

Inhibition of inflammatory cytokines by various strategies has worldwide been approved in the clinic. We will discuss the physiologic consequences of global IL-6 blockade versus selective blockade of IL-6 trans-signaling in the treatment of chronic inflammatory diseases and inflammation-associated cancer.

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TNF-dependent signaling pathways in liver cancer

Tom Lüdde, M.D./Ph.D.
Department of Internal Medicine III, University Hospital RWTH Aachen, Pauwelsstr. 30, 52074 Aachen, Germany

HCC in most cases arises in a chronically inflamed liver due to e.g. chronic viral hepatitis. However, at present molecular mechanisms linking inflammation and tumour development in the liver are not fully understood yet. Tumour necrosis factor (TNF) is a cytokine essentially involved in the pathogenesis of a variety of liver diseases. Next to activation of caspases, ligand binding of TNF to its receptor also leads to the activation of several inflammatory signaling cascades, such as the Jun-(N)-terminal Kinase (JNK)-, the p38 (MAPK)- and the NF-κB-pathway. Studies previously conducted in genetically modified mouse models have highlighted that all these different signaling axes play important roles in the progression from chronic liver disease to liver cancer and show close functional interaction in this process. The molecule TAK1(TGF-β-activated kinase 1) represents a central signaling nexus connecting TNF-dependent signals with NF-κB, JNK and p38 and thus has been in the centre of our recent interest. Data on the functional role of TAK1 and its interaction with other TNF-dependent signaling cascades in hepatocarcinogenesis will be in the focus of this presentation.

Adipokines and HCC

H. Tilg
Innere Abteilung, Landeskrankenhaus Hall i.T., Austria

The adipose tissue has emerged as an important endocrine organ releasing many mediators including adipokines, classical cytokines and others. Adiponectin, a major products of adipocytes, is an anti-diabetic adipokine, which actions are exerted by the activation of AMP-activated kinase and peroxisome proliferator-activated receptor alpha. Adiponectin has many anti-inflammatory activities and suppresses tumour necrosis factor-alpha (TNFα, a cytokine of key relevance in non-alcoholic fatty liver disease (NAFLD). The anti-inflammatory effects of adiponectin are also mediated via induction of the anti-inflammatory cytokines interleukin-10 (IL-10) or IL-1 receptor antagonist and up-regulation of heme-oxygenase-1.

Liver regeneration involves many growth factors, cytokines and metabolic pathways. Adiponectin may be involved as adiponectin KO mice demonstrate decreased liver mass growth after partial hepatectomy. Obesity and associated-related disorders including metabolic syndrome and NAFLD are established risk factors for the development of HCC. Overweight might also influence the incidence of HCC in other liver diseases such as chronic HCV infection. Hypoadiponectinemia could promote tumour formation in the liver. These authors used a choline-deficient mouse NASH model and studied liver histology and oxidative stress markers in KO and wild-type mice. After 24 weeks, the majority of KO mice developed liver cirrhosis and hepatic tumours, whereas wild type mice showed simple steatosis. In another study, adiponectin increased apoptosis of HCC cells through activation of caspase-3 and blocked liver tumorigenesis in nude mice. Analysis of adiponectin expression levels in tissue microarray of human HCC patients showed an inverse correlation of adiponectin expression with tumor size. Adiponectin treatment abrogated leptin-induced cell proliferation of HCC cells and adiponectin treatment effectively blocked leptin-induced invasion of HCC cells in invasion assays. Importantly, adiponectin significantly reduced leptin-induced tumor burden in nude mice. In HCC samples, leptin expression significantly correlated with HCC proliferation, whereas adiponectin expression correlated with increased disease-free survival and inversely with tumour size and local recurrence. These data support the evidence that certain adipokines might be involved in the pathogenesis of liver cancer.

Session IV

Growth factors and regeneration

Accelerated carcinogenesis following liver regeneration

Eithan Galun
Goldyne Savad Institute of Gene Therapy, Hadassah Hebrew University Hospital, Ein Karem, Jerusalem, Israel

Chronic inflammation is a common underlying condition associated with tumor development, accounting for approximately 20% of human cancers. This association is especially apparent in hepatocellular carcinoma (HCC), which often develops on the background of chronic hepatitis, hepatic fibrosis and chronic inflammation. HCC is one of the most common tumors worldwide, exhibiting a very poor prognosis and high mortality rate with limited available therapeutic tools. The etiology of liver cancer is well known, however there is still a lack of precise knowledge about pathogenesis of HCC. Both IL-6 and one of its downstream signaling mediators, STAT3, have been shown to be of importance for liver protection and prevention of liver injury in animal models of acute sclerosing cholangitis and correlate with increased HCC in human patients. Using a murine model of chronic cholangitis based on the ablation of the Mdr2 gene, this study has examined the role of IL-6 and STAT3 signaling in chronic hepatitis and in the subsequent development of liver cancer. In addition, this study has examined the role of IL-6 in accelerated tumorigenesis on the background of chronic hepatitis following PH in Mdr2-KO mice. The results of the study testify to the existence of a complex, cell type and gender-dependent relationship between chronic injury-driven IL-6/STAT3 signaling, liver fibrosis and the emergence of HCC. The main observations of this study are that IL-6 signaling in male Mdr2-KO mice protects from the development of liver injury and fibrosis, but simultaneously promotes tumor initiation. In contrast, in female mice IL-6 signaling does not substantially affect the levels of liver injury, but acts to profoundly reduce tumor initiation and progression. In addition we show that STAT3 activation in hepatocytes of male Mdr2-KO mice acts to reduce the persistence of liver injury as well as tumorigenesis. We also demonstrated that IL-6 signaling in Mdr2-KO mice plays a crucial role in regenerative stress accelerated tumorigenesis associated with chronic inflammation-induced DNA instability. Understanding the role of IL-6 and STAT3 in the development of chronic inflammation associated HCC and the role of IL-6 in the accelerated carcinogenesis following PH may be of importance in the development of clinical tools for the treatment of HCC.

TGFβ in onset and progression of HCC

S. Dooley
II. Medizinische Klinik, UMM Universitätsmedizin Mannheim, Universitätsklinikum Mannheim, Germany

Transforming growth factor-β (TGF-β is a central regulator in chronic liver disease contributing to all stages of disease progression from initial liver injury, through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma. Liver damage-induced levels of active TGF-β enhance hepatocyte destruction, mediate hepatic stellate cell (HSC) and fibroblast activation resulting in a wound healing response, including myofibroblast generation and extracellular matrix deposition. Further evidences point to a decisive role of cytostatic and apoptotic functions mediated on hepatocytes, which is critical for the control of liver mass with loss of TGF-β activities resulting in hyperproliferative disorders and cancer. This concept is based on studies that describe a bipartite role of TGF-β with tumor suppressor functions at early stages of liver damage and regeneration, whereas during cancer progression TGF-β may turn from a tumor suppressor to a tumor promoter that exacerbates invasive and metastatic behaviour. We have delineated this molecular switch of the pathway from cytostatic to tumor promoting in further detail and identify activation of survival signaling pathways in hepatocytes as most critical requirement.

Targeting the TGF-β signaling pathway has been explored to inhibit liver disease progression. While interfering with TGF-β signaling in various short-term animal models has demonstrated promising results, liver disease progression in human is a process of decades with different phases, in which TGF-β or its targeting may have both beneficial and adverse outcomes. We emphasise that in order to achieve therapeutic effects, targeting TGF-β signaling in the right cell type at the right time is required.

Growth factors in hepatocellular carcinoma: Focus on the EGFR pathway

Matías A. Avila
Division of Hepatology and Gene Therapy, CIMA, University of Navarra, 31008 Pamplona, Spain, E-Mail: maavila@unav.es

Hepatocellular carcinoma (HCC) slowly unfolds on a background of chronic liver injury, fibrosis and inflammation triggered by exposure to infectious agents, toxic compounds, or metabolic impairment. Mounting evidence indicates that the pro-inflammatory milieu participates not only in hepatic fibrogenesis, but also plays a role in tumor development. The molecular links that connect inflammation, fibrosis and cancer are not completely known, although recent studies are beginning to define more precise mechanisms. A central role for growth factors like TGF-β, Wnt family members, insulin-like growth factors and the epidermal growth factor receptor (EGFR) signalling system in the progression from chronic inflammation to cancer is currently being established. Given the complex nature of hepatocarcinogenesis the implication and crosstalk between different molecular pathways is likely to occur. Furthering our knowledge of these mechanisms is needed to design more effective therapies. We have identified a novel role for the EGFR ligand amphiregulin (AR), in the progression of liver disease. AR is not expressed in normal liver, but it is detected in experimental models of acute liver damage and in patients with chronic liver injury and cirrhosis, as well as in a significant proportion of human HCCs. AR expression is induced by inflammatory mediators in macrophages and hepatocytes, and promotes the growth and survival of normal and transformed liver parenchymal cells, as well their resistance to anti cancer drugs. In addition, we have also characterized the relevant contribution of AR to hepatic fibrogenesis and HCC progression. The pro-tumorigenic mechanisms triggered by AR overexpression are not completely known, however we have recently observed that this growth factor promotes the expression of oncogenic splice variants of the tumor suppressor protein p73. Novel crosstalks of the EGFR system with protumorigenic pathways such as the YAP oncogene, and its transcriptional target connective tissue growth factor (CTGF), will be presented.

Stem cells and liver cancer

Tania Roskams, M.D., Ph.D.
Head Translational Cell and Tissue Research, University of Leuven, Leuven, Belgium

According to the cancer stem cell concept, hepatocellular carcinoma (HCC) consists of a hierarchy of cell populations, of which the very small cancer stem cell population is the one that has the growth and metastatic potential of the tumour. The other neoplastic cells are offspring of the cancer stem cells and each can differentiate a little differently, according to the local microenvironment in each part of the tumor, hence explaining the enormous phenotypic heterogeneity of a neoplasm.

Current therapeutic strategies mostly target rapidly growing differentiated tumour cells. However the results are often unsatisfactory because of the chemoresistance of HCC. New therapies targeting cancer stem cells should therefore be developed. A prerequisite is a good understanding of the mechanisms of activation and differentiation of normal stem/progenitor cells in normal and diseased liver. Hepatocytes and cholangiocytes have stem cell features, but also progenitor cells, located in the smallest branches of the biliary tree. These cells are especially activated in the cirrhotic stage of chronic liver diseases, the stage in which HCC develops. HCC with progenitor cell features, possibly reflecting a progenitor cell origin, have a very bad prognosis and therefore should be recognized and treated accordingly.

Keywords: liver stem cells, liver progenitor cells, hepatocellular carcinoma, cholangiocarcinoma, chemoresistance, cancer stem cells, side population

List of Chairpersons, Speakers and Scientific Organizers

PD Dr. Hana Algül
II. Medizinische Klinik
Klinikum rechts der Isar
der Technischen Universität
Ismaninger Str. 22
81675 München
Germany

Dr. Matías A. Avila
Clinica Universitaria Pamplona
Terapia Génica y Hepatologia
CIMA
Avda. Pio XII 55
31008 Pamplona
Spain

Prof. Dr. Ralf Bartenschlager
Molekulare Virologie
Universitätsklinikum Heidelberg
Im Neuenheimer Feld 324
69120 Heidelberg
Germany

Prof. Dr. Rudi Beyaert
Department for Molecular
Biomedical Research
VIB – Ghent University
VIB Research Building FSVM
Technologiepark 927
9052 Gent
Belgium

Prof. Dr. Steven Dooley
II. Medizinische Klinik
UMM Universitätsmedizin Mannheim
Universitätsklinikum Mannheim
Theodor-Kutzer-Ufer 1-3
68167 Mannheim
Germany

Prof. Dr. Eithan Galun
Hadassah University Hospital
Ein Kerem
Goldyne Savad Institute of
Gene Therapy
P.O. Box 12000
91 120 Jerusalem
Israel

Tim F. Greten, M.D.
Professor of Medicine
National Cancer Institute, NIH
Gastrointestinal Cancer Section
Med. Oncology, Bldg.10/Rm 12N226
9000 Rockville Pike
Bethesda, MD 20892
USA

Prof. Dr. Mathias Heikenwälder
Institut für Virologie
Helmholtz Zentrum München
Ingolstädter Landstr. 1
85764 Neuherberg
Germany

Prof. Dr. Frank Lammert
Klinik für Innere Medizin II
Universitätsklinikum des Saarlandes
Kirrberger Str.
66424 Homburg
Germany

Prof. Massimo Levrero
Sapienza Università di Roma
Policlinico Umberto I
I Clinica Medica
00161 Roma
Italy

Prof. Dr. Ansgar W. Lohse
Medizinische Klinik I
Universitätsklinikum Eppendorf
Martinistr. 52
20251 Hamburg
Germany

PD Dr. Tom Lüdde
Medizinische Klinik III
Universitätsklinikum Aachen
Pauwelsstr. 30
52074 Aachen
Germany

Prof. Dr. Markus F. Neurath
Medizinische Klinik 1
Universitätsklinikum Erlangen
Ulmenweg 18
91054 Erlangen
Germany

Prof. Dr. Ulrike Protzer
Virologie
Technische Universität München
Trogerstr. 30
81675 München
Germany

Prof. Dr.Dr.h.c. Giuliano Ramadori
Gastroenterologie
Universitätskliniken Göttingen
Robert-Koch-Str. 40
37075 Göttingen
Germany

Prof. Dr. Stefan Rose-John
Biochemie
Universität Schleswig-Holstein
Campus Kiel
Olshausenstr. 40
24118 Kiel
Germany

Prof. Dr. Tania Roskams
Department of Pathology
Catholic University of Leuven
University Hospital St. Rafael
Minderbroederstraat 12
3000 Leuven
Belgium

Prof. Dr. Antonio Sica
Istituto Clinico Humanitas IRCCS
Via Manzoni, 56
20089 Rozzano
Italy

Prof. Dr. Robert Thimme
Innere Medizin II
Universitätsklinikum Freiburg
Hugstetter Str. 55
79106 Freiburg
Germany

Prof. Dr. Gisa Tiegs
Experimentelle Immunologie
Universitätsklinikum Eppendorf
Martinistr. 52
20251 Hamburg
Germany

Prof. Dr. Herbert Tilg
Interne Abt.
Landeskrankenhaus
Hall i.T.
Milserstr. 10
6060 Hall/Tirol
Austria

Prof. Dr. Christian Trautwein
Medizinische Klinik III
Universitätsklinikum Aachen
Pauwelsstr. 30
52074 Aachen
Germany

POSTER ABSTRACTS

Poster Numbers 1 – 55

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Age and gender affect BMP6-mediated hepcidin expression and iron homeostasis

Stephanie Arndt[1], Eva Wacker[1], Ulrike Maegdefrau[1], Christoph Dorn[2], Katharina Schardt[1], Claus Hellerbrand[2] & Anja-Katrin Bosserhoff[1*]
[1]Institute of Pathology, and [2]Department of Internal Medicine I, University Regensburg, 93042 Regensburg, Germany

Introduction: Intestinal iron absorption is critical for normal iron balance, and it is regulated by hepcidin which is synthesized predominantly in the liver. Dysregulation of hepcidin production induces many iron related disorders, and increased (hepatic) iron levels have been shown to play a critical role in hepatocancerogenesis. We have shown that bone morphogenetic protein 6 (BMP6) is an important endogenous regulator of hepcidin and that it is produced predominantly in the small intestine but not in the liver (Gastroenterology. 2010; 138 [1]: 372–382). We expanded these studies to a comparison between male and female mice of different age to investigate whether there are differences that may also account for age and gender dependent variation in the progression of chronic liver disease.

Methods and results: Interestingly, BMP6 deficient mice ($BMP6^{-/-}$) on a 129Sv/Ev background fed with normal diet developed severe hepatic iron accumulation and reduced hepcidin expression with increasing age. It is important to note that the phenotype of hepatic iron accumulation and reduced hepcidin expression could be triggered in younger mice by feeding iron-supplemented diets or parenteral application of iron-dextran. Both treatments induced a marked upregulation of BMP6 expression in the small intestine and higher BMP6 serum levels. These results suggest that intestinal BMP6 is transported via the bloodstream to the liver for hepcidin regulation. One further interesting new finding was that male mice showed significantly lower hepcidin levels and higher hepatic iron-overload after feeding an iron-supplemented diet than female mice of the same age.

Conclusion: We observed age and gender dependent differences with regards to BMP6 and hepcidin expression under basal conditions as well as in response to iron overload. We speculate that these findings accounts at least in part for age and gender dependent differences in the progression of chronic liver diseases and cancerogenesis.

2

Induction of heme oxygenase 1 in Mdr2ko mice prevents fibrosis progression, supports fibrolysis and delays tumor development

Roja Barikbin[1], Daniel Neureiter[3], Jan Wirth[2], Johannes Kluwe[2], Thomas Ernst[4], Gisa Tiegs[1], Gabriele Sass[1]
[1]Department of Experimental Immunology and Hepatology, University Medical Center Hamburg Eppendorf, Hamburg, Germany
[2]Department of Internal Medecine, University Medical Center Hamburg Eppendorf, Hamburg, Germany
[3]Institute of Pathology, Paracelsus Medical University, Salzburg, Austria
[4]Center for Radiology and Endoscopy, University Medical Center Hamburg Eppendorf, Hamburg, Germany

Introduction: Heme oxygenase 1 (HO-1) has been shown to protect mice from acute hepatitis and chronic inflammation. We now investigated effects of HO-1 induction in a mouse model of chronic liver inflammation with progression to fibrosis and hepatocellular carcinoma (HCC) (Mdr2 knockout; Mdr2ko; FVB.129P2-Abcb4^{tm1Bor}) with respect to effects on fibrosis formation and long term progression to HCC.

Methods: HO-1 was induced in Mdr2ko mice for 7 consecutive weeks to investigate fibrosis. Fibrosis formation was monitored by CAB staining, hepatic stellate cell (HSCs) activation, hydroxyproline levels and zymography for activity of MMP-9. Activity of HSCs was determined in *in vitro* experiments. Real time RT-PCR was used to quantify expression levels of fibrosis markers, e.g. a-SMA. For long term experiments HO-1 was induced for 9 consecutive weeks and tumor development was monitored by magnetic resonance imaging (MRI).

Results: Induction of HO-1 interfered with fibrosis formation by reducing connective tissue assembly and hydroxyproline levels as well as collagen I and III expression and MMP-9 activity. The amount of quiescent and activated HSCs was reduced. *In vitro* HSCs showed reduced activation upon HO-1 induction. MRI monitoring showed that early HO-1 induction decreased incidence of tumor formation in 60 week old Mdr2ko mice.

Discussion/Conclusion: Induction of HO-1 reduced fibrosis formation and partially reverted fibrosis in Mdr2ko mice by regulating number and activity of HSCs. As a long term effect of early HO-1 induction progression to HCC seems to be delayed.

Characterization of B-cell activation and exhaustion markers in patients with chronic viral infections (HBV, HCV)

C. Beisel[1], I. Tóth[2], C. Scheurich[2], V. Matzat[2], S. Kummer[2], S. Lüth[1], J. van Lunzen[2], P. Hartjen[2], A.W. Lohse[1], J. Schulze zur Wiesch[1,2]
[1]Zentrum für Innere Medizin, I. Medizinische Klinik und Poliklink, Universitätsklinikum Hamburg-Eppendorf, Germany
[2]Heinrich Pette Institut – Leibniz Institut für Experimentelle Virologie (HPI), Hamburg, Germany

Introduction: Infections with hepatitis B virus (HBV) or hepatitis C virus induce an acute necroinflammatory liver disease that may result in clearance of the virus in some patients, while evolving into a chronically persisting viral infection in other patients. An effective B-cell response is essential to eliminate the virus in an acute stage of infection. Relevant secondary diseases, such as cryoglobulinemia or non-Hodgkin lymphomas, are likely to evolve due to chronic B-cell activation. So far, the role of B-cells, their phenotype and the expression of different activation and exhaustion markers in chronic viral infections are insufficiently characterized.

Methods: Peripheral blood of untreated, chronic hepatitis B (n = 25) and hepatitis C (n = 25) infected patients was analyzed by multicolour flow cytometry to determine the frequency and phenotype of B-cells. The frequency of CD20+ B-cells, activation markers (CD80, CD86, CD71, CD69), exhaustion markers (BTLA, FcRL, CTLA4) and their proliferation (via Ki67) were analyzed.

Results: Compared to healthy controls, we detected a significant higher frequency of CD20+ B-cells ($p > 0.001$) in patients with chronic viral hepatitis. Beside an increased expression of Ki67 ($p < 0.0063$), a dramatically high expression of BTLA (*B and* T lymphocyte *attenuator*), a member of the B7 CD28 family, was observed in HBV and HCV-infected patients ($p < 0.0001$).

Discussion/Conclusion: Our experiments showed an increased frequency and proliferation of B-cells, as well as an upregulation of BLTA in HBV/HCV-infected patients. Further functional experiments in different stages of HBV/HCV infection will need to be performed. Moreover, our results raise the question wether BTLA expression is correlated with the clinical course of infection or additional immunological markers.

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Impact of tumor markers in diagnosis of patients with hepatocellular carcinoma (HCC) and their clinico-pathologic correlation: An interim analysis

Dominik Bettinger[1], Michael Schultheiß[1], Jonas Bürk[2], Elisabeth Panther[1], Eva Knüppel[1], Robert Küper [3], Robert Thimme[1], Hubert E. Blum[1], Hans Christian Spangenberg[1]
[1]University Hospital Freiburg, Department of Medicine II, Hugstetter Str. 55, 79106 Freiburg
[2]University Hospital Freiburg, Department of Diagnostic Radiology and Medical Physics, Hugstetter Str. 55, 79106 Freiburg
[3]Wako Chemicals GmbH, Fuggerstr. 12, 41468 Neuss, Germany

Introduction: Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Diagnosis is mainly based on imaging performance whereas alpha-fetoprotein (AFP) measurement is not recommended according the current AASLD guidelines. The aim of this study is to determine the usefulness of AFP, AFP-L3% and des-γ-carboxyprothrombin (DCP) in diagnosis of HCC and their correlation to clinico-pathologic parameters.

Methods: Serum samples of 75 patients with confirmed HCC and of 175 patients with liver cirrhosis were prospectively collected. Tumor markers were measured using a micro-total analysis system. Diagnostic accuracy was determined by ROC analysis reporting sensitivity, specificity and the area under the curve (AUC) with 95% confidence interval. Differences in marker values were calculated using non-parametric tests.

Results: Optimal cut-off values for AFP, AFP-L3% and DCP were 10 ng/ml, 10% and 2.87 ng/ml with an AUC of 0.745, 0.784 and 0.777, respectively. Combination of all markers showed superior diagnostic accuracy with an AUC of 0.912 yielding sensitivity and specificity of 93% (87–99%) and 70% (63–77%). In patients with AFP < 20 ng/ml AFP-L3% showed sensitivity and specificity of 68% (54–83%) and 89% (84–94%). AFP, AFP-L3% and DCP were significantly higher in patients with multifocal HCC ($p = 0.008$, $p = 0.004$, $p = 0.011$ respectively). Larger tumors and higher BCLC stage were associated with higher DCP values.

Discussion/Conclusion: Combination of all markers showed better diagnostic accuracy than using them alone. In patients with AFP < 20 ng/ml AFP-L3% may be useful in diagnosing HCC. DCP yielded a better association with clinico-pathologic parameters than AFP and AFP-L3% and may provide further information concerning disease state.

Can alpha-fetoprotein (AFP) be replaced by other tumor markers in patients with inflammatory active liver cirrhosis in HCC surveillance programs?

Dominik Bettinger[1], Michael Schultheiß[1], Jonas Bürk[2], Elisabeth Panther[1], Eva Knüppel[1], Robert Küper[3], Robert Thimme[1], Hubert E. Blum[1], Hans Christian Spangenberg[1]
[1]University Hospital Freiburg, Department of Medicine II, Hugstetter Str. 55, 79106 Freiburg
[2]University Hospital Freiburg, Department of Diagnostic Radiology and Medical Physics, Hugstetter Str. 55, 79106 Freiburg
[3]Wako Chemicals GmbH, Fuggerstr. 12, 41468 Neuss, Germany

Introduction: The incidence of hepatocellular carcinoma (HCC) is rising in the Western world due to chronic hepatitis C infection. AFP is often false positive in patients with viral hepatitis and inflammatory active liver cirrhosis resulting in impaired usefulness in HCC surveillance. The aim of this study is to determine the impact of AFP, AFP-L3% and des-γ-carboxyprothromin (DCP) in these patients.

Methods: 60 patients with liver cirrhosis (CI) and 24 patients with HCC were prospectively included in this study. All patients had viral hepatitis. AFP, AFP-L3% and DCP were measured using a micro-total analysis system. Diagnostic accuracy was determined by ROC analysis reporting sensitivity, specificity and the area under the curve (AUC).

Results: 36/60 patients with CI had elevated aspartat aminotransferases (AST) and alanin aminotransferases (ALT) indicating hepatic inflammation. These showed higher median AFP values compared to those without elevation of AST and ALT (13.4 U/l vs. 5.1 U/l; p = 0.001) whereas AFP-L3% and DCP did not differ significantly. Using a threshold of 10 ng/ml specificity of AFP was lower in patients with elevated AST and ALT than in patients with normal values (59% vs. 72%). The AUC of AFP was lower in patients with elevated aminotransferases (0.59 vs. 0.72) whereas the AUCs for AFP-L3% and DCP were similar.

Discussion/Conclusion: Hepatic inflammation results in elevated AFP values leading to an insufficient HCC screening tool. AFP-L3% and DCP are not influenced by hepatic inflammation and may provide better diagnostic accuracy for diagnosing HCC in patients with viral hepatitis associated CI.

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Protective effects of LCN2 in acute experimental liver injury

E. Borkham-Kamphorst, E. Van de Leur, L. Tiha and R. Weiskirchen
Institut für Klinische Chemie und Pathobiochemie, Aachen, Germany

Introduction: Lipocalin-2 (LCN2) is a 25-kDa protein belonging to the lipocalin family. It preferably binds to lipophilic substances and plays a role in iron metabolism, regulates haematopoiesis, modulates inflammatory processes, and has recently been implicated in epithelial-to-mesenchymal transition and restoration of tissue homeostasis following LPS-induced injury. Our liver injury models showed rapid induction of well sustained LCN2 expression throughout. Immunohistochemistry and cell-based experiments identified injured hepatocytes as the main source of LCN2 production, but the biological function of LCN2 in liver injury requires further elucidation.

Methods: $LCN2^{-/-}$ mice were used in experimental models for acute liver injury e.g. CCl_4, BDL, LPS and ConA models. Liver injury was assessed by liver function analysis, real time PCR for chemokine and cytokine expression, liver tissue Western Blot, histology and Tunel assay for apoptosis.

Results: Acute single dose CCl_4 intoxication showed increased liver damage in $LCN2^{-/-}$ mice as indicated by significant higher levels of AST and ALT, increased expression of liver injury associated- and inflammatory cytokines and chemokines such as IL-1beta, TNFalpha and CCL2. $LCN2^{-/-}$ mice further showed a marked lipid droplet accumulation in hepatocytes by oil red O staining and increased hepatocyte apoptosis. In the ConA model, $LCN2^{-/-}$ animals reflected increased hepatocyte apoptosis. Similar findings were obtained in LPS and 5 day-BDL models.

Conclusion: Upregulation of LCN2 in response to liver injury is not only an indicator of liver damage but LCN2 also has an endogenous hepato-protective effect on tissue homeostasis.

Precision-cut liver slices as a useful in vitro tool for the investigation of metabolic activation via oxidative phase I metabolism

Britta Burkhardt[1/2], Judith Wittenauer[1], Erika Pfeiffer[1], Manfred Metzler[1]
[1]Karlsruhe Institute of Technology (KIT), Institute of Applied Biosciences, Department of Food Chemistry, Karlsruhe, Germany
[2]Department of Traumatology, Eberhard Karls University, Tübingen, Germany

Introduction: One major mechanism leading to carcinogenicity of xenobiotics is the generation of electrophilic metabolites via phase I metabolism. As an example, CYP-mediated oxidation of aflatoxin B_1, a potent liver carcinogen, is responsible for the carcinogenicity of this mycotoxin. For investigation of the oxidative metabolism, in vitro methods which sufficiently reflect the in vivo situation are required. In this study, we present precision-cut liver slices as a useful tool to clarify the oxidative metabolism of the genotoxic mycotoxin Alternariol (AOH) under in vivo-like conditions. CYP-mediated hydroxylation of this compound leads to reactive catechols that might be of toxicological relevance.

Methods: Precision-cut rat liver slices were prepared from the freshly isolated liver of a male Sprague Dawley rat using a Vitron Tissue Slicer (Vitron, Tucson, AZ, USA). The slices were incubated with 50 µM AOH in Waymouth´s medium for 5 and 24 h, followed by LC-MS/MS analysis.

Results: After 5 h, all four monohydroxylated metabolites were detectable, together with several *O*-methylation products (MPs) formed by catechol-*O*-methyltransferase (COMT). However, after 24 h, the oxidative main metabolite (10-hydroxy-AOH) could not be detected anymore, suggesting its reactivity. Incubations with simultaneous inhibition of COMT led to a total decrease of MPs with only a slight increase of the catechol metabolites. These findings indicate that *O*-methylation is a major detoxification pathway; without this, AOH catechols might undergo redox cycling, leading to oxidative stress and/or covalent protein or DNA adducts.

Discussion/Conclusion: Our investigations have shown that hydroxylation of AOH occurs under in vivo-like conditions and must therefore be expected in vivo. Because of their catechol structure, the oxidative metabolites may be of toxicological relevance.
Precision-cut liver slices not only represent a useful tool for the investigation of the oxidative metabolism; in future studies, we will focus on the induction of oxidative stress and the formation of DNA adducts by oxidative AOH metabolites.

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Non-alcoholic fatty liver disease does not affect early stage of liver regeneration after partial hepatectomy in rats

Z. Červinková, T. Garnol, H. Lotková, O. Kučera
Department of Physiology, Faculty of Medicine, Charles University in Prague, Hradec Kralove, Czech Republic

Introduction: Non-alcoholic fatty liver disease (NAFLD) is a common hepatic disorder which refers to a wide spectrum of liver damage, ranging from simple steatosis to steatohepatitis, advanced fibrosis, cirrhosis, and liver cancer. Fatty infiltration of the liver is associated with an increased incidence of complications and mortality after liver resection. The aim of our study was to evaluate whether steatosis impairs course of liver regeneration after 2/3 hepatectomy (PH) in rats.

Methods: Male Sprague-Dawley rats were fed ad libitum a standard pelleted diet (ST-1, 10% of energy from fat) or high-fat gelled diet (HFGD, 71% of energy from fat) for 6 weeks and then PH after Higgins and Anderson was performed. Animals were sacrificed 24 and 48 hours after PH; serum activities of ALT, AST, and concentrations of bilirubin, glucose, urea, triacylglycerols and cholesterol were measured. Respiration of isolated liver mitochondria was assessed using high-resolution respirometry (Oroboros 2k). Malondialdehyde (MDA) contents in the liver (HPLC) and tissue cytokines (ELISA; TNF-α, IL-6) were assessed; histopathological samples were prepared (H+E, Sudan III). The extent of liver regeneration was evaluated by incorporation of bromodeoxyuridine (BrdU).

Results: Feeding with HFGD caused a decrease in urea concentration ($p < 0.05$ and $p < 0.01$ 24 and 48 h after PH) in comparison with ST-1 group. There were no significant differences among groups in total serum cholesterol, glycaemia, and respiration of isolated mitochondria. HFGD increased production of MDA in the liver 48 h after PH ($p < 0.01$).

Discussion/Conclusion: Regeneration of the liver with simple steatosis was not significantly affected as documented by incorporation of BrdU.

Hepatocyte-specific IKKγ (NEMO)-mediated liver damage is induced through distinct signaling pathways in TRAIL and TNFR1-knockout mice

Francisco Javier Cubero[1*], Anjana Singh[1*], Erawan Borkham-Kamphorst[2], Yulia A. Nevzorova[1], Malika Al Masaoudi[1], Mark Boekschoten[3], Nikolaus Gassler[4], Ralf Weiskirchen[2], Tom Luedde[1], Mathias Heikenwalder[5], Michael Müller[3], Christian Liedtke[1], Christian Trautwein[1]
[*]Equal contribution
[1]Department of Internal Medicine III, University Hospital, RWTH Aachen, Germany
[2]Institute of Clinical Chemistry and Pathobiochemistry, University Hospital, RWTH Aachen, Germany[†]
[3]Division of Nutrition, Metabolism & Genomics, Wageningen University, Wageningen, The Netherlands
[4]Institute of Pathology, University Hospital, RWTH Aachen, Germany
[5]Institute of Virology, TU Munich, Munich, Germany

Introduction: Hepatocyte-specific IKKγ knockout mice ($Nemo^{\Delta hepa}$) show high susceptibility to apoptosis. In this context, tumor necrosis factor receptor 1 (TNF-R1) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) play a pivotal role in the complex regulation of the apoptotic machinery in liver cells. Therefore, we aimed to better characterise the involvement of death receptors in the progression of liver injury in $Nemo^{\Delta hepa}$ mice.

Methods: We generated $Nemo^{\Delta hepa}/TRAIL^{-/-}$ and $Nemo^{\Delta hepa}/TNFR1^{-/-}$ double knockout mice and analyzed the progression of liver injury.

Results: $Nemo^{\Delta hepa}/TRAIL^{-/-}$ displayed a similar phenotype to $Nemo^{\Delta hepa}$ mice characteristic of high apoptosis, infiltration of immune cells, proliferation, steatohepatitis, fibrosis, and appearance of dysplastic nodules at 8 weeks. These pathophysiological features were significantly ameliorated in $Nemo^{\Delta hepa}/TNF\text{-}R1^{-/-}$ livers. Reduced apoptotic cell death concomitant accompanied with a strong reduction in JNK activation was observed after deletion of TNF-R1. Cell cycle parameters such as Cyclin A, Cyclin D and p21 were significantly less activated in $Nemo^{\Delta hepa}/TNFR1^{-/-}$ livers. Additionally, markers of liver fibrosis such as collagen IA1 and αSMA and indicators of tumor progression were significantly decreased in absence of TNF-R1.

Discussion/Conclusion: Our present data demonstrate that the death receptor TNFR1 but not TRAIL is essential in determining progression of liver injury in hepatocyte-specific NEMO knockout mice, which opens a new therapeutic gate for the treatment of chronic liver disease.

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Monoacylglycerol lipase expression is down regulated in hepatocellular carcinoma

Barbara Czech, Daniela Valletta, Claus Hellerbrand
Department of Internal Medicine I, University Hospital Regensburg, Germany

Introduction: Cancer cells connect finely tuned lipogenesis with lipolysis to produce fatty acid networks that support malignancy. Monoacylglycerol lipase (MAGL) plays a principal role in lipolysis by converting monoglycerides, including the endo-cannabinoid 2-arachidonoylglycerol, to free fatty acids, recent study has shown that MAGL plays a critical role in the regulation of fatty acid networks that promotes cancer pathogenesis in cancer cell lines from multiple tumors of origin (Cell 140, 49–61, 2010). The liver is the central organ of lipid metabolisms, but so far no studies assessed MAGL expression in malignant liver disease.

The aim of this study was to analyze MAGL expression in hepatocellular carcinoma (HCC).

Methods and results: We screened MAGL mRNA expression in primary human hepatocytes (PHHs) and four different HCC cell lines (HepG2, Hep3B, HuH-7, PLC) by quantitative PCR analysis. Of note, MAGL mRNA expression was significantly higher in PHH compared to all four HCC cell lines. This result was confirmed by Western Blot analysis. While PHH revealed clear MAGL protein expression, no band was visible in cellular extracts of malignant liver cells.

Conclusion: Our data indicate a marked downregulation of MAGL expression in hepatocellular carcinoma, and herewith, this tumor varies from multiple cancers of different origin. Further studies have to unravel whether this relatively low MAGL levels still promotes HCC progression or whether the functional role of MAGL in HCC generally differs from other types of cancers.

Use of resveratrol as an anti-liver cancer drug cause damage on primary human hepatocytes

Georg Damm[1], Liping Hao[2], Sabrina Ehnert[3], Antje Blankenstein[1], Andreas K. Nüssler[3], Daniel Knobeloch[1], Matthias Glanemann[1]
[1]Department of General-, Visceral- and Transplantation Surgery, Campus Virchow Clinic, Medical University Berlin, Germany
[2]Department of Nutrition and Food Hygiene, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[3]Department of Traumatology, Eberhard Karls University Tübingen, Germany

Introduction: Apart from cardiac diseases, cancer is a major cause of morbidity and mortality worldwide. The resection of the tumor is a common therapeutic treatment. Alternative therapy with chemotherapeutics often leads to various side effects. Therefore there is growing interest in improving anticancer drugs. Resveratrol (RES) has already proven an apoptotic effect on liver cell lines. Aim of the present study was the investigation of its effect on primary human hepatocytes (pHH).

Methods: PHH were isolated from human liver resectates using a two step collagenase perfusion technique. PHH and SkHep1 cells were treated with RES for 24 h in concentrations up to 300 µM. Toxic influence of RES and/or 5-FU was evaluated by LDH and XTT assay. Influence of RES on the cell cycle was investigated using FACS analysis. Additionally the phosphorylation levels of Akt, ERK1/2 and p53 were measured with Western blot analysis.

Results: Between 50–300 µM RES caused a strong LDH release from SkHep1 cells, while pHH showed an increasing loss in membrane integrity starting at 100 µM. In combination with the widely used chemotherapeutic agent 5-fluorouracil (5-FU), RES could not lower the IC_{50} value significantly. Furthermore, RES only induced the known cell cycle arrest in the S-phase of SkHep1 cells in low concentrations and up to 48h of treatment. In SkHep1 cells the phosphorylation of ERK1/2 was activated, while phosphorylation of Akt decreases in a concentration-dependent manner. In both SkHep1 cells and pHH no cell death was detected, regardless of p53 activation.

Discussion/Conclusion: The distinct effect of RES on tumor cell lines is not selective and concerns pHH as well. This complicates the use as a liver tumor therapeutic drug due to a small therapeutic non-toxic window. Pre-treatment and 5-FU incubation did not lead to a synergistic effect. Thus, it is unlikely that RES can be used as a possible anti-liver cancer drug.

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Xanthohumol preferentially targets less differentiated colon carcinoma cells *in vitro*

Christoph Dorn, Michael Saugspier, Claus Hellerbrand
Department of Internal Medicine I, University Hospital Regensburg, Germany

Introduction: Xanthohumol is the major prenylated chalcone found in the female inflorescences of the hop plant and is known to exhibit multiple antitumoral effects. However, most studies did not differentiate between effects on highly malignant and less malignant cells.

The aim of this study was to compare the effects of xanthohumol on HT-29M3 cells and on the more malignant parental colon carcinoma cell line HT-29.

Methods and results: Several studies have identified HT-29M3 cells as more differentiated than the parental HT-29 colon cancer cell line. We further characterized both cell lines by quantifying the migratory capacity as well as by analyzing *snail* and *e-cadherin* expression, both markers for epithelial-mesenchymal transition. HT-29M3 cells showed a lower migratory capacity, a significantly reduced *snail* expression and a significantly higher *e-cadherin* expression, indicating a reduced malignancy compared to HT-29 cells. Xanthohumol induced apoptosis in both colon carcinoma cell lines at a concentration of 75 µM. Interestingly, xanthohumol mediated apoptosis was stronger in HT-29 cells than in HT-29M3 cells. Furthermore, already at concentrations as low as 2.5 µM, xanthohumol significantly hampered spheroid formation in a 3-dimensional cell culture model. Again, this effect was more pronounced in HT-29 cells compared to the more differentiated HT-29M3 cells. In further experiments we could demonstrate that xanthohumol could reduce the migratory capacity of HT-29 cells at concentrations which did not affect HT-29M3 cells (2.5 µM and 5 µM).

Conclusion: Xanthohumol preferentially targets the more malignant and less differentiated cell line HT-29 compared to the HT-29M3 cell line. These data underscore the safety as well as the potential of xanthohumol as a functional nutrient for both the prevention and treatment of colon cancer.

Encapsulation of primary human hepatocytes into alginate beads preserves their functionality for long-term culture

S. Ehnert[1], J.J. Martinez Sanchez[1], G. Damm[2], A.K. Nüssler[1]
[1]BG Unfallklinik, Eberhard Karls Universität Tübingen, Tübingen, Germany
[2]Department of General-, Visceral- and Transplantation Surgery, Campus Virchow Clinic, Medical University Berlin, Berlin, Germany

Introduction: One of the biggest problems in pre-clinical drug-development is the lack of suitable human *in vitro* models. Primary human hepatocytes (hHeps) have a limited availability and rapidly lose their metabolic activity under conventional culture-conditions. Thus, substances e.g. acetaminophen and diclophenac only become metabolized in the organotypic culture of a bioreactor. However, as this type of culture required a huge amount of cells it is not suitable for pharmacologic screenings. Thus, aim of this study was to evaluate hHep-culture in Alginate beads as possible alternative.

Methods: Immediately after isolation hHeps were mixed with alginate and dripped into a gelling-solution. Application of an electric current defined the size of the Alginate beads to be 0.5 µm, which allows supply of nutrients to the cells. After 1, 7, 14, 21 and 28 days we measured viability (resazurin conversion), phase I and II enzyme activities, urea and glucose metabolism in the cells.

Results: Encapsulation did not affect viability of the cells. Resazurin conversion remained stable over the first 14 days of culture and decreased by 15% up to day 28 of culture. Urea and glucose metabolism was significantly improved by the encapsulation in alginate (3- to 4-fold) as compared to 2D-culture. While under conventional (2D) culture phase I and II enzyme activities rapidly decreased in the first 7 days of culture, they remained stable over the entire culture period in the alginate beads. This was confirmed by measuring the toxicity of acetaminophen and diclophenac, which showed strong toxicity in the alginate beads up to day 28 of culture.

Discussion/Conclusion: Our results show that encapsulation of hHeps in alginate beads preserves their viability and metabolic function over a time-period of at least 21 days. The small size of the beads enables down-scaling of the culture to micro-titer-plate format and can thus be used for screenings. Thus representing a time- and money-effective method for pharmacological testings.

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Undesirable side effects in stem cell transplantation in experimental chronic hepatic disease

Nagwa Elkhafif[1], Olfat Hammam[2], Hanan El Baz[3], Soheir Mansy[1], Hoda Yehia[1], Soheir Mahmoud[4]
Theodor Bilharz Research Institute, Deparments of [1]Electron Microscopy, [2]Pathology, [3]Immunology and [4]Parasitology, Warak El Hadar, Imbaba, Giza, Egypt

Introduction: In this study we investigated the potential of transplanted human umbilical cord blood derived hematopoietic stem cells and human Wharton's Jelly derived mesenchymal stem cells in enhancing hepatic repair in a chronic hepatic fibrosis experimental model of murine schistosomiasis.

Methods: The engraftment of the human cells in the mice livers was detected by immunohistochemical techniques using antihuman monoclonal antibodies.

Results: Our results showed an improved liver pathology in the experimental chronic liver fibrosis model after stem cell transplantation. However, the percentage of hepatocytic differentiation of the transplanted human cells within mice hepatic tissue was relatively low. Moreover, increased angiogenesis was seen upon transplantation of hematopoietic stem cells and increased fibrosis was observed upon transplantation of mesenchymal stem cells.

Discussion/Conclusion: Stem cell therapy in hepatic disease may contribute to hepatic regeneration through a minor direct contribution to the functional hepatocyte population as well as through an indirect contribution to the promotion of endogenous regenerative processes through paracrine factors. However the development of adverse cell types is not excluded.

Prediction of clinical outcome in chronic hepatitis due to HCV genotype 4 after combination therapy with pegylated interferon-α2a and ribavirin

Nagwa Elkhafif[4], Nihal M. El Assaly[1], Naema El Ashri[1], Omnia El Bendary[1], Shendy M. Shendy[2], M. Ali Saber[3], Ehab El Dabaa[3]
[1]Clinical Chemistry Department, [2]Gastroenterology and Hepatology Department, [3]Biochemistry and Electron Microscopy Department of Theodor Bilharz Research Institute (TBRI), Giza, Egypt

Introduction: Combined therapy using Interferon-α (IFN) and ribavirin (RIB) represents the standard treatment in patients with chronic hepatitis C. However, the percentage of responders to this regimen is still low, while its cost and side effects are elevated. Therefore, the possibility to predict patient's response to the above treatment is of paramount importance.

Aim of this work is to estimate the clinical and prognostic role of TNF-αR (P55), IL-1ra and TNF receptors related to inflammatory cytokines and, GH and Insulin hormones metabolized in the liver in HCV infection, cirrhotic and non-cirrhotic. Also to find their significance as non-invasive biochemical markers that may correlate with HCV infection on predicting the outcome of interferon-α2a therapy in patients with chronic HCV infection.

Methods: 54 patients infected by HCV genotype 4 were enrolled in this study. They were classified into two groups according to the liver histology**.** Group A of 42 chronic compensated HCV patients with no cirrhosis, Group B of 12 chronic HCV patients with established cirrhosis and 12 healthy controls. Patients were treated by pegylated IFN-α2a (180 µg for group A and 130 µg for group B) once weekly & 1200 mg ribavirin/day in two doses. Tested parameters have been done by ELISA method before and after treatment for group A, group B and control group.

Results: There was a significant increase of serum insulin ($p < 0.01$) of group A after treatment compared to group A before treatment, group B and control group. On the other hand, serum TNF-R (P55) showed significant decrease ($p < 0.05$) in group A after treatment compared to group A before treatment, group B and control group. TNF-αR (P55) showed positive correlation with sALT and sAST. Also, serum GH level decreased in group A after treatment compared to the other studied groups; but, this decrease was not statistically significant.

Discussion/Conclusion: Pegylated IFN-α2a and ribavirin are effective combination in treatment of chronic HCV genotype 4. Insulin and TNF-αR (P55) correlate with HCV infection and could be used as a marker of peg-INF-α2a and ribavirin response while IL-1ra and GH are of no value.

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Hierarchy and immunodominance profile of naturally occurring, tumor-specific $CD8^+$ T cells in patients with hepatocellular carcinoma

Tobias Flecken, Nathalie Schmidt, Sandra Hild, Hans Christian Spangenberg, Christoph Neumann-Haefelin, Oliver Drognitz*, David A. Price[#] and Robert Thimme
Department of Internal Medicine II and *Department of Surgery, University Hospital Freiburg, Germany
[#]Department of Infection, Immunity and Biochemistry, Cardiff University School of Medicine, Cardiff, United Kingdom

Introduction: Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and has one of the highest mortality rates. This is partly due to a limited number of available therapeutic approaches. Since several studies found that the prognosis of HCC patients depends on the antitumoral immune response, immunotherapy is a potential future way of treatment. However, to develop immuno therapies, a better understanding of the natural immune response against HCC is obligatory.

Methods: We used overlapping peptides covering the entire sequence of four prominent tumor-associated antigens (TAAs) of HCC, i.e. α-fetoprotein (AFP), Glypican-3, melanoma-associated gene-A1 (MAGE-A1) and NY-ESO-1. $CD8^+$ T cells were obtained from blood and tumor samples of a large cohort of HCC patients (n = 96) as well as from the blood of controls (n = 19). These $CD8^+$ T cells were expanded and stimulated with the respective peptides and analyzed for the production of interferon-γ by flow cytometry.

Results: We found that TAA-specific immune responses were readily detectable in HCC patients at a significantly higher frequency than in controls ($p < 0.0001$). We could identify a clear hierarchy between the individual antigens and several immunodominant regions within the respective TAAs. In addition, several responses were fine-mapped, leading to the identification of novel dominant tumor-specific epitopes. A subsequent tetramer-analysis revealed that the tumor-specific $CD8^+$ T cells could expand specifically *in vitro* but remained dysfunctional with respect to effector functions.

Discussion/Conclusion: In sum, our results indicate that HCC induces a broad immune response against several antigens and epitopes. However, tumor-specific $CD8^+$ T cells in HCC patients are clearly functionally impaired. These results indicate that insufficient immune-mediated control of HCC is not due to a lack of priming of $CD8^+$ T cell responses but rather associated with impaired effector functions of these cells. These results have important implications for the further development of immune-based therapies for HCC.

CD40ligand-co-stimulation improves immunotherapy with α-fetoprotein pulsed dendritic cells towards established orthotopic hepatocellular carcinoma *in vivo*

Maria A. Gonzalez-Carmona[1], Annabelle Vogt[1], Tiyasha H. Ayub[1], Georges Decker[1], Yildiz Yildiz[1], Tilman Sauerbruch[1] and Wolfgang H. Caselmann[1,2]
[1]Department of Internal Medicine I, University of Bonn, Bonn, Germany
[2]Bavarian State Ministry of the Environment, Public Health and Consumer Protection, Munich, Germany

Introduction: Dendritic cells (DC) as professional antigen presenting cells can be used to prime T-cells against α-fetoprotein (AFP) for immunotherapy of hepatocellular carcinoma (HCC). However, lack of co-stimulation and a immunosuppressive tumor-environment seem to limit their efficacy in patients. In this study, the impact of co-stimulation with CD40ligand (CD40L) was analyzed on improving immunotherapies with murine (m)AFP-pulsed DC towards HCC.

Methods: Murine DC cultured with GM-CSF and IL-4 were transduced with Ad-mAFP, Ad-CD40L or Ad-LacZ. Hepa129-mAFP-cells were injected into the right flank or the liver of C3H-mice to induce subcutaneous (s.c.) and orthotopic AFP-expressing HCC. For treatments, 10^6 mAFP-pulsed DC were inoculated s.c. followed by 10^6 CD40L-expressing DC injected intratumorally (i.t.).

Results: The i.t.-injection of CD40L-DC elicited complete tumor remissions in 37.5% of tumor-bearing animals. Additional s.c.-inoculation of mAFP-DC enhanced further the tumor remission rate to 62.5%, suggesting synergistic effects of both approaches. The combination was also effective towards orthotopic HCC, inducing significantly prolonged survival of tumor-bearing mice. Analysis of tumors after i.t.-injection with CD40L-DC revealed enhanced expression of Th1-cytokines as well as activated $CD4^+$-, $CD8^+$-T-cells and DC but also higher caspase-3-activity, suggesting induction of tumor-apoptosis.

Discussion/Conclusion: I.t.-injection of CD40L-DC achieved a strong apoptotic and inflammatory tumor-environment, causing effective regression of s.c. and orthotopic HCC. Additional s.c.-inoculation with mAFP-DC synergizes with CD40L-DC, increasing the antitumoral effects, probably through activation of tumor-specific effector cells which can be attracted into the tumor. Thus, CD40L-co-stimulation may be a promising tool for improving DC-based immunotherapies of HCC.

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Common variants in the NOD2 gene are associated with elevated IL-8 levels and ascitic decompensation in patients with advanced liver cirrhosis

F. Grünhage[1], B. Appenrodt[2], T. Sauerbruch[2], F. Lammert[1], C. Reichel[3]
[1]Department of Medicine II, Saarland University Hospital, Homburg, Germany, [2]Department of Interal Medicine I, University Hospital Bonn, Bonn, Germany, [3]Rehabilitation Center Bad Brückenau, Clinic Hartwald, German Pension Insurance, Federal Office, Bad Brückenau, Germany

Introduction: Recently we reported an association of common variants (p.R702W, p.G908R and c.3020insC) in the nucleotide-binding oligomerisation domain containing 2 (*NOD2*) gene with the occurrence of spontaneous bacterial peritonitis and survival in cirrhosis (Grünhage et al. Hepatology. 2010; epub). Our aim now was to confirm these findings in an independent sample of patients with advanced cirrhosis, who we studied previously for the association of soluble tumor necrosis factor receptors (sTNF-R) 55 and 75 serum levels and survival (Grünhage et al. Clin Gastroenterol Hepatol. 2008; 6: 1255–1262).

Methods: A total of 92 patients (66% male) with liver cirrhosis were included in the study. Clinical (age, gender, BMI) and survival data were recorded prospectively, and data on ascitic decompensations (CPS 3) were obtained retrospectively by analysis of patient files. *NOD2* gene variants were genotyped by employing 5'-exonuclease assays with fluorescent dye-labelled probes. Biochemical parameters including bilirubin, creatinine and INR were determined using standard methods. In addition, we determined plasma levels of cytokines (IL-6, IL-8, IL-12) which have been functionally linked to *NOD2* variants, by ELISA.

Results: The majority of patients presented with advanced cirrhosis (80.5% Child B/C) and a median MELD score of 14 (range 6–35). The mean overall survival time was 243 ± 203 days. During follow-up, 40 (44%) patients died, and 5 (4%) received a liver transplant. Carriers of the p.R702W risk allele presented more often with ascitic decompensation as compared to patients who carried the wild-type (1.0 vs. 1.5 episodes; $p < 0.05$). However, we did not detect a difference in survival between carriers of *NOD2* variants and other patients. Of note, carriers of the p.R702W mutation showed lower basal IL-8 levels in comparison to patients with wild-type *NOD2* (123.0 ± 121.7 vs. 69.4 ± 76.4 ng/ml; $p = 0.03$).

Discussion/Conclusion: Cirrhotic patients with *NOD2* mutations display increased levels of IL-8 and develop ascitic decompensations more frequently. In contrast to our previous prospective study, survival was not compromised by *NOD2* variants in the present cohort, which might be related to lower sensitivity or patient selection.

Identification of systemic and organ-specific genetic determinants of fibrogenesis in liver and heart

Rabea Hall[1], Andrey Kazakov[2], Ulrich Laufs[2], Michael Böhm[2], and Frank Lammert[1]
[1]Department of Medicine II, Saarland University Medical Center, Homburg, Germany
[2]Department of Medicine III, Saarland University Medical Center, Homburg, Germany

Introduction: Various chronic human diseases result in organ fibrosis, i.e. excessive accumulation of extracellular matrix. Fibrosis is a multifactorial trait determined by both genetic and exogenous factors such as xenobiotics. Our **aim** is to compare fibrogenesis in liver and heart in a systemic fibrosis model to dissect common and organ-specific mechanisms of fibrosis. Furthermore, we employed a systems genetics approach to identify genetic modifiers of hepatic and cardiac fibrosis.

Methods: We tested the parental strains (C57**B**L/6J [**B**] and **D**BA/2J [**D**], 6/group) for the following fibrosis models: (i) N^G-nitro-L-arginine-methylester (L-NAME), a NO synthase inhibitor, provided in drinking water (5 weeks, ad libitum); (ii) CCl_4, administered i.p. (0.7 mg/kg, 12 injections for 6 weeks). Collagen accumulation was quantified in histological liver and heart sections after Sirius red staining. The proliferation marker Ki-67 and fibroblast marker fibronectin were determined by immunohistochemistry. Thirty BxD recombinant inbred lines, genotyped for > 13,000 markers, served as genetic reference population for the identification of genetic risk factors.

Results: Whereas L-NAME treatment did not induce hepatic fibrosis, CCl_4 induced fibrosis in liver and heart, a hitherto unknown phenomenon. We observed marked differences for both hepatic and cardiac fibrosis between C57BL/6J and DBA/2J strains. Cardiac fibroblast proliferation and collagen accumulation was enhanced in DBA/2J mice (collagen area [%] in heart: B = 0.6%, D = 1.2%, $p = 0.006$; in liver B = 2.5%, D = 7.2%, $p = 0.0002$). CCl_4-challenged BxD lines also differed in fibrosis susceptibility. Collagen levels in livers and hearts of BxDs were correlated significantly ($R = 0.39$, $p < 0.002$), suggesting common genetic determinants of fibrogenesis. However, significant quantitative trait loci controlling fibrosis could not yet be identified.

Discussion/Conclusion: CCl_4 induces systemic fibrosis in liver and heart. In the genetically heterogeneous genetic reference population, hepatic and cardiac fibrosis susceptibility were correlated significantly. CCl_4-induced fibrosis in BxD lines provides a basic experimental framework to dissect common and organ specific modifiers of fibrogenesis.

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PGE_2 promotes the *circulus vitiosus* of prostaglandin- and cytokine-dependent hepatic insulin resistance

Janin Henkel, Nancy Schanze, Lindsey Brevick, Daniela Gärtner, Gerhard P. Püschel
University of Potsdam, Department of Nutritional Biochemistry, Arthur-Scheunert-Allee 114–116, 14558 Nuthetal, Germany

Introduction: Hepatic insulin resistance is a major contributor to hyperglycemia in metabolic syndrome and type II diabetes, which both are often accompanied by NAFLD. There is a close correlation between hepatic steatosis and insulin resistance. Obesity is associated with a low-grade chronic systemic and intrahepatic inflammation that contributes in part to hepatic insulin resistance. In the course of this inflammation cytokines and prostaglandins are produced in abdominal adipose tissue and by non-parenchymal liver cells, primarily resident macrophages, which modulate functions of parenchymal cells in a paracrine mode but may also alter their own function in an autocrine mode. We have recently shown that PGE_2 can interrupt insulin receptor signalling by a distinct but synergistic mechanism with IL-6. Aim of this study was to investigate, whether PGE_2 in addition can affect hepatic insulin resistance indirectly by increasing the release of cytokines from macrophages.

Methods: Primary hepatocytes (rat) or HepG2-cells (human) were incubated with conditioned media of PGE_2-stimulated Kupffer cells (rat), peritoneal macrophages (mouse) and macrophage cell lines (murine, human) and subsequently stimulated with insulin. Cytokine expression and activation of insulin- and cytokine signalling were quantified by westernblot and RT-qPCR.

Results: PGE_2 induced expression of IL-6 and oncostatin M (OSM) in murine and human macrophages while TNFα-expression was unchanged or reduced. IL-6, OSM, TNFα and PGE_2 increased expression of key-enzymes of PGE_2 synthesis and synergistically with PGE_2 decreased insulin-dependent induction of glucokinase in hepatocytes. Incubation of hepatocytes or HepG2-cells with conditioned media of PGE_2-treated macrophages increased STAT3-phosphorylation, SOCS3-expression and ERK1/2-activation while insulin-stimulated expression of glucokinase was diminished. This effect was abolished after simultaneous incubation with an OSM-neutralising antibody.

Discussion/Conclusion: In addition to reducing hepatic insulin sensitivity by directly interrupting insulin signalling pathway in hepatocytes, PGE_2 induced OSM-production in macrophages by an autocrine feed-forward loop as an additional, so far unrecognized, mechanism contributing to hepatic insulin resistance.

Complement 5a receptor (C5R1) deficiency ameliorates liver fibrosis in mice

Katrin Hochrath[1], Susanne N. Weber[1,] Reinhild Goebel[1], Ursula Pieper-Fürst[2], Sebastian Huss[3], Hans-Peter Fischer[3] and Frank Lammert[1]
[1]Department of Medicine II, Saarland University Medical Center, Homburg, Germany
[2]Department of Medicine I, University Hospital Bonn, Bonn, Germany
[3]Institute of Pathology, University Hospital Bonn, Bonn, Germany

Introduction: Previously we identified a trait locus that confers susceptibility to hepatic fibrosis in inbred mice and showed that the gene encoding complement factor C5 underlies this locus (*Nat Genet* 2005). C5a, the proinflammatory fragment of C5, binds to the G protein-coupled C5aR1 and suppresses the expression of members of the IL-12 cytokine family, which may modulate fibrogenesis via alteration of the Th1/Th2-balance (*Immunity* 2005). Our **aim** now was to dissect whether the profibrogenic effects of C5 are mediated via C5aR1.

Methods: We characterised hepatic fibrogenesis in *C5aR1* knockout (*C5aR1*$^{-/-}$) and C57BL/6J mice (n = 20) after fibrosis induction (CCl_4 1.4 mg/kg/week i.p., 6 weeks). Liver enzyme activities were measured in plasma. Hepatic mRNA of cytokines were determined by quantitative PCR. Histological stages of fibrosis (F-score) and relative collagen areas were assessed.

Results: The liver fibrosis progresses to the maximum score F2 in *C5aR1*$^{-/-}$ and controls. *C5aR1*$^{-/-}$ mice show higher ALT (248 ± 40 vs. 100 ± 5 U/l; p = 0.004) but decreased AP activities (249 ± 40 vs. 80 ± 7 U/l; p = 0.002) compared to wild-type animals. Fibrosis stages are lower in *C5aR1*$^{-/-}$ mice (1.27 ± 0.27) as compared to controls (1.89 ± 0.11; p = 0.07), which are reflected by lower collagen areas in male mice (1.0 ± 0.4 vs. 2.2 ± 0.2%; p = 0.02). Whereas 89% of wild-type mice develop fibrosis-score F2, only 55% of *C5aR1*$^{-/-}$ mice display this phenotype. Male *C5aR1*$^{-/-}$ mice display decreased expression of IL-23 (p < 0.001), and IL-12 and IL-27 levels tend to be lower, in comparison to controls.

Conclusion: *C5aR1*$^{-/-}$ mice, in particular males, are less susceptible to hepatic fibrosis as compared to wild-type mice, which is associated with reduced expression of cytokines. The observation is in line with our previous results demonstrating antifibrotic effects of peptidic inhibitors of C5aR1 in *C5*$^{-/-}$ mice, indicating that the profibrogenic effects of C5 are mediated via C5a/C5aR1. C5-dependent mechanisms potentially modulating hepatic fibrosis such as the second C5 receptor, C5L2, are being investigated.

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Inflammatory gene products of colon cancer cells are blocked by dietary tea flavonoids

Harald P. Hoensch, Dolores Müller*, Elke Richling*
Marienhospital Darmstadt, Germany
*Technische Universität Kaiserslautern, Lebensmittelchemie und Toxikologie, Erwin-Schrödinger-Str. 52, 67663 Kaiserslautern, Germany

Introduction: Tea flavonoids derived from camomile and green tea such as apigenin and epigallocatechin gallate (EGCG) can inhibit intestinal neoplasia. Recurrences of adenomas and cancers were reduced in patients with resected colorectal cancer by treatment with tea bioflavonoids after tumor operation (1).
To clarify the biomolecular pathway for suppression of neoplasia we investigated the anti-inflammatory effect of a nutritional supplement Flavo Natin® (FN) which had been used in the clinical study on tertiary tumor prevention and of EGCG in a colon tumor cell line. The aim of our study was to investigate if tea flavonoids are capable to suppress the inflammatory markers produced by tumor cells after cytokine stimulation.

Method: We studied the cytotoxicity of FN in the colon cancer cell line T-84 by resazurin fluorescence and compared it with the placebo supplement. Additionally, the T-84 cells were incubated with FN, EGCG or placebo and stimulated with TNF-alpha, IF-gamma and IL-1-beta. After the cytokine stimulation the mRNA expression of IP-10, IL-8 and TNF-alpha was measured by quantitative Real-Time PCR (qRT-PCR).

Results: Stimulation of T-84 cells increased the expression of IP-10 (gamma-interferon inducible protein 10), TNF-alpha and IL-8. By preincubation with FN at 10 µM the mRNA expression of IP-10 was strongly reduced (log2-ratio -14). The TNF-alpha mRNA was also but less decreased by FN. EGCG displayed an inhibition pattern similar to FN. Placebo did not influence the mRNA expression of the chemokines and TNF-alpha.

Discussion and conclusion: Clinically useful dietary tea bioflavonoids inhibit the expression of inflammatory genes in a colon cancer cell line. Blockage of inflammatory gene products could be achieved in vivo by botanicals without clinically relevant side effects.

Reference:

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Prenatally induced liver damage affects the maternal health and fetal development

Timo Keßler[1*], Kristin Thiele[2*], M. Emilia Solano[2], Petra Arck[2], Gisa Tiegs[1], Annette Erhardt[1]
[1]Institute of Experimental Immunology and Hepatology, [2]Department of Obstetrics and Fetal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[*]Both authors contributed equally to this work.

Introduction: During fetal development, the liver transiently functions as the main hematopoietic organ before the bone marrow takes over. In adults, oval cells (OC) represent a population of hepatic progenitor cells. In response to liver injury, OC are expanded and maturate into hepatocytes. Interestingly, a correlation between acetaminophen (APAP) usage during pregnancy and an increased risk of asthma development in the offspring has been demonstrated in human studies. Here we studied consequences of prenatal liver injury regarding maternal health and fetal development.

Methods: APAP was injected intraperitoneally into pregnant and non-pregnant mice on gestation day 12.5. Liver damage was quantified after 24 h by measurement of plasma transaminase activity. Fetal development was analysed by determination of body weight and total cell numbers of liver and thymus on gestation day 16.5.

Results: Upon APAP challenge, pregnant mice developed increased liver damage compared to non-pregnant mice indicated by significantly elevated transaminase activities. Moreover, fetal development was strongly delayed in prenatally APAP-challenged mice demonstrated by significantly reduced body weight of the fetuses. Furthermore, the number of total cells in the liver and thymus were dramatically reduced in the fetuses from APAP-treated mice suggesting an influence on liver progenitor cell reservoir and primary lymphoid organs.

Discussion/Conclusion: In summary, pregnant mice are more susceptible against APAP-induced liver damage. Moreover, prenatal liver injury also affects fetal development. Further studies are intended to identify whether maternal drug abuse resulting in liver damage and deregulation of maternal and fetal liver progenitor cell reservoir might increase the children's risk for allergic or autoimmune diseases.

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Gene expression of chemokines in KRAS and BRAF mutated colorectal cell lines: Role of cytokines

S. Khan, I. Malik, M. Blaschke, F. Moriconi, G. Ramadori
Universitätsmedizin, Göttingen, Germany

Background: KRAS and BRAF mutations represent an important step in development of carcinoma-adenoma-sequence in colon cancer. Survival and proliferation of tumors is influenced by immune cells of tumor environment and vice versa. Here we demonstrate the effect of pro-inflammatory cytokines (TNFα, IL-1β & IFNγ) on gene expression of their receptors, chemokines and chemokine-receptors in wildtype and mutated cell lines.

Methods: Cell lines DLD-1(KRAS), HT-29, Colo205(BRAF) and Caco-2, Colo-320, CX-1 (wildtype) were treated with cytokines, lysed at different time points (1–24 h) for mRNA and protein analysis.

Results: Expression of regulatory angiogenic CXC chemokines (CXCL1, CXCL2 & CXCL8) was significantly upregulated in DLD-1(KRAS), HT-29 & Colo205(BRAF) at base level. Furthermore DLD-1(KRAS) exhibited down regulation for CXCL10. Expression of pro-inflammatory cytokines (TNFα, IL-1β) and their receptors showed similar pattern in all cell lines.
TNFα strongly induced **CXCL1** mRNA expression in DLD1(KRAS) 310 ± 2.18-fold, HT-29(BRAF) 36.15 ± 3.28-fold and Colo-205(BRAF) 31.45 ± 2.64-fold (1 h). Caco-2(Wt) was down regulated (1 h). IL-1β induced gene expression of **CXCL1** in HT-29(BRAF) 46.42 ± 5.98-fold, DLD-1(KRAS) 21.19 ± 0.37-fold, Colo-205(BRAF) 18.07 ± 0.57-fold and significantly downregulated CXCL1 expression in Caco2(Wt) and Colo-320(Wt).
However, **CXCL8** gene expression was strongly induced by IL-1β in Caco2(Wt) 806.41 ± 19.76-fold, while in DLD1(KRAS) 353.22 ± 40.63-fold, Colo-205(BRAF) 99.58 ± 10.42-fold and HT-29(BRAF) 41.51 ± 0.72-fold expression was observed.
IFNγ significantly enhanced **CXCL10** mRNA expression in HT-29(BRAF) 15361.19 ± 2974.33-fold followed by DLD1(KRAS) 597.71 ± 64.62-fold in contrast to cell lines Caco2(Wt) 45.75 ± 1.44-fold and Colo-320(Wt) 48.90 ± 2.39-fold.
An effect through IFNγ stimulation in **TNFα Rec1** mRNA expression was observed in DLD1(KRAS) 3.9 ± 1.2-fold HT-29(BRAF) 3.5 ± 0.5-fold and Caco2(Wt) 8.29 ± 1.04-fold. TNFα also induced **IFNγ Rec1** in DLD1(KRAS) 6.09 ± 1.4-fold, HT-29(BRAF) 3.0 ± 0.4-fold and Caco-2(Wt) 5.50 ± 0.7-fold to the same extent. CX-1 showed different pattern.

Conclusion: At basal level chemokine gene expression is upregulated in mutated-compared to wildtype cell lines. CXCL1, CXCL8 and CXCL10 are induced differentially in KRAS or BRAF in comparison to wild type after cytokine stimulation. It proofed that micro environment is different in tumors consistent of wildtype or mutated cells.

Key words: KRAS, BRAF, CXCL1, CXCL10, CXCL8, TNFα, IL-1β, IFNγ.

Hepatocellular steatosis promotes tumorigenicity of cancer cells in vitro

Andreas Koch, Birgitta Ott-Rötzer, Anja Bosserhoff*, Claus Hellerbrand
Department of Internal Medicine I, University Hospital Regensburg, Germany
*Institute of Pathology, University Regensburg, Germany

Introduction: Although obesity is known as a risk factor for several human cancers, the association of obesity with cancer progression and metastasis remains to be characterized. Obesity frequently leads to (non-alcoholic) hepatic steatosis, and in general, the liver is the main metastatic niche of most tumors.

The aim of this study was to assess the effect of hepatic steatosis on cancer cells derived from malignant melanoma, a tumor which frequently metastasizes into the liver.

Methods and results: Incubation with free fatty acids induced a dose-dependant cellular lipid accumulation in HepG2 hepatoma cells and primary human hepatocytes (PHH) in vitro. Subsequently, conditioned medium (CM) from cells with and without steatosis was collected and used for stimulation of the human melanoma cell line Mel-Im. Functional analysis revealed that CM from steatotic hepatocytes significantly enhances proliferation and migration of Mel-Im cells compared to CM from control cells. Incubation with free fatty acids alone did not significantly affect proliferation and migration of Mel-Im cells in vitro, which indicates that soluble factors secreted by hepatocytes in response to cellular lipid accumulation are responsible for the functional effects on the tumorigenicity of melanoma cells.

Conclusion: These data indicate hepatic steatosis as a risk factor of metastasis to the liver. The novel in vitro model described here may be used to identify the secreted factors and underlying molecular mechanisms by which steatotic hepatocytes affect tumorigenicity, which may lead to innovative therapeutic strategies against hepatic metastasis.

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HBV infection promotes tumorigenicity of HCC cells in vitro

Leandra Koletzko, Claus Hellerbrand
Department of Internal Medicine I, University Hospital, Regensburg, Germany

Introduction: Chronic hepatitis B virus (HBV) infection is one of the most frequent liver diseases worldwide and harbors a high risk to progress to cirrhosis and hepatocellular carcinoma (HCC). HBV replication is the key motor of disease progression, while HBV elimination or suppression reduce the risk of disease-progression including HCC development. Further, some studies suggest that HBV promotes tumor progression and metastasis via indirect mechanisms as inhibitory effects on the immune system or remodeling of the extracellular matrix.

The aim of this study was to analyze whether HBV directly affects tumorigenicity of HCC cells in vitro.

Results: HepG2.2.15 cells revealed significantly higher proliferation rate than HepG2 cells as assessed by XTT-assays and the xCELLigence System (Roche Applied Science). Further, the xCELLigence System was used to assess cell-attachment, and we found that HepG2.2.15 cells attached significantly faster than parental HepG2 cells. In contrast, HepG2.2.15 and HepG2 cells showed similar migratory activity in Boyden chamber assays.

Discussion/Conclusion: Our in vitro data indicate that HBV directly affects tumorigenicity of HCC cells with various effects on individual pathophysiological mechanisms. If confirmed in vivo, these data may provide a rationale for antiviral therapy in patients with HCC to prevent HCC progression and metastasis.

Genetic risk analysis of *FGFR4* and *Klothobeta* variation in a large European cholangiocarcinoma cohort

Marcin Krawczyk[1], Aksana Höblinger[2], Florentina Mihalache[3], Monica Rusticeanu[1], Monica Acalovschi[3], Tilman Sauerbruch[2], Frank Lammert[1], Vincent Zimmer[1]
[1]Department of Medicine II, Saarland University Hospital, Homburg, Germany
[2]Department of Medicine I, University Hospital Bonn, Bonn, Germany
[3]Department of Medicine III, University Iuliu Hatieganu, Cluj-Napoca, Romania

Introduction: Previously, we have demonstrated that heterozygosity for the *alpha1-antitrypsin* Z allele modulates risk of cholangiocarcinoma (CCA)[1]. Recently, variants of the receptor of the bile acids (BA) feedback regulator fibroblast growth factor (FGF) 19 have been reported to determine BA synthesis and homeostasis[2]. As distorted BA metabolism may predispose to biliary malignancies, we now investigate these variants in a large European cohort of CCA patients.

Methods: We genotyped 206 individuals with CCA (males n = 101, age 28–70 years) originating from Germany (n = 170) and Romania (n = 36). The control group consisted of 359 CCA-free individuals (males n = 160, age 22–90 years). Three single nucleotide polymorphisms (SNPs) in the FGF receptor 4 (*FGFR4*; *rs351855* and *rs376618*) and the co-receptor *Klotho-beta* (*KLB*; *rs17618244*) were genotyped by PCR-based assays with 5'-nuclease and fluorescence detection (*TaqMan*).

Results: All genotype frequencies were in HWE ($p > 0.05$). The association tests did not provide evidence for genetic risk modulation by the *FGFR4* (rs351855, common OR = 0.972; rs376618, common OR = 1.046) or *KLB* (*rs17618244,* common OR = 1.233) variants (all $p > 0.05$). Similarly, separate analyses for individuals with intra- or extrahepatic tumors did not yield significant associations either.

Discussion/Conclusion: Although genetic variations in the *FGFR4-KLB* pathway may influence hepatic BA metabolism, our association study does not support a relevant role of *KLB* and *FGFR4* variants in genetic CCA risk.

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Impact of Genistein on hepatic lipid metabolism in an *in vitro* hepatic steatosis model

A. Krüger, S. Lünse, M. Glanemann, G. Damm
Department of General, Visceral, Vascular and Thoracic Surgery, Berlin, Germany

Introduction: Non-alcoholic fatty liver disease (NAFLD) is a pervasive disease often leading to steatosis, inflammation, fibrosis and cirrhosis. Epidemiologic studies suggest that Genistein (GEN) improves hepatic lipid levels. Our aim was to establish an *in vitro* steatosis model in primary human hepatocytes (PHH) in order to assess the effects of GEN on hepatic lipid metabolism.

Methods: PHH were isolated from liver resectates using a two step collagenase perfusion technique. They were treated with oleic acid and palmitate (1 mM, 2:1) for 24 h. Then, GEN was applied up to 100 µM for 24 h. Lipid content was measured by Oil Red O and SRB staining. Toxicity of FFA and GEN was evaluated by AST, LDH and XTT assay as well as Urea formation. Cytosolic and nuclear protein of the transcription factors PPARalpha and SREBP1c were measured using Western blot analysis.

Results: The steatosis showed a significant increase in number and size of lipid vesicles. XTT, AST and LDH revealed a slightly toxic effect of FFA in steatotic PHH compared to control, whereas Urea level was unchanged. GEN did not significantly decrease lipid content in this setting. Western blot analysis displayed that GEN elevated nuclear PPARalpha and decreased cleaved SREBP1c, both in a concentration dependent manner.

Discussion/Conclusion: The *in vitro* steatosis model showed an increased lipid content and a slight lipotoxicity, similar to *in vivo* conditions. GEN did not significantly lower hepatic lipid level. However, GEN altered hepatic lipid pathways by increasing PPARalpha and decreasing active SREBP1c, implying an increased catabolic and impaired anabolic lipid metabolism. Further investigation is needed to elucidate the entire effects of GEN and its potential to lower lipid content in PHH.

Bone morphogenetic protein 9 (BMP-9) induces epithelial-mesenchymal transition in hepatocellular carcinoma cells

Qi Li, Honglei Weng, Yan Liu, Xin Gu[1], Chunfang Gao[1], Johanna Dzieran, Iryna Ilkavets, Shahrouz Ghafoory[2], Stefan Wölfl[2], Steven Dooley, Katja Breitkopf-Heinlein
Molecular Hepatology – Alcohol Associated Diseases, II. Medical Clinic, Medical Faculty Mannheim at Heidelberg University, Mannheim, Germany
[1]Department of Laboratory Medicine, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China
[2]IPMB (Institute of Pharmacy and Molecular Biotechnology), Heidelberg University, Heidelberg, Germany

Introduction: It has been reported that BMP-9, a member of the Transforming growth factor-β (TGF-β) superfamily participates in the progression of ovarian, breast and prostate cancer, although its functional role in the different cancer types is variant. In liver, one report demonstrates that BMP-9 can indeed induce cell proliferation in HepG2 cells, a human hepatoma cell line. Abnormal cancer cell proliferation is an important characteristic of malignant diseases. This prompted us to further delineate the role of BMP-9 in hepatocellular carcinoma (HCC).

Methods: Tissue sections from 22 HCC patients were enrolled for BMP-9 expression with immunohistochemical staining. Two of these samples were further analyzed for BMP-9 mRNA expression by in situ hybridization. Matrigel assays were performed to detect the effects of BMP-9 on cell migration in 2 HCC cell lines HLE and HepG2. Protein and RNA expression was investigated by Western blot and conventional PCR. Immunofluorescence staining was used to display localization of pSmad1, expression of E-cadherin, ZO-1 and vimentin.

Results: Moderate to strong positive BMP-9 immunostaining was observed in (12/22) HCC tissues. At the RNA level we investigated 2 of these patients with strong positive BMP-9 staining in cancer cells and found correlating strong positive staining for BMP-9 with in situ hybridization. HLE and HepG2 cell lines responded to rhBMP-9 with Smad1 phosphorylation and nuclear translocation. In the presence of BMP-9, cell migration was increased by 1.8 and 3.0 fold in HLE and HepG2, respectively. Incubation with BMP-9 for 72 h down-regulated E-cadherin, reduced ZO-1 and up-regulated snail and vimentin.

Discussion/Conclusion: In summary our data point to a role of BMP-9 as a potential promoter of HCC invasiveness by inducing typical features of epithelial-mesenchymal transition, like cell migration, down-regulation of cell-cell-contact proteins such as E-cadherin and ZO-1, up-regulation of mesenchymal proteins like vimentin and up-regulation of snail, a transcriptional factor suppressing expression of E-cadherin.

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The effect of statins on the production of cytokines in the cholestatic liver induced by bile duct ligation in rats

H. Lotková, O. Kučera, T. Roušar, Z. Červinková
Department of Physiology, Faculty of Medicine, Charles University in Prague, Hradec Králové, Czech Republic

Introduction: Extrahepatic biliary obstruction is associated with oxidative stress, pro-inflammatory response and fibrosis. Antiinflammatory effects of statins mediated by the reduction of cytokine IL-6 have been reported in hepatocyte culture. The aim of this study was to verify the effect of fluvastatin on the inflammatory response of cholestatic liver.

Methods: Cholestasis was induced in Wistar rats by bile duct ligation (BDL). Fluvastatin (5 mg/kg; Lescol, Novartis) was administerd after surgery and then daily for 7 days by intragastric tube. Pro-inflammatory IL-6, TNF-alpha, pro-fibrogenic TGF-beta were measured by ELISA kits (Bender MedSystems, Austria) in the serum and the liver, respectively. Serum levels of ALT, AST, GGT, ALP, bilirubin, albumin were determined. Glutathione content in the liver was measured fluorometrically. Histological analysis was done.

Results: Administration of fluvastatin attenuated the production of IL-6 induced by BDL ($p < 0.05$) but markers of liver injury (ALT, AST, GGT, ALP; $p < 0.001$) were deteriorated. Increase in the production of TGF-beta was observed in BDL rats after fluvastatin. Glutathione content in the liver rised transiently after BDL, fluvastatin administration in BDL rats led to the depletion of glutathione. Histology confirmed sporadic progression of fibrosis after fluvastatin in BDL rats.

Discussion/Conclusion: Although the production of pro-inflammatory IL-6 was supressed by fluvastatin in the cholestatic liver, other markers of liver liver injury were deteriorated and fibrogenic response was more expressed. Decrease in GSH content can contribute to this aggravating effect.

Impact of Genistein on energy metabolism in a steatotic liver model

S. Lünse, A. Krüger, M. Glanemann, G. Damm
Department of General, Visceral and Transplantation Surgery, Campus Virchow Klinikum, Charité – University Medicine Berlin, Berlin, Germany

Introduction: Non-alcoholic fatty liver disease (NAFLD) is already the most common chronic liver disease in western countries. The complex metabolic disorder is strongly associated with insulin resistance. Epidemiological studies suggest beneficial effects of the natural soy isoflavone Genistein (GEN) on hepatic energy metabolism. Our aim was to establish a steatotic liver *in vitro* model to investigate the impact of GEN on energy metabolism focused on insulin signaling.

Methods: Primary human hepatocytes (PHH) were isolated from liver tissues via a two-step collagenase perfusion. Steatosis was induced by adding oleic and palmitic acid to the culture medium. The lipid content was determined photometrically after staining with Oil-Red-O and SRB. Assays of XTT, AST and LDH were used to evaluate cytotoxicity. Phosphorylation of ERK, AKT, FOXO1 and GSK3 was measured by Western blot analysis in PHH with and without insulin as well as after GEN incubation for 24 h.

Results: PHH treated with free fatty acids showed a significant increase of fat accumulation and a moderate lipotoxicity. Furthermore, in steatotic PHH we observed decreased insulin-induced phosphorylation levels of ERK, AKT, FOXO1 and GSK3. Additional treatment with GEN showed in control PHH increased insulin-induced phoshorylation levels of ERK, AKT and GSK3 whereas steatotic PHH revealed decreased levels of ERK and AKT in a dose dependent manner.

Discussion/Conclusion: Our data show that the steatotic liver *in vitro* model is a reliable reproduction of *in vivo* conditions regarding fat accumulation, insulin resistance and lipotoxicity. Insulin resistance was observed by an impaired phosphorylation of ERK, AKT, FOXO1 and GSK3. GEN leads only in control PHH to an improvement of insulin signaling by increasing phosphorylation levels. However, in steatotic PHH a beneficial effect was not observed. Therefore we recommend further studies to investigate signaling pathways related to energy metabolism in steatotic liver.

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Hepatocellular lipid accumulation enhances the susceptibility for oxaliplatin-induced hepatic injury

Abdo Mahli, Christoph Dorn and Claus Hellerbrand
Department of Internal Medicine I, University Hospital Regensburg, Germany

Introduction: Chemotherapy can affect non-tumor healthy hepatic tissues and cause histopathological and functional changes summarized as chemotherapy-associated steatohepatitis (CASH). Malignant tumors and the metabolic syndrome are frequent diseases worldwide. The majority of patients with obesity or type 2 diabetes develop hepatic steatosis, and in a subset of patients progressive inflammation leads to non-alcoholic steatohepatitis (NASH). Thus, in a significant number of cases chemo-therapy is given to patients with fatty livers.

The aim of this study was to evaluate whether steatosis makes the liver more vulnerable for CASH.

Methods and results: We applied an *in vitro* model of hepatic steatosis, which we have recently developed. Incubation of HepG2 hepatoma cells or primary human hepatocytes with free fatty acids led to a dose-dependant cellular lipid accumulation. Microscopical analysis, analysis of mitochondrial activity and LDH-leakage into the supernatant revealed that oxaliplatin caused cytotoxicity in a dose-dependent manner. Importantly, in cells with hepatocellular lipid accumulation the cytotoxic effects occurred at significantly lower oxaliplatin concentrations.

Conclusion: Our data indicate that hepatocellular lipid accumulation increases the susceptibility for oxaliplatin-induced hepatic injury. The presented novel *in vitro* model may be used to unravel the underlying mechanisms of this phenomenon as basis for therapeutic strategies to prevent CASH particularly in patients, who suffer from metabolic syndrome.

Melanocortin receptors in rat liver cells: Change of gene expression and intracellular localization during acute-phase response

Ihtzaz Ahmed Malik, Jakob Triebel, Jessica Posselt, Pierluigi Ramadori, Dirk Raddatz, Giuliano Ramadori§
Department of Internal Medicine, Division of Gastroenterology and Endocrinology, University Medical Center, Göttingen, Germany

Background: Melanocortin receptors (MCRs) are known to be expressed predominantly in the brain where they exert metabolic and anti-inflammatory functions. Furthermore, the brain MCRs are supposed to also mediate anti-inflammatory effects in other organs.

Materials and methods: As serum levels of MCRs-ligands are elevated in clinical situation (acute-phase response [APR]) of tissue damage, we studied hepatic gene expression of MCRs in a model of muscle tissue damage induced by turpentine-oil (TO) injection in rats.

Results: A significant increase in gene expression of all five MCRs (MC4R was the highest) in liver at the RNA and protein level was detected after TO-injection. A similar pattern of increase was also found in brain. Immunohistology showed MC4R in the cytoplasm but also in the nucleus of parenchymal and non-parenchymal liver cells whereas MC3R-postivity was mainly cytoplasmic. A time-dependent migration of MC4R protein from the cytoplasm into the nucleus was observed during APR in parallel with an increase in α-MSH and leptin serum level. An increase of MC4R was detected at protein level in Wild type mice, while such an increase was not observed in IL-6ko mice during APR. Moreover, treatment of isolated liver cells with melanocortin-agonists (α-MSH and THIQ) inhibited the endotoxin-induced upregulation of the acute-phase-cytokines (IL-6, IL1β and TNF-α) gene expression in Kupffer cells and of chemokines gene expression in hepatocytes.

Conclusions: MCRs are expressed not only in the brain but also in liver cells and their gene expression in liver and brain tissue is upregulated during APR. Due to presence of specific ligands in the serum, they may exert a protective effect in liver cells without involvement of the brain receptors.

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Matrix gels as an alternative method for long-time toxicity test for primary human hepatocytes

Juan J. Martínez Sánchez[1,2], Xueying Yan[1], Sabrina Ehnert[1], Georg Damm[3], Andreas K. Nüssler[1,2]
[1]Eberhard-Karls University, BG Trauma Clinic, Department of Traumatology, Tübingen, Germany
[2]TU Munich, Department of Traumatology, Munich, Germany
[3]Universitätsmedizin Berlin, Department of General Surgery, Campus Virchow, Berlin, Germany

Introduction: New strategies in drug development use primary human hepatocytes (hHeps) to reduce animal tests. hHeps rapidly lose their function under conventional culture conditions (2D). Classical approaches of 3D cultures, e.g. bioreactors, cannot be used for drug-testing due to their large need for cells. Therefore, aim of this work was to develop culture system for hHeps that allows down-scaling for screening purposes.

Methods: hHeps were isolated, by a standard two step collagenase perfusion technique, according to the ethical guidelines. Immediately after isolation hHeps were resuspended in collagen-gels, which were isolated from rat tails, and seeded into 96-well-plates. Viability was measured by Resazurin conversion and life-dead-staining. CYP and hepatic transporter channels expression were observed by PCR.

Results: The highest viability was observed with a cell density of 5000 cells per µl gel. With this concentration the cells could interact with each other, as in the organotypic culture conditions of a bioreactor. However, with the here presented technique we can fill approx. 175 wells using the same amount of hHeps needed to fill the smallest available bioreactor (25 mil.). Both hHeps cultures (2D and 3D) showed expression of CYP and hepatic transporter channels during the performance of PCR after 48 hours of culture. However, hHeps cultured in collagen-gels respond to acetaminophen and diclofenac stimulation with cellular damage with their viability reduced down to one fifth. This is a difference to hHeps in conventional cultures which barely respond to these well known *in vivo* toxins.

Discussion/Conclusion: The present work demonstrates the importance of a 3D structure for hHeps to maintain their differentiated state for longer time permitting more accurate cell studies.

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A role of AXL in EMT and hepatocellular carcinoma progression

Wolfgang Mikulits, Franziska van Zijl, Patrick Reichl, Heidemarie Huber, Markus Grubinger
Department of Medicine I, Division: Institute of Cancer Research, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria

Metastasis is the leading cause of cancer mortality and represents a multi-step process including tumor cell invasion. In hepatocellular carcinoma (HCC), intrahepatic metastasis frequently correlates with a preceding epithelial to mesenchymal transition (EMT) of malignant hepatocytes. EMT is therefore considered as a pivotal event in HCC progression. Several mechanisms have been elaborated to be essentially involved in hepatocellular EMT such as the collaboration of Ras signaling with transforming growth factor-β. Yet, the diversity of signaling cascades that govern EMT in HCC progression is still poorly understood. We established a unique cellular EMT model of human HCC which particularly allows to identify novel molecular mechanisms of HCC cell dissemination.

Expression profiling of epithelial versus mesenchymal HCC cells revealed that the receptor tyrosine kinase AXL is a major regulator of EMT and HCC progression. AXL is expressed at the cell surface of mesenchymal HCC cells, whereas epithelial cells are devoid of AXL expression. Negative interference with AXL expression showed abrogation of migratory abilities in HCC cells that have undergone EMT. These *in vitro* data could be verified by immunohistochemical evaluation of AXL expression in a large number of HCC patient samples. High AXL expression could be correlated with (i) advanced stages of liver cancer, (ii) elevated vessel invasion of HCC cells, (iii) higher risk of tumor recurrence after liver transplantation, and (iv) a significantly lower survival rate within 5 years after tumor resection. In conclusion, our data suggest that Axl might represent a promising target in HCC invasion and intrahepatic metastasis.

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In vitro response of primary hepatocytes to TGF-beta: How to separate the wheat from the chaff

Roman Müllenbach[1], Rabea Hall[2], Iryna Ilkavets[1], Steven Dooley[1], Frank Lammert[2]
[1]Molecular Hepatology – Alcohol Associated Diseases, II. Medical Clinic Faculty of Medicine at Mannheim, University of Heidelberg, Mannheim, Germany
[2]Department of Medicine II, Saarland University Medical Center, Homburg, Germany

Introduction: Hepatocyte damage is the primary event in all aetiologies of chronic liver disease. Injured hepatocytes release a cascade of inflammatory cytokines, of which TGF-beta is considered to represent the central pro-fibrogenic molecule. This "cytokine storm" triggers an avalanche of reactions in either neighbouring hepatocytes (autocrine signalling) or adjacent liver cells such as hepatic stellate cells or resident macrophages.

Hypothesis: Genetic variants resulting in different transcriptional regulation by TGF-beta might modulate the hepatocellular response to paracrine and autocrine cytokine signalling, and hence cause differential susceptibility to fibrogenesis in response to chronic hepatocellular damage.

Methods: We measured transcriptional response of primary hepatocytes to TGF-beta in two different inbred laboratory mouse strains, C57BL/6J (B6) and DBA/2J (D2), *in vitro* after TGF-beta stimulation (5 ng/ml). The expression of > 19,000 transcripts was quantified using Affymetrix MOE430.2 exon array. After normalisation and log-transformation of fluorescence readings, expression across 24 hours (mean vaue of 1 h, 6 h and 24 h) was compared between treated and untreated hepatocytes by t-test to identify TGF-beta regulated genes. Expression values were compared between strains to identify differentially regulated genes.

Results: Overall, 79 genes varied significantly across 24 h (treated vs. untreated, $p < 0.001$) in B6 hepatocytes, and 30 genes varied significantly in D2 hepatocytes. In total, 207 genes differed between TGF-beta treated B6 and D2 hepatocytes. In particular, CXCL15 was significantly downregulated by TGF-beta in B6 hepatocytes. Of note, NR0B2 was significantly induced in D2 hepatocytes *and* differed significantly between B6 and D2. CXCL14 and PPARgamma differed significantly between B6 and D2.

Discussion/Conclusion: Applying stringent analytical conditions we identified TGF-beta target genes showing varying transcriptional response. Averaging expression across 24 hours ensures robustness of the differential analysis. These genes are candidates for causing differential susceptibility to fibrogenesis in response to chronic liver damage.

TGF-β1 contributes to intrahepatic cholangiocarcinoma via Smad dependent and independent pathways

Stefan Munker[1], Qi Li[1], Yan Liu[1], Christoph Meyer[1], Steven Dooley[1], Jun Li[2], Hong-Lei Weng[1]
[1]Molecular Hepatology – Alcohol Associated Diseases, II. Medical Clinic Faculty of Medicine at Mannheim, University of Heidelberg, Mannheim, Germany; [2]General, Visceral Surgery and Transplantation, University Hospital Tübingen, Germany

Introduction: Incidence of intrahepatic cholangiocarcinoma (ICC) rises in western countries. So far, only few studies focus on the biological characteristics ICC. TGF-β1 plays a dual role in the progression of human cancer and has been implicated in ICC. In the present study, we investigated the role of TGF-β1 canonical and non canonical signaling pathways in ICC.

Methods: Immunohistochemical staining for TGF-β1 and P-Smad2/3 was performed in 25 paraffin-embedded cholangiocarcinoma specimens paired with adjacent non-cancerous tissue. TFK-1, a cholangiocellular cell line was used for assessing the impact of TGF-β1 on proliferation. Knockdown experiments by RNAi and Adenoviral overexpression of Smad2 and Smad3 were used to investigate the importance of the canonical signalling pathway. Specific inhibitors were used for blockage of the non-canonical pathways.

Results: Compared with surrounding non-tumor tissues, cancer cells showed strong positivity for TGF-β1 and P-Smad2/3C staining in all 25 ICC patients investigated. Further, Smad3C activation is stronger in samples with lower grading, whereas Smad2C activation correlates positively to cholangiocarcinoma with higher grading. In vitro, RNAi and Adenoviral experiments revealed that TGF-β1 dependent expression of p21, a marker of cell cycle arrest, is Smad3 dependent. However, TGF-β dependant downregulation of PCNA, a marker for proliferation, appeared independent of either Smad2 or Smad3.

Discussion/Conclusion: Smad2 activation appears to be connected to a better prognosis of cholangiocarcinoma, whereas Smad3 activation is connected to poorer grading and prognosis.

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Expression of prospero-related homeobox 1 (Prox-1) transcription factor and epithelial adhesion molecule (EpCAM) in a rat model of intrahepatic cholangiocarcinogenesis

N. Naz, I. Malik, F. Schultze, T. Mansuroglu, G. Ramadori
George August University Hospital, Göttingen, Germany

Deregulation of homeobox genes may give rise to tumorigenesis in target organs. Immuhistochemical studies have shown that the Prospero-related homeobox 1 (Prox-1) gene is expressed in mature hepatocytes but is not detectable in non-parenchymal liver cells. In the current study we investigated the expression pattern of Prox-1 in a rat model of damage, fibrosis, cirrhosis and intrahepatic cholangio-carcinoma (CC). Sprague dawley rats were given thioacetamide in drinking water (500 mg/l) until they developed tumors (for 18 weeks). Control rats were given normal tap water. Animals were sacrificed, liver was removed and RNA was isolated for real time PCR. Cryostat and Paraffin sections were used for histopathology. The mRNA expression levels of Prox-1, cytokeratin-19 (CK-19), and epithelial cell adhesion molecule genes were examined by RT-PCR.

Immunohistochemical analysis of the normal liver revealed that Prox-1 is expressed in hepatocytes, while it could not be detected in EpCAM and CK-19 positive biliary epithelial cells. A co-expression of Prox-1 with CK-19 and EpCAM was found in proliferating ductular cells, which were located within the fibrotic tissue of the cirrhotic liver after 12 and 16 weeks of TAA administration. Immunohistochemical analysis of the CC tissue demonstrated Prox-1 positivity in CK-19 and EpCAM positive tumoral cells as well. Compared to the normal liver tissue, no significant up-regulation of the Prox-1 gene-mRNA expression could be found during the development of CC whereas an up-regulation of CK-19 and EpCAM could be detected.

These data suggest that EpCAM and Prox-1 positive cells might be tumor initiating cells and Prox-1 might be involved in neoplastic changes of the biliary epithelium during cholangiocarcinogenesis.

Polymorphisms of nuclear hormone receptor peroxisome proliferator-activated receptor-γ gene might be associated with inflammation, atherosclerosis, endothelial dysfunction and colonic cancerogenesis

O.M. Plehutsa, L.P. Sydorchuk, A.R. Sydorchuk, R.I. Sydorchuk, I.I. Sydorchuk, I.M. Plehutsa, J.V. Ursuliak, O.A. Karliychuk, A.A. Sokolenko
Bukovinian State Medical University, Chernivtsi City Emergency Hospital, Chernivtsi, Ukraine

Introduction: It is known that the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) is not only related to metabolic disorders but inflammation, atherosclerosis, endothelial dysfunction, immunoregulation and cancerogenesis as well. Different somatic PPAR-γ mutations found in patients with colonic cancers have suggested that PPAR-γ might act as a tumor attenuator or suppressor. This statement is confirmed by successful experimental inhibition of cellular proliferation by several synthetic PPAR-γ agonists which were able to inhibit cell proliferation both in vitro and in vivo.

Methods: 104 patients with mild-severe hypertension (inflammation, atherosclerosis, endothelial dysfunction) were inspected: 48.1% (50) female and 51.9% (54) male, average age 53.2 ± 8.7 years. In 50 (48.1%) patients was concomitant IBD; 28 (26.9%) patients were complicated with development of colonic cancer. 26 patients without IBD and cancer formed control group. TNF-α, NF-κB and endotoxin-core antibodies IgG (EndoCAb) concentrations in plasma were defined by IEA. Data of colonic microbiology, mesenteric vessels sonography; PCR were compared.

Results: PPAR-γ2 gene (Pro/Pro12Ala/Ala) polymorphisms were respectfully observed in IBD/cancer group in 6.0%, 28.9% and 65.1% (in control – 16.0%, 34.0% and 50.0%, $p = 0.032$). NF-κB levels were higher in cancer and IBD group, $p < 0.05$. EndoCAb and TNF-α didn't differ significantly between groups. Colonic dysbiosis grades III–IV was observed: in cancer group in 26 (92.9%); in IBD group in 46 (92.0%) compared to 15 (57.7%) in control. There were no significant differences found in mesenteric ultrasonography between groups, but increased volume characteristics observed in IBD and cancer groups, while endothelial dysfunction was almost similar.

Discussion/Conclusion: Based on previous data we hypothesized that PPAR-γ gene polymorphism (Pro12Ala) contributes into development of colonic cancer and IBD. However, this is not sufficiently proved by our study, further attention has to be focused on PPAR-γ expression.

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Hop bitter acids inhibit tumorigenicity of hepatocellular carcinoma cells *in vitro*

Michael Saugspier[1], Christoph Dorn[1], Barbara Czech[1], Manfred Gehrig[2], Jörg Heilmann[3], and Claus Hellerbrand[1]
[1]Department of Internal Medicine I, University Hospital Regensburg, Germany
[2]Nateco$_2$, Wolnzach, Germany
[3]Department of Pharmaceutical Biology, Institute of Pharmacy, University of Regensburg, Germany

Introduction: Female inflorescences of the hop plant contain a variety of secondary metabolites with bitter acids as major component besides cellulose and lignin. Recent experiments revealed various potent biological properties with promising effects in cancer therapy and prevention.

The aim of this study was to assess the effect of bitter acids on the tumorigenicity of hepatocellular carcinoma cell lines.

Methods and results: Three different human hepatocellular carcinoma cell lines were incubated with two different hop bitter acid extracts purified with supercritical CO_2. At a concentration of 25 µg/ml, the beta but not alpha rich extract started to induce aspartate transaminase (AST) release and significant increase was detected with 50 µg/ml of both extracts. Already at lower concentrations both extracts led to a dose dependent inhibition of proliferation and migration was inhibited at a concentration as low as 5 µg/ml. Further, both extracts (5 µg/ml) inhibited the ERK-phosphorylation and reduced the activity of the transcription factors AP-1 and NFkappaB activity.

Conclusion: The analysis of functional effects of hop bitter acids on human hepatocellular carcinoma cell lines revealed an inhibition of tumorigenicity. Moreover ERK, AP-1 and NFkappaB which are important regulators of tumor development and progression were identified as targets of hop bitter acids. Thus, these data suggest the potential use of hop bitter acids as a functional nutrient for both the prevention and treatment of human hepatocellular carcinoma.

An oncogenic gp130 deletion mutant displays impaired maturation and induces an inflammatory phenotype in a transgenic mouse model

Dirk Schmidt-Arras[1], Silke Horn[1], Ruven Wilkens[1], Johannes Herkel[2], Stefan Rose-John[1]
[1]Institute for Biochemistry, Christian-Albrechts-University Kiel, Germany
[2]Department of Medicine I, University Medical Centre Hamburg-Eppendorf, Germany

Introduction: Recently deletion mutants of the interleukin-6 receptor signalling subunit gp130 have been found in hepatocellular adenoma. These mutants display ligand-independent constitutive active signalling of gp130. Although mutated in 60% of human inflammatory hepatocellular adenoma it is not clear if gp130 deletion mutants are sufficient to induce tumor formation. Furthermore the molecular mechanisms of gp130 activation and sustained STAT3 phoshphorylation are completely unknown.

Methods: Immunoblotting, mutagenesis, immunofluorescence, confocal microscopy, metabolic labeling, transgenic mice

Results: We found that the gp130 ΔY186-Y190 (gp130 ΔYY) deletion mutant is constitutively active and dimerized when transfected to HepG2 cells and can be detected predominantly in a lower molecular weight band.
Biochemical analysis including ^{35}S-Cys/Met metabolic labeling revealed that gp130 ΔYY displays an impaired maturation and predominates in a lower molecular weight highmannose form and shows an increased association to the ER chaperone Calnexin. Using confocal fluorescence microscopy we can show that gp130 ΔYY localises predominantly to the endoplasmic reticulum.and to early endosomes.
Activation of Janus kinases was dispensable for ER retention.
We also present preliminary data on transgenic C57BL/6N mice expressing EYFP-tagged gp130 ΔYY under the control of the liver-specific Albumin promoter. Transgenic animals displayed elevated acute phase protein levels in the serum and increased STAT3 phosphorylation in the liver. Histological analysis of liver tissue revealed a mild endothelitis comprising neutrophil and macrophage infiltrations.

Discussion/Conclusion: It is possible that an active conformation of the gp130 ΔYY extracellular domain is sensed by the ER quality control thereby leading to its retention in the endoplasmic reticulum. Activation of gp130 ΔYY from within the ER may cause aberrant signalling, contributing to oncogenesis, as we have seen before for the oncogenic receptor tyrosine kinase FLT3 ITD.
Finally we show that transgenic mice expressing EYFP-tagged gp130 ΔYY in hepatocytes might be a valuable mouse model for the study of inflammatory hepatocellular adenoma and inflammation induced carcinogensis.

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Structural analysis of an oncogenic gp130 deletion mutant

Antje Schuett[1], Silke Horn[1], Stefan Rose-John[1], Dirk Schmidt-Arras[1]
[1]Institute for Biochemistry, Christian-Albrechts-University Kiel, Germany

Introduction: Recently deletion mutants of the interleukin-6 receptor signalling subunit gp130 have been found in hepatocellular adenoma. These mutants display ligand-independent constitutive active signalling of gp130. Although mutated in 60% of human inflammatory hepatocellular adenoma it is not clear if gp130 deletion mutants are sufficient to induce tumor formation. Furthermore the structural mechanism of ligand-independent gp130 activation is completely unknown.

Methods: Immunoblotting, mutagenesis, immunofluorescence, confocal microscopy, metabolic labeling, transgenic mice

Results: We found that the gp130 ΔY186-Y190 (gp130 ΔYY) deletion mutant is constitutively active and dimerized when transfected to HepG2 cells.
Deletion of the entire D1 domain or the first N-terminal five aminoacids abrogated constitutive activity, pointing to a role of the N-terminus for the activation of gp130 ΔYY. Furthermore we identified residues at the D2-D3 interface that seem to be crucial for gp130 activation.

Discussion/Conclusion: Previous work and our data presented here suggest that gp130 exist as a preformed dimer. Here we present data that constitutive activation of gp130 deletion mutants involves the N-terminus of gp130 that has been shown in crystal structures to interact with IL-6 in the ligand-bound state. In addition we present data that hydrophobic interaction between D2 and D3 domain, as well as stabilization of the IL-6 contact site loop is important to keep gp130 in an inactive conformation.

Expression and function of hepatotrophic protein augmenter of liver regeneration, ALR, in fatty livers

S. Spieker[1,2], R. Dayoub[1,2], M. Lupke[1,2], C. Dorn[3], C. Hellerbrand[3], M. Melter[2], T.S. Weiss[1,2]
[1]Center for Liver Cell Research, University Medical Center, Regensburg, Germany
[2]Department of Paediatrics, University Medical Center, Regensburg, Germany
[3]Department of Internal Medicine, University Medical Center, Regensburg, Germany

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease worldwide and its spectrum ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. NASH has been thought to be a disease caused by triglyceride accumulation in hepatocytes with subsequent oxidant stress and lipid peroxidation causing inflammation and fibrosis. The protein ALR (augmenter of liver regeneration) was previously shown to have hepatoprotective properties against liver toxins. The aim of our study was to investigate the expression of ALR in murine NASH models and its potential protective role under conditions of fatty liver in vitro. Expression of ALR mRNA was increased in liver samples from mice treated with methionine-choline-deficient (MCD) and Paigen (lard, cholesterol, Na-cholate) diet. In livers from mice with Paigen diet, ALR expression was in correlation to fat uptake and addition of cholesterol to the diet. Primary human hepatocytes and hepatoma cells (HepG2, Huh-7) were treated with free fatty acids and showed dose and time depended accumulation of fat vacuoles and triglyceride levels. To elucidate the role of ALR under conditions leading to NASH we generated stable-transfected HepG2 and Huh-7 cells expressing human ALR protein. We cultured wildtype and stably ALR expressing cells with FFAs and after 24 h we observed a significant lower lipid-induced cell toxicity and caspase activity in ALR expressing cells compared to wildtype. Further, lipotoxicity of wt-HepG2 could be reduced to levels of HepG2-ALR after treatment with recombinant human ALR. In addition, intracellular triglyceride content was significantly decreased in HepG2-ALR cells compared to wt-HepG2 cells.

This data indicates that ALR is increased in murine models of NASH with distinct inflammation, diminish cellular lipid accumulation and therefore might reduce hepatic steatosis/NASH. In conclusion, we demonstrate that hepatotrophic factor ALR, besides its beneficial effect in liver regeneration, attenuates disease progression in NAFLD.

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Changes of LCN-2 gene expression in different organs in a rat model of tissue damage

Sadaf Sultan, Shakil Ahmad, Matteo Pascucci, Giuliano Ramadori
Gasteroenterology and Endocrinology, George-August University, Göttingen, Germany

Introduction: Lipocalin-2 (LCN-2) is a secretory protein exists as a 25 kDa monomer and known as neutrophil gelatinase associated lipocalin. It is shown to mediate an innate response to bacterial infection by sequestrating iron and known as a marker of kidney damage. We recently showed the LCN-2 expression in rat and mice as compared to different acute phase proteins. The main aim of this study is to analyze the kinetics of LCN-2 serum levels together with gene and protein expression liver in comparison to the other organs in a rat and mouse model of turpentine-oil (TO)-induced sterile abscess.

Methods: Turpentine oil was injected in both hindlimbs of male Wistar rat and was sacrificed at 1 h to 48 h along with control of every time point. Liver, kidney, heart, brain, spleen and lung were taken, rinsed, minced and stored at -80ºC for RNA and protein isolation for RT-PCR and western blot analysis respectively.

Results: Serum LCN-2 concentrations increased dramatically up to 200-fold (20 µg/ml) at 48 h after TO-injection. A strong elevation of LCN-2 *m*RNA in rat liver was observed starting from 4 h upto 48 h after injection, with a maximum (8738 ± 2104-fold) at 24 h, which was further confirmed by Western blot analysis. In contrast, the increases in LCN-2 expression in other organs i.e. kidney (5.12 ± 2.16-fold), heart (2.45 ± 0.12-fold), brain (35.59 ± 6.71-fold), spleen (8.63 ± 1.61-fold) and lung (21.63 ± 0.19-fold) was extremely less as compared to liver.

Discussion/Conclusion: In tissue damage the most dramatic changes in LCN-2 gene expression take place in liver. This can explain increased serum level described in kidney damage. LCN-2 can be considered as a new injury biomarker.

Association of chronic systemic inflammation, liver cirrhosis, cancerogenesis with T894G polymorphism of endothelial nitric oxide synthase gene (eNOS) and vascular injury

L.P. Sydorchuk, V.P. Prysyazhnyuk, O.I. Voloshyn, O.V. Kushir, A.R. Sydorchuk, A.A. Sokolenko, R.I. Sydorchuk, J.V. Ursuliak, I.I. Sydorchuk
Bukovinian State Medical University, Chernivtsi, Ukraine

Introduction: Whilst the role of proangiogenic and proinflammatory factors is well established in cancerogenesis, the association of T894G polymorphism of endothelial nitric oxide synthase (eNOS) gene with cytokines, cardiovascular injury in patients with hepatic carcinoma is unclear.

Methods: Study group included 50 patients with liver malignancies due to non-viral cirrhosis (20 female, 24 male, mean age 63.2 ± 8.7); control group included 10 healthy volunteers. All patients had vascular injury in a form of concomitant Arterial Hypertension (AH) and Chronic Heart Failure (CHF). IL-4, TNF-α, TGF-β1, pro-Atrial Natriuretic Peptide (proANP) plasma concentrations were defined by IEA; eNOS (T894G) gene polymorphism was assessed with PCR.

Results: Difference in genotypes distribution between groups was not significant. Presence of T-allele in patients with liver cirrhosis was associated with reliable increase of AST activity (27.4%, $p < 0.05$), urea concentration (33.3%, $p < 0.05$), creatinine (22.2%, $p < 0.05$) compared to GG-carriers. In T-allele carriers concentration of proANP was higher (89.2%, $p < 0.001$), as well as diameter of left atrium was larger (13.6%, $p < 0.01$), than in patients with GG-genotype. IL-4, TNF-α and TGF-β1 didn't differ reliably between genotypes, but TNF-α and proANP level were significantly higher in research patients than in control group ($p < 0.001$). In research group males T-allele also combined with the increased LVMI (by 12.2%, $p < 0.05$) compared to GG-genotype patients.

Discussion/Conclusion: eNOS is connected with hepatic circulation via changes of endothelial function, inflammation and metabolic syndrome and reflects widespread vascular damage as proved by cardiovascular changes. We hypothesized that oxidation, systemic inflammatory reaction and unregulated cellular proliferation depends on eNOS gene expression. T-allele associates with cytolysis, indirect fibrotic liver changes, cardiovascular failure progression (proANP) and may be a risk factor of liver cancer.

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Selective cyclooxygenase-2 inhibitor decreases pro-cancerogenic effect of endotoxin in experimental murine model

R. Sydorchuk[1], P. Fomin[2], L. Sydorchuk[1], I. Sydorchuk[1], O. Plehutsa[3], A. Sydorchuk[1], R. Knut[1], A. Palianytsia[1], O. Sydorchuk[1], O. Palianytsia[3], O. Polianskyy[3]
[1]Bucovinian State Medical University, Chernivtsi, Ukraine, [2]National State Medical University, Kyiv, Ukraine, [3]City Emergency Hospital, Chernivtsi, Ukraine

Introduction: Growing evidence indicates that bacterial infections and following inflammation is contributing to cancer growth. Recent studies showed promotional influence of endotoxin-lipopolysaccharide (LPS) on cancerogenesis and metastatic growth (MG) which in part is associated with involvement of cyclooxygenase-2 (COX-2). We hypothesized that inhibiting of COX-2 may decrease inflammation contributing to MG.

Methods: Murine model including 2 groups (25 each) of adolescent mice was used. Metastatic process was modeled by i/v injection of 200 µl spontaneously metastasizing mammary adenocarcinoma cell culture suspension. Both control and experimental group animals received 200 µl suspension of 10 µg LPS per mouse. Experimental group received selective COX-2 inhibitor celecoxib orally from day 1. MG evaluated hystochemically within pulmonary metastases. TNF-α, VEGF quantified with ELISA; COX-2 with Western Immunoblotting.

Results: Control group mice developed a mean number of 35.27 ± 6.19 macroscopic metastases by day 15. Both the weight and quantitative parameters of MG were significantly lower in study group compared to control ($p < 0.005–0.0001$). Experimental group MG was characterized by 48.8% lower mitotic index (MI) and 37.6% higher apoptotic index (AI). MI/AI ratio in the experimental group was 2.1 times lower ($p < 0.001$) than the ratio observed in control group mice. Systemic COX-2, TNF-α and VEGF were significantly lower in celecoxib group ($p < 0.005–0.001$) compared to control.

Discussion/Conclusion: Although expression of COX-2 has been associated with development and progression of numerous malignancies, its precise role in promotion of cancer cell dissemination is still poorly understood. TNF-α – a major proinflammatory cytokine is released in response to LPS associated increase of VEGF production via COX-2 pathway. Current study shows that pro-cancerogenic effect of endotoxin in experimental murine model could be inhibited by neutralizing COX-2 associated pathway. Treatment with celecoxib was effective in reducing metastasis volume, suggesting that COX-2 contributes to MG. However, there is no direct evidence that all metastases are could be sensitive to COX-2 inhibition.

Metastatic growth is experimentally potentiated by endotoxin-induced inflammatory response

R. Sydorchuk[1], P. Fomin[2], L. Sydorchuk[1], I. Sydorchuk[1], O. Plehutsa[3], A. Sydorchuk[1], R. Knut[1], S. Levites[3], O. Sydorchuk[1], O. Karliychuk[1], A Vynohradskyy[3]
[1]Bucovinian State Medical University, Chernivtsi, Ukraine, [2]National State Medical University, Kyiv, Ukraine, [3]City Emergency Hospital, Chernivtsi, Ukraine

Introduction: Existing data shows that endotoxin – lipopolysaccharide (LPS) is both angiogenic, inducing neovascularization and immunosuppressing, thus potentially promoting metastatic growth (MG) in colonic cancer. We hypothesized that increased LPS promotes MG.

Methods: Murine model including 2 groups (25 each) of adolescent mice was used. Metastatic process was modeled by i/v injection of 200 µl spontaneously metastasizing mammary adenocarcinoma cell culture suspension. Control group animals received 200 µl sterile saline intraperitoneal (i.p.), experimental group 200 µl suspension of 10 µg LPS per mouse. MG evaluated hystochemically within lung metastases.

Results: Experimental group showed significantly higher ($p < 0.001$) MG compared with the control group. MG was characterized by 61.2% higher mitotic index (MI) in the experimental group and 42.3% lower apoptotic index (AI). MI/AI ratio in the experimental group was 3.2 times higher ($p < 0.001$) than the ratio observed following saline injection. LPS injection resulted in reliably ($p = 0.002$) higher levels of serum VEGF than in control with strong positive correlation ($r = 0.971$) between circulating VEGF and LPS levels. However, Anti-Endotoxin Core AntiBodies (EndoCAB) levels remain almost similar in both experimental and control group ($p = 0.058$), following 18.6 ± 2.59% elevation due to injection.

Discussion/Conclusion: Bacterial translocation as an important pathogenesis factor cause massive release of LPS from endogenous gut flora, potentiating the MG and stimulating pro-inflammatory response. LPS, introduced into the circulation augments MG by increasing cell proliferation, decreasing apoptosis and increasing VEGF production as an important pro-angiogenic factor. Thus, increased MG following removal of primary colonic tumor may not only be due to the removal of angiogenesis inhibitors produced by the primary neoplasm.

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Objective response in the treatment of HCC with IL-2, BCG and melatonin

B. Tomov[1], D. Popov[1], R. Tomova[1], N. Vladov[2], Z. Krastev[1]
[1]Medical University Sofia, Clinic of Gastroenterology, Sofia, Bulgaria
[2]Military Medical Academy Sofia, Clinic of Liver-Pancreatic and Transplant Surgery, Sofia, Bulgaria

Introduction: Nowadays hepatocellular carcinoma is the third leading cause of cancer death worldwide. Resection may benefit certain cases, but many patients with advanced disease or deteorated hepatic function are not suitable candidates for such treatment.

Case report: We report the case of a 67-year-old female, in whom a single liver tumor (60 mm) without AFP elevation was diagnosed in 2007. The patient was HCV and HbsAg negative, anti-HBcor-total positive and HBV-DNA negative. Due to AFP rise up to 200 IU/ml in November 2008 liver resection was performed. Histological examination revealed hepatic adenoma and chronic hepatitis. The control CT in March 2009 was without liver tumors. During follow-up AFP rise continued, reaching 1000 IU/ml, without any liver lesion detection by ultrasound. Striking AFP rise was registered in June 2009 (5000 IU/ml) when CT scan and MRI revealed 7 liver lesions. The patient was considered inoperable. Immunotherapy with s.c. IL-2 (1.5 ME five consecutive days monthly), followed by BCG vaccination after a fortnight interval plus Melatonin 20 mg/daily was initiated. In March 2011 AFP dropped down. In October only one liver lesion was detected by CT scan with characteristics of adenoma and AFP level 30 IU/ml.

Discussion/Conclusion: When conventional therapy for HCC is not an option, combined immunomodulating therapy can be performed.

Atrophin 2 expression and function in hepatocellular carcinoma

Daniela Valletta, Barbara Czech, Claus Hellerbrand
Department of Internal Medicine I, University Hospital Regensburg, Germany

Introduction: Atrophin proteins have first been identified in dentatorubral-pallidoluysian atrophy. Atrophin family proteins have been identified as nuclear receptor corepressors. They are involved in the regulation of various biological processes including migration and orientation, and altered atrophin expression has been shown in neurodegenerative disease and cancer. Atrophin2 (ATN2) has critical functions in normal mouse embryonic development whereas Atrophin1 appears to be dispensable.

The aim of this study was to analyze the expression and function of ATN2 in hepatocellular carcinoma (HCC).

Methods and results: Quantitative RT-PCR analysis showed an upregulation of ATN2 expression in three different HCC cell lines (PLC, Hep3B, HepG2) compared to primary human hepatocytes. Similarly, analysis of ATN2 mRNA expression in tumorous liver tissue of 28 HCC patients revealed an increase of ATN2 expression in 22 cases compared to corresponding non-neoplastic hepatic tissue. To assess the functional role of ATN2 in HCC, HCC cells were transiently transfected with specific siRNA against ATN2, which leads to approximately 50% downregulation of ATN2 compared to mock-transfected cells. Functional analysis applying the xCELLigence System (Roche) revealed that ATN2 downregulation affects attachment and proliferation of HCC cells in vitro.

Conclusion: Our data indicate ATN2 as a functionally relevant transcriptional regulator in hepatocellular carcinoma. Targeting ATN2 or the molecular mechanism responsible for ATN2 upregulation in HCC, respectively, may be a viable method to inhibit the progression of this highly aggressive tumor.

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Combined immunotherapy with IL12-expressing and AFP-pulsed dendritic cells towards established hepatocellular carcinoma *in vivo*

Annabelle Vogt[1], Carlo Schneider[1], Georges Decker[1], Wolfgang H. Caselmann[2], Tilman Sauerbruch[1], Maria A. González-Carmona[1]
[1]Department of Internal Medicine I, University of Bonn, Bonn, Germany
[2]Bavarian State Ministry of the Environment and Public Health, Munich, Germany

Introduction: Dendritic cells (DC) are able to prime T-cells against tumor-associated antigens (TAA), such as α-Fetoprotein (AFP) for hepatocellular carcinoma (HCC). IL-12 as Th1 cytokine is essential for inducing cellular immune responses. In this study, the impact of DC engineered to express IL-12 on tumor-environment and on tumor-growth was analyzed alone or in combination with a conventional vaccination using AFP-pulsed DC in a poor immunogenic murine HCC-model.

Methods: DC were obtained from the bone marrow of C3H-mice and transduced with Ad-mIL12, Ad-mAFP and Ad-LacZ. Hepa129-mAFP-cells were injected into the right flank or the liver of C3H-mice to induce subcutaneous (s.c.) and orthotopic HCC. For vaccination, 10^6 mAFP-pulsed DC were inoculated s.c. IL-12-expressing DC were injected intratumorally (i.t.).

Results: I.t. but not s.c. injection with IL12-expressing DC elicited complete tumor eradication in 75% of mice with pre-established HCC. The combination with an s.c. immunization using AFP-pulsed DC was even more effective with 90% complete tumor elimination. i.t. injection of IL12-DC induced crucial changes in the immunological tumor-environment with increased expression of Th1-polarized cytokines (IL-12 and IFN-γ) as well as chemokines. The induced inflammatory tumor-milieu was associated with recruitment of $CD4^+$-, $CD8^+$-T-cells and NK-cells within the tumor.

Discussion/Conclusion: IL12-expressing DC were able to change the immunosuppressive tumor-environment of pre-established HCC into an inflammatory milieu, eliciting an effective tumor-regression of pre-established HCC due to activation of both, acquired and innate immune responses. Combining this approach with a vaccination using AFP-pulsed DC further enhanced the antitumoral effect. This seems to represent a promising approach for immunotherapy of HCC.

Strain-specific differences influence hepatocellular carcinoma initiation in Abcb4-deficient mice

Susanne N. Weber, Annika Bohner, Frank Lammert
Department of Medicine II, Saarland University Hospital, Homburg, Germany

Introduction: In patients, the development of hepatocellular carcinoma (HCC) is usually a consequence of advanced liver fibrosis but the mechanisms are still poorly understood. It has been shown that the initiation of HCC can be modelled in mice by the administration of a single dose of diethylnitrosamin (DEN, Naugler et al., Science 2007). Mice that lack the hepatocanalicular phospholipid transporter Abcb4 develop biliary fibrosis spontaneously and can be used as a model to study tumor formation in a pre-damaged liver. The aim of this study was to examine HCC initiation in Abcb4-deficient mice of different genetic backgrounds.

Methods: Abcb4-deficient mice on the FVB/NJ genetic background were crossed to three distinct genetic backgrounds (Balb/cJ, C3H-HeN, C3H-HeJ) for at least ten generations. Congenic knockout and wildtype (WT) mice at the age of 7 and 16 weeks were treated with a single dose of DEN. After 48 hours, mice were sacrificed and the livers were compared to untreated controls. Phenotypic differences were determined by analyzing apoptosis (TUNEL) rates, expression of the inflammatory marker interleukin 6 (IL-6) and serum activities of liver enzymes.

Results: All parameters were significantly increased in Abcb4-deficient mice compared to WT mice and DEN-treated mice as compared to untreated controls. Interestingly, FVB-Abcb4-deficient mice showed the lowest apoptosis rates and IL-6 expression levels at the age of 7 weeks and the lowest apoptosis rates at the age of 16 weeks compared to all other Abcb4-deficient mice.

Discussion/Conclusion: This study indicates that HCC initiation upon DEN challenge depends on pre-existing fibrosis and genetic background. As HCCs are known to occur spontaneously in FVB-Abcb4-deficient mice, the high apoptosis rates in the other strains might confer a protective mechanism against HCC development. Further characterisation of the genetic differences between congenic lines is necessary to understand the molecular mechanisms underlying HCC initiation and formation.

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Regulatory T cell-attracting chemokines in hepatocellular carcinoma

Gabriela Wiedemann[1], Moritz Rapp[1], Lydia Kriegl[2], Doris Mayr[2], Enrico de Toni[3], Veit Gülberg[4], Stefan Endres[1], David Anz[1,4]
[1]Division of Clinical Pharmacology, [2]Institute of Pathology, [3]Medizinische Klinik II, [4]Medizinische Klinik Innenstadt, Ludwig-Maximilian University Munich, Germany

Introduction: Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and has poor prognosis. HCC tissues are infiltrated by cytotoxic CD8+ T cells that can eliminate cancer cells through recognition of tumor antigens. However, in most tumors the presence of CD4+FoxP3+ regulatory T cells (Treg), a population of immunosuppressive T cells, leads to an inhibition of anti-tumor immunity. For HCC it has been shown that the number of intratumoral Treg is associated with tumor progression and poor survival. It is so far unclear which factors mediate the recruitment of Treg to the HCC tissue. An important chemokine known to attract Treg to peripheral sites is CCL22.

Methods: To test whether CCL22 is expressed by HCC and how this affects FoxP3+ Treg infiltration, we established an immunohistological staining on paraffin-embedded tissue sections. HCC specimens from 102 patients were screened for expression of CCL22 and FoxP3. Additionally, we transfected Hepa 1.6 cells with Lentivirus to generate a novel hepatoma cell line with inducible CCL22 expression.

Results: We could show CCL22 expression in the majority of all analyzed HCC tissues. In addition, FoxP3+ cells were found at high numbers in many HCC tumors. CCL22- and FoxP3-expressing cells were distributed heterogeneously in the tumor and peritumoral tissues. Induced CCL22 expression in vitro by hepatoma cells did not affect tumor cell morphology and survival, but CCL22 induced the migration of regulatory T cells.

Discussion/Conclusion: In conclusion we identify here the expression of CCL22 in human HCC. CCL22 is thus a molecule that may serve as target for cancer therapy.

Loss of Smad2 correlates with poor differentiated gastric cancer

Yijun Wu[1*], Qi Li[2*], Jiren Yu[1], Yunchuan Mu[1], Stefan Munker[2], Chengfu Xu[3], Zhe Shen[3], Roman Müllenbach[2,4], Yan Liu[2], Li Li[5], Norbert Gretz[5], Derek Zieker[6], Jun Li[6], Kouichi Matsuzaki[7], Youming Li[3], Matthias Ebert[2], Steven Dooley[2*], Hong-Lei Weng[2*]
[1]Department of General Surgery, The First Affiliated Hospital, Medical School, Zhejiang University, Hangzhou, China; [2]Molecular Hepatology – Alcohol Associated Diseases, II. Medical Clinic Faculty of Medicine at Mannheim, University of Heidelberg, Mannheim, Germany; [3]Department of Gastroenterology, The First Affiliated Hospital, Medical School, Zhejiang University, Hangzhou, China; [4]Department of Medicine II, Saarland University Hospital, Saarland University, Homburg, Germany; [5]Medical Research Center, Medical Faculty Mannheim, University of Heidelberg, Germany; [6]General, Visceral Surgery and Transplantation, University Hospital Tübingen, Germany; [7]Departments of Gastroenterology and Hepatology, Kansai Medical University, Osaka, Japan

Introduction: TGF-β plays a dual role in the progression of human cancer. During early stages of cancinogenesis, TGF-β functions as a tumor suppressor. During late stages of tumor develpment, however, TGF-β can promote tumor growth and metastasis. A shift in Smad2/3 phosphorylation from the carboxy terminus to linker sites is a key event determining biological function of TGF-β in colorectal and hepatocellular carcinoma. In the present study, we investigated the potential role of differential Smad2/3 phosphorylation in gastric adenocarcinoma.

Methods: Immunohistochemical staining with anti-P-Smad2/3C and p-Smad2/3L was performed on 130 paraffin-embedded gastric adenocarcinoma specimens. The relationship was analyzed between P-Smad2/3C and P-Smad2/3L immuno-histochemical score and clinicopathologic characteristics of patients. Real time PCR was used to measure mRNA expression of Smad2 and Smad3 in cancer and surrounding non-tumor tissue.

Results: No significant P-Smad2L and/or P-Smad3L positive staining was detected in the majority of specimens (positive staining in 18/130 samples). Positive P-Smad2/3L staining was not associated with loss or decrease in carboxyterminal staining. Loss of P-Smad2C remarkably correlated with depth of tumor infiltration and differentiation of cancer cells in patients with gastric cancer. No correlation was detectable between P-Smad3C and clinicopathologic characteristics of gastric adenocarcinoma. However, co-staining analysis revealed that P-Smad3C co-localised with α-SMA and collagen I in gastric cancer cells, indicating a potential link between P-Smad3C and epithelial-to-mesenchymal transition of cancer. Real time PCR demonstrated reduced mRNA expression of Smad2 in gastric cancer when compared with surrounding non-tumor tissue in 15/16 patients.

Discussion/Conclusion: Loss of P-Smad2 tightly correlated with cancer invasion and poor differentiation in gastric cancer. Contrary to colorectal and hepatocellular carcinoma, canonical C-terminal, but not linker, phosphorylation of Smad2 is critical for gastric cancer.

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Chemical modification epigenetically 'renews' old human adipose-derived mesenchymal stem cells and improves their differentiation into hepatocytes lineage

X. Yan[1,3], S. Ehnert[1], J.J. Martinez Sanchez[1], U. Stöckle[1], P.A. De Sousa[2], A.K. Nüssler[1,3]
[1]BG Trauma Hospital Tübingen, Eberhard Karls University Tübingen, Tübingen, Germany [2]Scottish Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, United Kingdom
[3]Departement of Traumatology, Klinikum rechts der Isar, TU Munich, Germany

Introduction: Adipose derived mesenchymal cell (Ad-MSCs) is a promising source for autologous cell therapy. We previously found an age-related loss of differentiation capacity, which had a link to the epigenetic and pluripotency status. The aim of the present study was to find out whether old Ad-MSCs could be modified epigenetically by 5-azacytidine (AZA) or BIX 01294 (BIX) to improve their differentiation capacity into hepatocyte like cells.

Methods: Ad-MSCs were isolated from abdominal adipose tissue of young (≤ 45 yrs) and old (> 45 yrs) donors. Each group was pre-treated with AZA (5, 20 µM) and BIX (0.1, 0.2 µM) over 24 h and 48 h, then analysed for genomic distribution of 5-methylcytosine and 5-hydroxymethylcytosine and expression of pluripotency genes (Oct4, Nanog, Sox2, c-Myc) and TET oncogenes (TET1, 2, 3) responsible for hydroxymethylation. To verify differentiation potential into hepatic lineage, assessment of CYP activity, urea and glucose production were tested.

Results: Both AZA and BIX treatment significantly decreased the global DNA methylation of Ad-MSCs, most prominent in old donors (AZA decrease by 30%, BIX by over 60%). At 48 h treatment of AZA and BIX led to an increase of TET1 expression and a higher 5-hmC level in Ad-MSCs, which associated with expression changes of pluripotency genes: BIX treatment led to a significant elevated Nanog, c-Myc and Oct4 expression in “old” Ad-MSCs. After differentiated into hepatocyte-like cells, AZA or BIX pre-treated ”old” Ad-MSCs demonstrated an elevated urea and glucose metabolic capacity as well as Phase I/II enzymatic activity. In some cases AZA/BIX-treated cells even reached similar CYP450 activities and urea levels as seen in primary human hepatocytes.

Conclusion: AZA and BIX treatment seems to be a promising approach to modify human Ad-MSCs from old donors up to the level of cells from young donors. This approach would be safer than iPSC technology also has less risk of tumorogenesis.

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The PSC susceptibility variant in the *MST1* locus increases genetic cancer risk in European cholangiocarcinoma cohort

Vincent Zimmer[1], Marcin Krawczyk[1], Aksana Höblinger[2], Florentina Mihalache[3], Monica Acalovschi[3], Frank Lammert[1]
[1]Department of Medicine II, Saarland University Hospital, Homburg, Germany
[2]Department of Medicine I, University Hospital Bonn, Bonn, Germany
[3]Department of Medicine III, University Iuliu Hatieganu, Cluj-Napoca, Romania

Introduction: Lately, the single nucleotide variant (SNV) rs3197999 in the potent tumor suppressor *MST1* gene[1], has been linked with PSC[2]. Here we test this *MST1* polymorphism as genetic determinant of cholangiocarcinoma (CCA).

Methods: We genotyped 226 CCA patients (126 males, age 28–90 years; PSC < 5%) from Germany (n = 170) and Romania (n = 56). The control group consisted of 359 CCA-free individuals (160 males, age 22–90 years). The *MST1* SNV rs3197999 was genotyped using a PCR-based assay with 5'-nuclease and fluorescence detection.

Results: We detected a deviation from the Hardy-Weinberg-equilibrium in cases ($p = 0.04$), but not in controls ($p > 0.05$), and a trend for a higher frequency of the [G] allele in cases (31% vs. 26% $p = 0.09$). Carriers of the [GG] genotype were at higher CCA risk as compared to [AA] individuals (OR = 1.95, 95% CI: 1.08–3.52, $p = 0.02$). In the whole cohort, gender (OR = 1.53, 95% CI: 1.10–2.14, $p = 0.12$) and age ($p = 0.03$) were associated with CCA development. In a multivariate model, including solely carriers of the genotypes [GG] and [AA], gender (OR = 1.59, 95% CI: 1.04–2.45, $p = 0.03$) and the [GG] genotype (OR = 1.95, 95% CI: 1.07–3.56, $p = 0.03$) proved to be risk factors for CCA.

Discussion/Conclusion: The presented data suggest that the PSC risk variant of the *MST1* gene contributes to the genetic risk of sporadic cholangiocarcinogenesis.

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