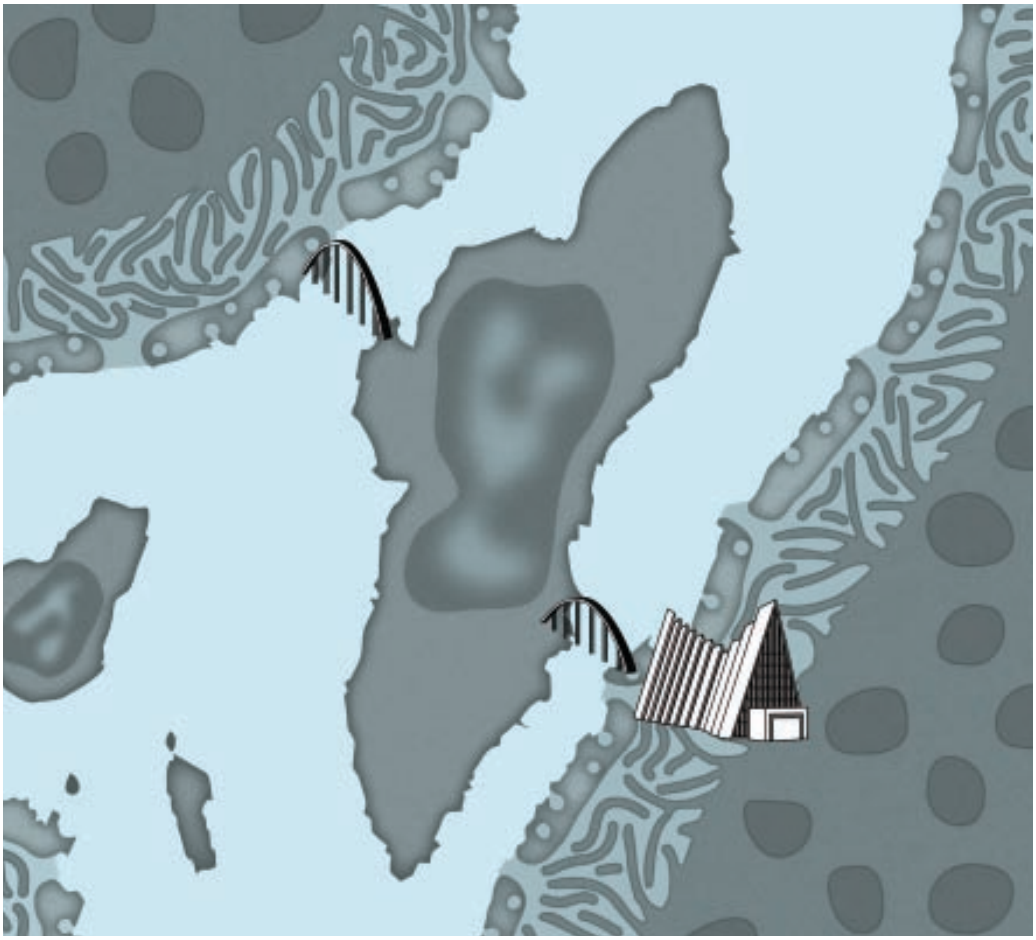
14th International Symposium on Cells of the Hepatic Sinusoid



Abstract Book

Tromsø, Norway, August 31 - September 4, 2008

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A welcome from the Chairman

Welcome to the 14th International Symposium on Cells of the Hepatic Sinusoid in Tromsø, Norway, Aug 31 - Sep 4, 2008! The International Scientific Committee has prepared for a scientific program that promises interesting presentations and discussions. I believe that the motto of the Symposium: "Integrating basic and clinical hepatology" will be clearly reflected throughout the scientific program. For the first time since these symposia were initiated in 1977, we will bring up a proposal of establishing a formalized society. The preparation towards transforming the series of symposia into a society has been taken care of by Prof. Hidekazu Tsukamoto on behalf of the International Scientific Committee. Please be present during the business meeting (Tuesday Sep 2, 12.20 - 13.00) when this issue will be discussed. The 15th ISCHS in Coronado, California, USA, Aug 29 - Sep 1, 2010, will also be presented during the business meeting.

The Local Organizing Committee has worked hard to establish optimal logistics and an interesting social program. The Committee realizes that it is hard to control weather, but the planning of the rest of the meeting events has been down to the detail.

I believe this Symposium will enable plentiful scientific and social communication that will spur ideas and collaborations within the topic of liver sinusoidal cells.

I also want to thank The Norwegian Research Council, The University of Tromsø, The City Government of Tromsø, and Olympus Norway for their generous sponsoring of this symposium.

Open up, participate in discussions, meet new colleagues, and experience Tromsø!

Bård Smedsrød
Chairman of the 14th International Symposium on Cells of the Hepatic Sinusoid

PROGRAM

SUNDAY August 31, 2008

15:00 Registration at Rica Ishavs Hotel

19:30 Welcoming remarks: Tromsø City Hall

19:45 Welcome reception in the Tromsø City Hall

MONDAY September 1, 2008

			Abstract
08:30	***Bård Smedsrød***	Welcome	
08:45	***Eddie Wisse**:*	Differences in sinusoids and sinusoidal cells in rat, mouse, rabbit, pig and human liver (key note)	#1

Session I Liver sinusoidal endothelial cells: Endocytosis

Chair Eddie Wisse and Bård Smedsrød

09:30	***Peter McCourt***	Liver sinusoidal endothelial cells - mega-endocytes (tutorial)	#2
10:00	***Sergij Goerdt***	Molecular biology of hepatic sinusoidal endothelium (tutorial)	#3

10:30 Coffee break

Session II Liver sinusoidal endothelial cells: Endocytosis and comparative biology

Chair Masaya Oda and Clark Anderson

11:00	***Clark Anderson***	FcγRIIb2 expression in endothelium of liver and placenta (tutorial)	#4
11:30	***Trond Berg***	Endocytosis and recycling of immune complexes mediated by the FcgammaRIIb2 in rat liver sinusoidal endothelial cells	#5
11:50	***Clive Crossley***	Invertebrate analogues of hepatic sinusoidal scavenger systems (tutorial)	#6
12:20	***Montserrat Martin-Armas***	Uptake of oligonucleotides by scavenger endothelial cells in the Atlantic cod (*Gadus morhua, L.*)	#7
12:40	***Vijay K. Kalra***	Hypoxia inducible factor (HIF-1α) and related miRNAs in the regulation of ethanol induced inflammatory genes in liver sinusoidal endothelial cells	#8
13:00	***Iñigo Martinez***	The influence of oxygen tension on the structure and function of isolated liver sinusoidal endothelial cells	#9

13:15	***Jaione Simon-Santamaria***	Age-related changes in scavenger-receptor mediated endocytosis in rat liver sinusoidal endothelial cells.	#10

13:30 Lunch at Rica Ishavs Hotel

Session III Liver sinusoidal endothelial cells: Cytoskeleton

Chair Robert McCuskey and David LeCouteur

14:30	***David LeCouteur***	Old age and the hepatic sinusoid (tutorial)	#11
15:00	***Robert McCuskey***	Age-related dysfunction in the hepatic microcirculation	#12
15:20	***Robin Fraser***	Atherosclerosis, lipoproteins and the liver sieve	#13
15:40	***Masaya Oda***	Different expressions of Aquapoint-1 in normal and cirrhotic human liver – Demonstrating the capillarization of hepatic sinusoids	#14

16:00 Coffee break

Session IV Liver sinusoidal endothelial cells: Cytoskeleton continued

16:30	***Frederic Saltel***	Implication of cortactin, a regulator of actin cytoskeleton assembly, in the control of fenestration in liver sinusoidal endothelial cells	#15
16:50	***Katsuhiko Enomoto***	Involvement of signaling of VEGF and TGF-β in differentiation of sinusoidal endothelial cells during culture of fetal rat liver cells	#16
17:10	***Beatriz Arteta***	Integrin LFA-1 enables colon carcinoma cells to upregulate sinusoidal endothelial cell mannose receptor via interleukin-1 dependent mechanism: implications on anti-tumor immunity inhibition during hepatic metastasis	#17

Free evening

TUESDAY September 2, 2008

Session V Kupffer cells

Chair Hartmut Jaeschke and Laura Nagy

09:00	***Laura Nagy***	Innate immune responses and alcoholic liver disease: Role of Kupffer cells (tutorial)	#18
09:30	***Takashi Yamamoto***	Kinetics and replacement of Kupffer cells after bone marrow transplantation in mice and rats	#19
09:50	***Nicolas Lanthier***	High fat feeding induces hepatic insulin resistance and preferential distribution of Kupffer cells around lipid loaded hepatocytes	#20
10:10	***Joan Clária***	Arachinodate 5-lipoxygenase modulates hepatocyte fatty acid uptake and microsomal triglyceride transfer protein activity	#21

10:30 Coffee break

Session V Kupffer cells continued

11:00	***Daniela Gärtner***	Cross talk between PGE2- and IL-6-mediated signaling pathways in cultured rat Kupffer cells	#22
11:20	***Hartmut Jaeschke***	The inflammatory response during drug-induced liver injury: Toxicity versus regeneration	#23
11:40	***Maria K. Dahle***	Cecal ligation and puncture sepsis is associated with a shift in adenylyl cyclase expression in sinusoidal liver cells	#24

12:00 – 13:00 Business meeting

13:00 – 14:00 Lunch at Rica Ishavs Hotel

18:00-23:30: Excursion: Boat to Sommarøya, and dinner there

WEDNESDAY September 3, 2008

Session VI Stellate cells: Signaling and gene regulation

Chair Kenjiro Wake and Jelena Mann

09:00	***Norifumi Kawada***	Splanchnic vitamin A-storing cell system and organ fibrogenesis (tutorial)	#25
09:30	***Erica Novo***	Modulation of human hepatic stellate cells chemotaxis by intracellular ROS generation and c-Jun N-terminal kinase activation	#26
09:50	***Gunter Maubach***	Cysteine cathepsins in hepatic stellate cells	#27
10:10	***Hidekazu Tsukamoto***	Canonical Wnt signaling and hepatic stellate cells	#28
10:30	***Ilse Bockx***	Proliferation of hepatic stellate cells in rats with acute hepatitis is independent on vagal innervation via muscarinic acetylcholine receptor subtype 2	#29

10:50 Coffee break

Session VII Stellate cells: Signaling and gene regulation continued

Chair Haruki Senoo and Hidekazu Tsukamoto

11:20	***Jelena Mann***	Epigenetic regulation of hepatic stellate cells (tutorial)	#30
11:50	***Yoshihiro Mezaki***	Rat hepatic stellate cells acquire retinoid responsiveness after activation in vitro	#31
12:10	***Kiwamu Yoshikawa***	Involvement of ADRP and TIP47 in lipid droplets formation of hepatic stellate cells	#32
12:30	***Richard George Ruddell***	Hepatic stellate cells express Tim-2: A possible mediator of ferritin-induced NfκB activation and proinflammatory gene expression	#33
12:50	***Keisuke Nagatsuma***	Cellular distribution of lecithin: retinol acyltransferase (LRAT) and cellular retinol binding protein-1 (CRBP-1) in human normal and pathological livers	#34

13:30 – 14:30 Lunch at Rica Ishavs Hotel

14:30 –15:30	**Poster session**

Session VIII Stellate cells: Modulators of liver fibrosis

Chair Trond Berg and Kenichi Ikejima

15:30	***Michele Pritchard***	Expression of the transcription factor, early growth response (Egr)-1, is a critical regulator of carbontetrachloride-induced fibrosis in mice	#35
15:50	***Noriko Yamaguchi***	Vitamin E suppressed the proliferation and induced apoptosis of ”activated” hepatic stellate cells	#36
16:10	***Peter Witters***	Blood platelets in cholestatic liver disease	#37
16:30	***Tomohiro Ogawa***	Study on a rabbit model of steatohepatitis that develops liver cirrhosis	#38
16:50	***Kenichi Ikejima***	C-reactive protein minimizes experimental hepatic fibrosis by inhibiting transactivation of hepatic stellate cells	#39
17:10	***Soichi Kojima***	Detection and prevention of hepatic fibrosis targeting TGF-β activation reaction	#40

20:00 Conference dinner at Rica Ishavs Hotel

THURSDAY September 4, 2008

Session IX Stellate cells: comparative morphology and methods

Chair Norifumi Kawada and Soichi Kojima

09:00	***Haruki Senoo***	Extracellular matrix in sinusoidal wall of frozen mammoths	#41
09:20	***Mitsutaka Miura***	A study of morphological changes in lamprey liver during the course of metamorphosis with special reference to hepatic stellate cells	#42
09:40	***Anna Nakamura***	Cryopreservation for long-tem storage of human primary cultured hepatic stellate cells	#43

10:00 Coffee break

Session X Immunology/Tumor Biology

Chair Makoto Naito and Fernando Vidal-Vanaclocha

10:30	***Percy Knolle***	Immunobiology of liver sinusoidal endothelial cells (tutorial)	#44
11:00	***Fernando Vidal-Vanaclocha***	The prometastatic environment of the liver (tutorial)	#45
11:30	***Aritz Lopategi***	Nerve growth factor expression by hepatic parenchymal and non-parenchymal cells during metastatic colorectal development in human and murine liver	#46
11:50	***Tatyana Korolenko***	Tumor-associated macrophages in experimental murine tumors	#47

12:10 Closing remarks by Bård Smedsrød

Abstracts: oral presentations

DIFFERENCES IN SINUSOIDS AND SINUSOIDAL CELLS IN RAT, MOUSE, RABBIT, PIG AND HUMAN LIVER

E Wisse[1, 3], G Koek[1], F Braet[3], B De Geest[2], and P Frederik[1]

Departments of Pathology (EM unit), Surgery and Internal Medicine, University of Maastricht[1], Center for Molecular and Vascular Biology, University of Leuven[2] and Australian Key Centre for Microscopy and Microanalysis, University of Sydney, Australia[3].

In order to study the morphology of hepatic sinusoidal cells, it is obligatory to use the better resolution and higher magnification of electron microscopes (TEM and SEM). As a result, it is necessary to apply perfusion-fixation to preserve fine cellular detail. With this approach, it has been possible to distinguish four different types of sinusoidal cells and study their function, topography, interaction and behaviour in experiments and disease. Structural and functional differences between endothelial cells and Kupffer cells were not well distinguished in textbooks and publications written before the year 1970. EM observations made clear that the sinusoid is a microenvironment special to the hepatic lobule, responsible for a specific segment of liver function. Endothelial endocytosis and filtration by fenestrae; endotoxin clearance, phagocytosis, and secretion of biologically active products by Kupffer cells; fat-storing cell storage of vitamin A and collagen synthesis, and the killing of invading tumor cells by pit cells (NK cells) were described and confirmed in numerous experiments, mostly performed in rats. Some of the functions found in situ, were extended with in vitro observations, obtained after isolation, purification and culture of the different types of sinusoidal cells. In as much as the in vitro results posed the question whether they equalled in vivo conditions, the question was also asked whether observations in the rat liver are relevant for other species, including the healthy and diseased human liver. Indeed, the histology and fine structure of many organs, tissues and cells does not differ much when different species, including Homo sapiens, are compared. This appeared also true for a comparative EM study of well-fixed livers of the animal species mentioned in the title of this abstract. Sinusoids and their cells showed remarkable resemblance.

In the present survey, we will show details of sinusoids and sinusoidal cells, for which whole livers or wedge biopsies (appr. 1 x 1 x 1 cm) were fixed by perfusion- or injection-fixation seconds after laparotomy. In the case of wedge biopsies, glutaraldehyde was injected from different sides into the tissue. Discoloration and hardening of the tissue indicated well-perfused and well-fixed areas. After postfixation in osmium, dehydration in ethanol series and embedding in Epon, these tissues showed in many cases an almost perfect quality of fixation. Differences in the structure of the sinusoids and their cells in rats, mice, rabbits, pigs and human livers are subtle and only concern details, suggesting that differences in function could also be assumed minimal.

Rat fenestrae in ultrathin plastic sections in TEM have an average size of 147 ± 0.9 nm in centrolobular areas, whereas periportal fenestrae measured 175 ± 1.0 nm. Fenestrae in mice (C57BL/6) have an average diameter of 141 ± 5.4 nm, whereas three rabbit strains showed different size distributions of fenestrae: NZW rabbits 103 ± 1.3 nm, DB rabbits measured 124 ± 3.4 nm, whereas FdB rabbits measured 105 ± 2.6 nm. In both mice and rabbit strains, a large size of fenestrae correlated well with good transgene expression of hepatocytes after adenoviral gene transfer, small fenestrae showed the opposite effect. Adenoviral capsules measure 93 ± 0.29 nm with protruding, flexible fibers of 30 nm. In well-fixed human liver biopsies, taken from livers without specific liver pathology, the diameter of fenestrae was 107 ± 1.5 nm, in a group of 12 patients undergoing liver surgery for colon cancer metastasis, the diameter of fenestrae was found to be 103 nm ± 0,15 nm, suggesting that adenoviral gene transfer vectors will be unsuccessful in the transfection of human liver parenchymal cells. This might explain the unsuccessful clinical attempts.

During this investigation a few other details of endothelial and other cells came to light and will be presented. These concern defenestration centers, saccular SER and coated pits near sieve plates, transendothelial channels and fenestrae labyrinths.

LIVER SINUSOIDAL ENDOTHELIAL CELLS - MEGA-ENDOCYTES

Peter McCourt
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Every day the body turns over enormous amounts of biological molecules vital for the maintenance of life. Liver sinusoidal endothelial cells (LSEC) play a pivotal role in this removal/recycling process, daily clearing gram amounts (i.e. > 20 times their own weight) of spent connective tissue molecules (e.g. hyaluronan and denatured collagen) and waste products (e.g. atherogenic lipids, coagulation products and damaged proteins) from the circulation, and recycling them to energy and new building blocks (1). LSEC are thus a central player in homeostasis and are clearly not a sedate endothelium with filter holes, simply connecting the portal and central veins in the mammalian liver lobule.

This endocytosis of such a wide range of different macromolecules is actually mediated by only a few categories of endocytosis receptor; namely the mannose receptor, the Fc-gamma receptor and the LSEC scavenger receptors stabilin-2 and its homologue stabilin-1 (1-4). Mannose and scavenger receptors are traditionally regarded as pattern recognition receptors (PRRs) in macrophages. Interestingly, this "endocytic profile" appears to be preserved in the LSEC analogues of all vertebrates (albeit in other organs in some non-mammals; (5)), and possibly in insects as well. Furthermore, LSEC are also exceptionally richly equipped with early and late endosomes and lysosomes (6) to degrade all the material internalised by this massive uptake. LSEC also use their massive endocytic capacity to recruit circulating lysosomal enzymes, via the mannose receptor, thus reducing their own need to devote resources to enzyme synthesis (7).

The aim of this tutorial is summarise the known endocytic pathways mediated by LSEC to maintain homeostasis, plus describe some preliminary studies indicating the wider role LSEC has in keeping our insides clean.

1. Smedsrod B. Clearance function of scavenger endothelial cells. Comp Hepatol 2004;3 Suppl 1:S22.
2. Malovic I, Sorensen KK, Elvevold KH, et al. The mannose receptor on murine liver sinusoidal endothelial cells is the main denatured collagen clearance receptor. Hepatology 2007;45:1454-1461.
3. McCourt PA, Smedsrod BH, Melkko J, Johansson S. Characterization of a hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship to scavenger receptors. Hepatology 1999;30:1276-1286.
4. Politz O, Gratchev A, McCourt PA, et al. Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. Biochem J 2002;362:155-164.
5. Seternes T, Sorensen K, Smedsrod B. Scavenger endothelial cells of vertebrates: a nonperipheral leukocyte system for high-capacity elimination of waste macromolecules. Proc Natl Acad Sci U S A 2002;99:7594-7597.
6. Wisse E. An ultrastructural characterization of the endothelial cell in the rat liver sinusoid under normal and various experimental conditions, as a contribution to the distinction between endothelial and Kupffer cells. J Ultrastruct Res 1972;38:528-562.
7. Elvevold, K., Simon-Santamaria, J., Hasvold, H., et al. Liver sinusoidal endothelial cells depend on mannose receptor mediated recruitment of lysosomal enzymes for normal degradation capacity. Accepted for publication in Hepatology, 2008

Abstract #3

MOLECULAR BIOLOGY OF HEPATIC SINUSOIDAL ENDOTHELIUM

Sergij Goerdt

FCγRIIB2 EXPRESSION IN ENDOTHELIUM OF LIVER AND PLACENTA

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Fc receptors for IgG (FcγR) are expressed in the endothelium (EC) of the body at only two sites, the liver sinusoids (LSEC) and the placental villus; and at both sites the FcγR is of the same special sort, namely, FcγRIIb2. Such expression presents three striking features. First, FcγRIIb2 is the only FcγR of many (8 FcγR genes in man; 4 in mouse) that transduces an inhibitory signal; the others are agonists. Is this compatible with the simple function of immune complex (IC) disposal or does it suggest a different activity? Second, FcγRIIb2 at these two sites is apparently unopposed by an interacting agonistic receptor as it is at all other sites of expression in the body. Have we missed finding an agonist receptor or does the receptor cluster with itself upon IC engagement? Third, the isoform expressed is the truncated 'b2' isoform found in macrophages rather than the longer 'b1' isoform found in B lymphocytes. Is this important? So far the function of FcγRIIb2 at both of these sites is unknown.
Despite the similarities in FcγRIIb2 expression, one must be wary of carrying too far the parallel between EC of both liver and placenta, for the EC at the two sites are distinctive in many respects.
Having analyzed in depth the expression and function of FcγRIIb2 in the placenta EC, we are extending these studies to the LSEC.

ENDOCYTOSIS AND RECYCLING OF IMMUNE COMPLEXES MEDIATED BY THE FcγRIIb2 IN RAT LIVER ENDOTHELIAL CELLS

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Introduction: Liver sinusoidal endothelial cells (LSECs) are equipped with receptors that mediate endocytosis of harmful molecules from blood. The receptors in question are, among others, scavenger receptors (ScR), mannose receptors and Fc-receptors. Earlier studies showed that an IC (DNP-BSA in complex with rabbit anti DNP-IgG) is removed almost mainly by the liver following intravenous injection in rats. Kupffer cells and LSECs are responsible for the uptake. The antigen (DNP-BSA) is a ligand for ScRs and was found to reach the lysosomes and degraded more rapidly than the IC[1].
Methods and Results: Four different classes of FcγRs have been identified in rodents (FcγRI-IV)[2]. RT-PCR and western blot analyses indicated that LSECs express only the FcγRIIb. As expected, a monoclonal antibody against FcγRIIb prevented binding of ^{125}I-ICs completely. Two splice variants exist for FcγRIIb[3] (FcγRIIb1 and FcγRIIb2); only FcγRIIb2 is able to mediate endocytosis of ICs. Using a deglycosylation analysis of FcγRIIb isoforms revealed that only the FcγRIIb2 is expressed in LSECs. By measuring binding of a monoclonal antibody against rat FcγRIIb to LSECs it was found that each cell expressed about 500,000 receptors. The cells internalise both ICs and antigens effectively, but the antigens (e.g. DNP-BSA) are degraded much more rapidly than the ICs, compatible with the *in vivo* data. IC was also found to be internalized at a slower rate than the Ag. Two reasons for these differences are suggested: First, the ICs seemed to be associated with detergent-resistant membranes on cross-linking before internalization via clathrin-coated pits, secondly the ICs were found to be recycled to the plasma membrane to a very large extent. Both these incidents will of course delay receptor-ligand transport to later endocytic compartments.
Discussion and Conclusion: The present data shows that the ICs are degraded, albeit at a lower rate than the Ag. What happens to the receptor? It was found that during four hours the cells internalise ICs corresponding to about 5 pools of FcγRIIb in the cells. Moreover, western blot analysis performed before and after incubation of the cells in presence of saturating concentrations of ICs over night did not reduce receptor concentration. These results are compatible with the finding that cross-linking of FcγRIIb does not lead to tyrosine phosphorylation. In conclusion, the FcγRIIb2 in LSECs effectively removes ICs from the circulation. It is perpetually available to mediate endocytosis of IC, as it is not downregulated. Maybe the FcγRIIb2 in LSECs, similar to its role in dendritic cells, is able to present antigen to B cells.
References: (1) Johansson AG, Løvdal T, Magnusson KE, Berg T and Skogh T: Hepatology 1996; 24:169-175. (2) Nimmerjahn F and Ravetch JV: Advances in Immunology 2007; 96:179-204. (3) Miettinen HM, Rose JK and Mellman I: Cell 1989; 58:317-327.

INVERTEBRATE ANALOGUES OF HEPATIC SINUSOIDAL SCAVENGER SYSTEMS

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Cells specialised for receptor-mediated endocytosis comprise an important part of invertebrate soluble waste management and innate immune systems. These cells can be visualised using the same vital dyes used in classical methods for identifying cells of the vertebrate RES, such as lithium carmine. Ultrastructural, molecular and genetic analyses reveal many analogies between invertebrate and vertebrate scavenger systems such as those of the hepatic sinusoid, including distinct processing systems for insoluble and soluble materials. In insects the pinocytic scavenger cells are termed nephrocytes, and are distinct from circulating and fixed phagocytic macrophages. Nephrocytes are central to homeostasis of the blood by recycling foreign, disfunctional or surplus proteins and metals. They are also important in innate immunity, responsible for bacteriolysis and uptake of viruses. Nephrocytes can be maintained *in vitro* allowing analysis of endocytic processes, independently of macrophage phagocytosis, and their large size facilitates microscopy. Nephrocytes show infolding of the plasma membrane, which increases the surface available for coated vesicle endocytosis, and have an extraordinarily active endocytic system. Receptor-mediated endocytosis is coupled to a rapid ligand-detachment and membrane-recycling system of tubular elements, and an active lysosomal degradation system. Unlike hepatic sinusoidal endothelial cells, nephrocytes are sheathed in a basal lamina, and a have podocyte, rather than a fenestrated, configuration. The podocyte structure allows filtration of lymph before it reaches vesicular receptors, and resembles the primary urine filter seen in the glomerular cells of the mammalian kidney. Insect nephrocytes have scavenger receptors for uptake of formaldehyde treated albumen, collagen, and hyaluronan, but no mannose receptor. The insect receptors appear not to be homologous with vertebrate scavenger endothelial cell receptors. However, as in scavenger endothelial cells, energy derived from endocytic catabolism is returned to the blood as lactate. Genetic analysis of *Drosophila* has revealed the sequence of genes controlling differentiation of heart, nephrocytes, and haemopoietic tissue from oligopotent haemangioblasts, and a similar sequence may be conserved in vertebrates.

UPTAKE OF OLIGONUCLEOTIDES BY SCAVENGER ENDOTHELIAL CELLS IN THE ATLANTIC COD (*Gadus morhua, L.*)

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Introduction*:* The term 'scavenger endothelial cells' (SECs) was introduced by Smedsrød *et al.* in 1999. It describes a specialized type of endothelium that is highly and uniquely active in endocytosis of an array of waste macromolecules and colloids. In land-based vertebrates (mammals, birds, reptiles and amphibians) SECs make up the liver sinusoidal endothelial cells (LSECs). In bony fish SECs are located in heart (endocardium) or kidney (kidney sinusoids), but not in liver. In the Atlantic cod, SECs line the inner aspects of the chambers of the heart. Studies in our laboratory have shown that Atlantic cod SECs share the high endocytic capacity and specificity that characterize their mammalian counterpart. These cells eliminate a broad spectrum of own and foreign waste material, including LPS.
The elimination of blood borne DNA fragments by mammalian LSECs has been previously shown by others. Fragments of DNA, especially bacterial DNA may act as immunomodulators. The immune stimulatory effects of bacterial DNA are a consequence of the presence of unmethylated CpG dinucleotides in particular base contexts, termed CpG motifs. In the present study we describe the elimination of DNA oligonucleotides from the circulation by SECs in the Atlantic cod.
Methods*:* Radiolabelled and FITC-CpG-oligonucleotides were injected intravenously into Atlantic cod. The radioactivity was measured and organ sections were studied to elucidate the site(s) of accumulation after different time points. Co-injection of radiolabelled oligonucleotides with excess amounts of formaldehyded-serum albumin (FSA, a much used test ligand to study scavenger-mediated endocytosis) was performed to determine the possible uptake of oligonucleotides via the scavenger receptor.
Primary cultures of heart SECs were prepared to investigate the route of cellular uptake of the oligonucleotides. We also tested the effect that pre-incubation with CpG-oligonucleotides, has on the endocytosis via the mannose- and scavenger-receptors by checking *in vitro* uptake of radiolabelled FSA, mannan and α-chains of collagen.
Results and conclusion: The results suggest that intravenously injected radiolabelled FITC-oligonucleotides accumulate mainly in the heart of Atlantic cod. Intravenously injected FITC-oligonucleotides were found concentrated in vesicles of heart SECs. Moreover, studies using in vitro cultures of cod SECs revealed efficient uptake of FITC-oligonucleotides after incubation for 1h. Injection of surplus amounts of FSA along with radiolabelled oligonucleotides diverted the accumulation of radioactivity from heart to liver and intestines. This indicates that uptake of these oligonucleotides in heart is scavenger receptor mediated. Additionally, *in vitro* studies showed that incubation of oligonucleotides in the presence of excess amounts of scavenger receptor ligands, such as FSA and hyaluronan, inhibits the uptake by Atlantic cod SECs. These observations strengthen the notion that endocytosis of CpG in cod SECs is a scavenger receptor mediated process.
Preincubation for 24 h of SECs with CpG-oligonucleotides (5 and 20 μg/ml) selectively down-regulated the scavenger receptor mediated endocytosis in SECs, while only marginal effects were noted on endocytosis via the mannose receptor. In conclusion, Atlantic cod SECs are the main elimination site of blood borne oligonucleotides and this elimination occurs via the scavenger receptor. Moreover, SECs cultured in the presence of CpG-oligonucleotides exhibit a profoundly reduced scavenger receptor mediated endocytic capacity.

HYPOXIA INDUCIBLE FACTOR (HIF-1α) AND RELATED miRNAs IN THE REGULATION OF ETHANOL INDUCED INFLAMMATORY GENES IN LIVER SINUSOIDAL ENDOTHELIAL CELLS (LSECs)

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Chronic ethanol consumption leads to liver inflammation and injury. Studies have shown that high blood alcohol levels are accompanied by hypoxia and activation of HIF-1α in the liver. To delineate the role of HIF-1α in regulating inflammatory genes in LSECs, rats were fed ethanol (9-15 gm/kg/day) by intragastric feeding for 9 weeks. Sinusoidal endothelial cells were isolated from the liver, which were >95% pure as assessed by LSEC markers. RNA was isolated from LSECs derived from control (C-LSEC) and ethanol fed rats (E-LSEC) followed by qRT-PCR. We show that the mRNA expression of chemokine genes (MCP-1, RANTES and MIP-2), vasoconstrictor molecule (ET-1) and HIF-1α increased 2-4-fold in E-LSEC vs. C-LSEC. Cultured rat LSECs, (which do not express fenestrae,) that were treated with ethanol (50-100mm) showed upregulation in the expression of the same genes as well as HIF-1α, as was observed in E-LSEC. Next, we examined whether the effect of ethanol was similar or different in endothelial cells derived from other vascular beds. Treatment of human dermal microvascular endothelial cells (HDMVECs) or cell line with ethanol resulted in increased mRNA expression of MCP-1, RANTES, MIP, ET-1 and HIF-1α. Thus, we used HDMVECs for ease of transfection and for mechanistic cell signalling studies. We examined whether ET-1 expression was regulated by HIF-1α, as hypoxia is known to regulate the expression of ET-1 via HIF-1α. We observed that ethanol caused a time-dependent increase in the expression of HIF-1α protein. Transfection of HDMVECs with wt-ET-1-luc plasmid (-669 bp) or truncated ET-1 promoter-luc plasmid (-176 bp) showed several fold increase in ET-1 promoter activity, which was attenuated when the hypoxia response element was mutated in the ET-1 promoter. Furthermore, silencing of HIF-1α protein with siRNA in HDMVECs attenuated ethanol-mediated ET-1 expression along with other genes. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis support the role of HIF-1α in the ethanol mediated transcription of ET-1 in response to ethanol.

Since ethanol increased the HIF-1α mRNA and protein levels in LSECs, we hypothesized that HIF-1α related microRNAs (miRNAs) may play a role in HIF-1α mRNA expression and concomitantly HIF-1α regulated genes. We isolated miRNA from E-LSEC and C-LSEC. Several putative HIF-1α miRNAs were identified including miR-20, miR-155, miR-199, miR-17-3p and miR-203. The expression levels of miR-155, miR-199 and miR-17-3p, as determined by qRT-PCR, were significantly reduced while the expression of miR-20 increased significantly in E-LSEC as compared to C-LSEC. Data will be presented on the cell signalling pathways for the expression of specific HIF-1α related miRNAs and their functions. These studies show for the first time that ethanol activates ET-1 via HIF-1α, independent of hypoxia. The ethanol mediated release of ET-1 may activate HSCs and exaggerate vasoconstriction and hepatic blood flow, and inflammation in the liver. *Supported by NIH-P50-AA11999, R24-AA12885 and training grant (T32-AA07578) fellowship to SY.*

THE INFLUENCE OF OXYGEN TENSION ON THE STRUCTURE AND FUNCTION OF ISOLATED LIVER SINUSOIDAL ENDOTHELIAL CELLS

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Background: Liver sinusoidal endothelial cells (LSECs) are specialized scavenger cells, with crucial roles in maintaining hepatic and systemic homeostasis. Under normal physiological conditions, the oxygen tension encountered in the hepatic sinusoids is in general considerably lower than the oxygen tension in the air; therefore cultivation of freshly isolated LSECs under more physiologic conditions with regard to oxygen would expect to improve cell survival, structure and function. In this study LSECs were isolated from rats and cultured under either 5% (normoxic) or 20% (hyperoxic) oxygen tensions, and several morpho-functional features were compared.

Results: Cultivation of LSECs under normoxia, as opposed to hyperoxia improved the survival of LSECs and scavenger receptor-mediated endocytic activity, reduced the production of the pro-inflammatory mediator, interleukin-6 and increased the production of the anti-inflammatory cytokine, interleukin-10. On the other hand, fenestration, a characteristic feature of LSECs disappeared gradually at the same rate regardless of the oxygen tension. Expression of the cell-adhesion molecule, ICAM-1 at the cell surface was slightly more elevated in cells maintained at hyperoxia. Under normoxia, endogenous generation of hydrogen peroxide was drastically reduced whereas the production of nitric oxide was unaltered. Culture decline in high oxygen-treated cultures was abrogated by administration of catalase, indicating that the toxic effects observed in high oxygen environments is largely caused by endogenous production of hydrogen peroxide.

Conclusions: Viability, structure and many of the essential functional characteristics of isolated LSECs are clearly better preserved when the cultures are maintained under more physiologic oxygen levels. Endogenous production of hydrogen peroxide is to a large extent responsible for the toxic effects observed in high oxygen environments.

AGE-RELATED CHANGES IN SCAVENGER-RECEPTOR MEDIATED ENDOCYTOSIS IN RAT LIVER SINUSOIDAL ENDOTHELIAL CELLS

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In spite of the generally accepted notion that liver sinusoidal endothelial cells (LSECs) play a pivotal role in the clearance of waste substances from the circulation via different types of scavenger receptors, very little is known about the effect of ageing on the activity of these receptors. Recently, distinct age-related morphological changes have been described in the liver sinusoids of human, rat, mouse and baboons (1-5). These changes have been named pseudocapillarization because they resemble those occurring in cirrhosis, including loss of fenestrae, thickening of the endothelium, and formation of basal lamina and collagen deposits in the Space of Disse. The aim of this study was to compare scavenger-receptor-mediated endocytosis in LSECs from young and old rats and determine if it is associated to the morphological changes seen in pseudocapillarization.

Hybrid F344/BN F1 rats, aged 4-8 months and 26-30 months of age, were chosen as animal model to avoid the effect of inbreeding and homozygocity on ageing. Freshly isolated LSECs in monolayer cultures were incubated for 2 hours at 37°C under serum free conditions with different concentrations of radiolabelled formaldehyde-treated serum albumin (FSA), a ligand for the LSEC scavenger receptors. All experiments commenced 2 hours after seeding, and the average cell density was equal in cultures from young and old rats. Parallel cultures of LSEC were fixed at similar time points for Scanning Electron Microscopy (SEM) analysis of culture quality and cell fenestration (using the Image-J software). Liver tissues from old and young rats were also subjected to light and transmission electron microscopy (TEM) for analysis of liver sinusoidal morphology in this rat strain.

Main results and conclusions: 1) With increasing concentrations of FSA there was a significant reduced endocytic capacity in LSECs from old rats compared to in cells from young individuals. 2) Morphometrical analysis of SEM images showed a marked porosity reduction in LSEC from old animals compared to young animals. 3) A significant correlation between LSEC porosity and endocytosis was not found, suggesting that the reduced porosity seen with ageing is not linked to the observed reduction in endocytosis.

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OLD AGE AND THE HEPATIC SINUSOID

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Morphological changes in the hepatic sinusoid with old age are increasingly recognized. These include thickening and defenestration of the liver sinusoidal endothelial cell, sporadic deposition of collagen and basal lamina in the extracellular space of Disse and increased numbers of fat engorged, non-activated stellate cells. In addition, there is endothelial upregulation of von Willebrands factor and ICAM-1 with reduced expression of caveolin-1. These changes have been termed age-related pseudocapillarization. The effects of old age on Kupffer cells are inconsistent but impaired responsiveness is likely. There are functional implications of these aging changes in the hepatic sinusoid. There is reduced sinusoidal perfusion, which will impair the hepatic clearance of highly extracted substrates. Blood clearance of a variety of waste macromolecules takes place in LSEC. Previous studies indicated either that aging had no effect, or reduced the endocytic capacity of LSEC. However, a recent study in mice showed reduced endocytosis in pericentral regions of the liver lobules. Reduced endocytosis may increase systemic exposure to potential harmful waste macromolecules such as advanced glycation end products Loss of fenestrations leads to impaired transfer of lipoproteins from blood to hepatocytes. This provides a mechanism for impaired chylomicron remnant clearance and post-prandial hyperlipidemia associated with old age. Given the extensive range of substrates metabolized by the liver, age-related changes in the hepatic sinusoid and microcirculation have important systemic implications for aging and age-related diseases.

AGE-RELATED DYSFUNCTION IN THE HEPATIC MICROCIRCULATION

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Ageing of the liver is associated with an impaired metabolism of drugs and potential adverse drug interactions and susceptibility to toxins. Reduced hepatic blood flow is suspected to be the cause. As a result, we examined age-related alterations in hepatic microcirculation and their causes. Livers of C57Bl/6 mice were examined at 0.8 (pre-pubertal), 3 (young), 14 (mature) and 27 (senescent) months of age using *in vivo* and electron microscopic methods. The results demonstrated a 14% reduction in the numbers of perfused sinusoids between 0.8 and 27 month mice associated with 35% reduction in sinusoidal blood flow measured by a Laser Doppler flowmeter. This was accompanied by a hepatic microvascular inflammatory response evidenced by a 5-fold increase in leukocyte adhesion in 27 month mice, up-regulated expression of ICAM-1, and increases in intrahepatic macrophages. Sinusoidal diameter decreased 6-10%. Evidence of sinusoidal endothelial cell (LSEC) dysfunction was seen as early as 14 months when there was a 3-fold increase in the numbers of swollen LSEC. The endocytosis of the FITC-FSA, a ligand for the scavenger receptor on LSEC was suppressed at 14 months and further reduced at 27 months. This could be mimicked in young animals by injecting advanced glycosylated end-products (AGEs). The sinusoidal endothelium in 27 month old mice exhibited early capillarization with reduced fenestration together with deposition of basal laminae and collagen in the Space of Disse. In conclusion, the results suggest that leukocyte accumulation in the sinusoids and narrowing of sinusoidal lumens reduce sinusoidal blood flow in aged livers due to early capillarization and dysfunction of SEC perhaps resulting from increased circulating AGEs during aging. This study was supported by the National Institute Health/ National Institute of Aging, grant No. R21 AG-02582, and the Norwegian Research Council, grant No. 153483/V50.

ATHEROSCLEROSIS, LIPOPROTEINS AND THE LIVER SIEVE

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Atherosclerosis, localized thickenings or plaques of the intima of arteries from cholesterol deposition and cellular hyperplasia causing thrombosis and obstruction, is a major killer due to ischaemic heart failure, strokes, gangrenous feet and aortic aneurysm. In 1976 in Tokyo, at the third International Symposium on Atherosclerosis, we first presented data confirming that the fenestrated liver sinusoidal endothelial cells (LSEC) filtered chylomicrons and their remnants (intestinal lipoproteins transporting cholesterol) according to their size.[1] We postulate, with implications in atherogenesis, that sieving controls the balance between exogenous (dietary) and endogenous (hepatic-synthesized) cholesterol in tandem with hepatocyte receptor-mediated uptake.[2]

The possibility that the size and composition of chylomicrons influences atherogenesis had long intrigued us.[3] Chylomicrons, in cholesterol-fed and atherosclerotic-prone New Zealand white rabbits increased in diameter from about 50-100nm to 100-800 nm as triglycerides were added to the cholesterol in their diet,[4] as well as increasing the ratio of free to esters of cholesterol.[5] Zilversmit and others have shown chylomicron lipids, especially the esters of cholesterol and retinol are the major lipids in atherosclerotic plaques.[6]

Despite fat of dietary origin in early fatty plaques, Wissler's group showed that the concurrent hyperplasia of the arterial smooth muscle cells (SMC), at least in tissue culture, was triggered not by chylomicrons, but by low density lipoproteins transporting liver-synthesized cholesterol.[7] It is known that dietary cholesterol, if it enters the hepatocytes, inhibits their synthesis of cholesterol.[2]

Florén in 1984 demonstrated that *in vitro* hepatocytes bound chylomicron-cholesterol, but *in vivo* large native chylomicrons did not bind, this only occurring when smaller chylomicron remnants entered the hepatic circulation. He suggested this was due to "steric hindrance" of chylomicron-uptake.[8] Wisse's seminal hypothesis in 1970, that the 100nm pores or fenestrae of the LSEC endothelium might filter chylomicrons, explains this "steric hindrance".[9] In 1978 both our group and Naito and Wisse published to confirm this sieving, as shown by comparing the sizes of chylomicrons in the sinusoidal lumen compared to the space of Disse, as well as the trapping of radio- labeled chylomicrons and remnants of small size within the liver. [10, 11]

Since then our major research thrust has been testing the hypothesis that a low porosity liver sieve predisposes to post-prandial hyperlipidaemia (consisting especially of chylomicrons and their remnants, but also of liver synthesized cholesterol in LDL, since the latter's synthesis would not be switched off by dietary cholesterol[2]). These experiments confirmed a low porosity in species of high sensitivity to dietary cholesterol (e.g. NZ white rabbits[12] and chickens[13]). In rats and primates with higher porosity sieves, the effects of drugs such as nicotine,[14] excess alcohol,[15] and various pro-atherogenic-diseases, such as diabetes[16] which reduce their porosity, lead to hyperlipoproteinaemia. Rabbit sub-species with differing porosities (which were shown to differ in ability to sieve a 70-90nm adenovirus) might in future make interesting experimental subjects if fed cholesterol.[17]

The new millennium heralded a change of direction, as instigated by our Sydney colleagues with their interest in old age [18]. Coronary artery disease and post-prandial hyperlipidaemia increase with age[19] and we found that these correlated with a decrease in sinusoidal porosity in many species including humans.[20, 21,] Whether this decrease is purely an age factor, or is related to long-term diets, toxins or concurrent disease states, has yet to be determined. It has also been shown that the semi-starvation of the Methuselah diet increases porosity and also leads to longevity,[22] while some detergents or surfactants decrease porosity and increase atherosclerosis.[23]

Our dream is to find an enjoyable lifestyle or a safe drug to increase LSEC porosity and longevity.

For reference Google **Liver Sieve**

DIFFERENT EXPRESSIONS OF AQUAPORIN-1 IN NORMAL AND CIRRHOTIC HUMAN LIVER – DEMONSTRATING THE CAPILLARIZATION OF HEPATIC SINUSOIDS

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In liver cirrhosis, defenestration of hepatic sinusoidal endothelial cells concomitant with extensive collagen deposition in the space of Disse causes an increase in peripheral portal microvascular resistance, leading to portal hypertension. In general, aquaporins (AQPs) are key regulators in water channels, contributing to the control of microcirculation by regulating water transport across the microvascular endothelial cells. A couple of immunohistochemical studies have been directed to the localizations of AQPs in the hepatobiliary system in mammals. However, little is known about the localizations of AQPs in the hepatic microvascular system including sinusoids and about their alterations in liver cirrhosis. The aim of the present study is to clarify the immunohistochemical and immunoelectron microscopic expressions of AQP-1 in the microvessels in normal human liver and primary biliary cirrhosis (PBC).

As human normal liver samples (controls), wedged liver biopsy specimens were obtained from normal portions of the livers of 10 patients who underwent surgical resection for metastatic liver carcinoma (8 colonic carcinomas, 2 gastric carcinomas). Fifteen wedged liver biopsy specimens of PBC patients (5 cases each of stage I-II and stage III) and 5 samples of cirrhotic livers removed from PBC stage IV patients for liver transplantation were subjected to the following studies. Immuno-staining was performed on the serial 5 μm sections of control and PBC liver tissues fixed in PLP solution. For immunohistochemistry, semi-thin liver sections were incubated overnight at 4°C with anti-AQP-1 rabbit polyclonal antibody as primary antibody, followed by incubation with peroxidase-conjugated anti-rabbit IgG antibody according to the Envision method for light microscopy. For immunoelectron microscopy, semi-thin liver sections were incubated with anti-AQP-1 antibody as above followed by incubation with 1.4nm colloidal gold-conjugated anti-rabbit IgG antibody for 40 min at room temperature. Then the ultrathin sections were observed by transmission electron microscopy. Western blotting was conducted using fresh control and PBC liver tissues. For histochemical in situ hybridization (ISH), human AQP-1 RNA probes were used with a CSA system. By focusing on the microvascular system in control liver tissue, the immunoperoxidase-positive reaction products showing the presence of AQP-1 were found to be mainly localized along the endothelial lining cells in the portal venules, hepatic arterioles and arterial capillaries in the portal tract, while these AQP-1 expressions were hardly detected in the hepatic sinusoids. By immunoelectron microscopy, the electron dense immuno reactive gold particles showing the presence of AQP-1 were evident on the plasma membranes of endothelial cells in the microcirculator vasculature except hepatic sinusoids as above. In PBC stage I-II liver tissue, AQP-1 distributions on the microvascular vessels were similar to those in control liver tissue. On the contrary, AQP-1 was aberrantly expressed on the sinusoidal endothelial lining cells in the periportal zone and in the periphery of the regenerating hepatic nodules circumscribed with the fibrotic septa in PBC stage IV cirrhotic liver tissue. By immunoelectron microscopy, the immunoreactive gold particles specifically demonstrating the existence of AQP-1 were proved on the endothelial cell plasma membranes of hepatic sinusoids surrounded not only with excessive collagen fibers in the perisinusoidal space of Disse, but also with the basement membranes just beneath the sinusoidal endothelial cell linings. Overexpressions of AQP-1 at protein and mRNA levels were demonstrated by Western blot and ISH, respectively.

In conclusion, AQP-1 protein molecules were proved to be aberrantly expressed on the plasma membranes of sinusoidal endothelial cells (SECs) in cirrhotic liver of PBC. These new findings indicate that AQP-1 would induce the transformation of the fenestrated SEFs into “the defenestrated capillary endothelial cells” in association with excessive collagen deposition in the perisinusoidal space, further increasing the defenestrated sinusoidal microvascular resistance, and thus contributing to the progression of portal hypertension in PBC.

IMPLICATION OF CORTACTIN, A REGULATOR OF ACTIN CYTOSKELETON ASSEMBLY, IN THE CONTROL OF FENESTRATION IN LIVER SINUSOIDAL ENDOTHELIAL CELLS

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Liver sinusoidal endothelial cells (LSECs) possess fenestrae, open pores approximately 100 to 200 nm in diameter, arranged in sieve plates. Alterations in their number or diameter in liver diseases have important implications for hepatic microcirculation and function. Sieve plates and fenestrae are clearly delineated by cytoskeleton elements, including actin and microtubules. In addition, from the use of actin binding drugs, which stabilize or disassemble actin microfilaments, it was learned that actin dynamics govern the appearance of these structures. Although decades of studies using SEM approaches have characterized sieve plates and fenestrae at the ultrastructural level, much remains to be discovered on the mechanisms by which these structures form.

Since cortactin constitutes an important molecular scaffold for actin assembly of cytoskeletal structures involved in cellular processes such as endocytosis, adhesion, migration and invasion, we sought to explore its role in the formation of fenestrae. We first developed an *in vitro* assay, which, by measuring the cell surface occupied by fenestrae though IRM (Interference Reflection Microscopy) enables us to study their dynamics. Next, we examine cortactin expression and subcellular distribution in LSEC. Immunohistochemical staining for cortactin in liver tissue sections revealed an intense staining in LSEC. Cortactin was also detected by immunofluorescence in primary LSEC isolated from mice livers. In these cells, cortactin presented an unusual pattern, which manifested by colocalization of the protein with the microtubule network. Consequently, cortactin was found excluded from sieve plates areas.

LSEC brought in culture de-differentiate, a process associated with cytoskeleton remodelling and loss of fenestration. Dedifferentiation induced the dissociation of cortactin from microtubules and its relocalization to the cytosol, where the protein was found evenly distributed after 3 days of culture. Cytochalasin D treatment restored the fenestration status and this was accompanied by relocalization of cortactin to microtubules. Profibrogenic cytokines are known to alter LSEC characteristics. The dedifferentiation process and the relocalisation of cortactin were accelerated upon TGF-β1 treatment and delayed in the presence of SB-431542, an inhibitor of Alk-5 TGF-β1 type I receptor. Analysis of TGF-β1 signalling pathways are in progress to determine how TGF-β1 affects cortactin localization and especially whether TGF-β1 is responsible for post-translational modifications of coractin.

Altogether, the correlation between cortactin subcellular localisation and the fenestration status of LSEC, combined with the known function of cortactin in cytoskeletal organization, identify cortactin as a prime candidate to explain the dynamics of LSEC fenestrae.

INVOLVEMENT OF SIGNALING OF VEGF AND TGF-β1 IN DIFFERENTIATION OF SINUSOIDAL ENDOTHELIAL CELLS DURING CULTURE OF FETAL RAT LIVER CELLS

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Introduction: Embryonic development of the liver is closely associated with vascular organization. Our previous study showed that the maturation of sinusoidal endothelial cell (SEC) occurred between E13.0 to E15.0 of the fetal rat liver. However, little is known about the mechanisms of SEC differentiation during liver development. To investigate factors related to the differentiation of SEC, we examined the effects of VEGF and SB-431542 (an inhibitor of TGF-β1 receptor kinase; ALK-5) on the cultured fetal rat liver cells. Appearance of the endothelial cell sheets with fenestration structures and expression of the maturation markers of SEC, SE-1 and stabilin-2, were examined during the culture.
Materials and Methods: Frozen sections obtained from E13.5 to 21.5 fetal rat livers were stained with antibodies against SE-1 and stabilin-2. Whole liver cells from E13.5 rat fetus were cultured in EBM-2 medium containing VEGF and/or SB-431542 for 4 and 7days. Endothelial sheet formation and expression of the two markers in the cultured cells were investigated. Phosphorylation of Smads, the down-stream molecules of TGF-β1, was examined.
Results and Conclusions: SE-1 and stabilin-2 were co-expressed in SEC in the late stage of liver development (E15.5-17.5), although stabilin-2 was expressed in almost all vascular endothelial cells in the early stage. After culturing E13.5 fetal liver cells for 7 days under the presence of VEGF, proliferated-endothelial sheets were observed. But cells of the sheets expressed neither SE-1 nor stabilin-2. Under the presence of both VEGF and SB-431542, the endothelial sheets became to express stabilin-2 and contained some SE-1 co-expressing cells. These findings suggest that VEGF plays a role in the endothelial sheet formation, and block of TGF-β1 signaling may involve in differentiation of SEC.

The present study indicates that co-expression of SE-1 and stabilin-2 is an adequate marker for the differentiated-SEC. Signaling of VEGF and TGF-β1 is involved in differentiation of SEC in culture of fetal rat liver cells. Further analysis on genes related to the SEC differentiation is currently in progress using the DNA micro array.

INTEGRIN LFA-1 ENABLES COLON CARCINOMA CELLS TO UPREGULATESINUSOIDAL ENDOTHELIAL CELL MANNOSE RECEPTORVIA INTERLEUKIN-1-DEPENDENT MECHANISM: IMPLICATIONS ONANTI-TUMOR IMMUNITY INHIBITION DURING HEPATIC METASTASIS

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Interleukin-1 (IL-1) up-regulates the endocytic activity of mannose receptor (ManR) expressed by liver sinusoidal endothelial cells (LSEC), a 175 kDa transmembrane glycoprotein involved in homeostatic processes and endogenous defence. ManR binds and uptakes mannosylated molecules, C-terminal procollagen peptides and denatured collagen from blood, and contributes to the clearance of circulating infectious agents, but also to the adhesion of cancer cells to liver sinusoid endothelial cells (LSEC). Interestingly, ManR is involved in antigen uptake, processing and presentation to T cells by LSEC and it has been suggested that this process diminishes local immune response of the liver under physiological conditions. However, at present it is unknown how expression and function of ManR are regulated during the hepatic microvascular infiltration of metastatic cancer cells. Furthermore, the possible contribution of ManR to the regulation of anti-tumor immunity during hepatic metastasis development is also unknown.

We examined ManR regulation during C26 colorectal cancer cell interaction with LSEC, and its immunological implications in the hepatic metastasis microenvironment. Expression and activity of ManR were determined by both immunohistochemistry and labelled mannan or ovalbumin uptake. Anti-ManR antibodies were used to suppress ManR activity in primary cultured LSEC. Anti-IL-1 receptor type I antibody, IL-1 receptor antagonist and IL-1beta converting enzyme inhibitor were used to analyze the role of IL-1 in the regulation of ManR.

ManR expression and endocytic activity concomitantly increased in LSEC upon direct interaction with metastatic C26 cells *in vitro* and *in vivo*. The mechanism involved three steps: (1) activation of LFA-1-expressing cancer cells by intercellular adhesion molecule (ICAM)-1 expressed and secreted by LSEC; F(2) release ofcyclooxygenase-2 (COX-2)-dependent IL-1-stimulating factor(s) by cancer cells enhancing LSEC-derived IL-1 by 2-fold; and (3) widespread increase of ManR expression and function in LSEC induced by paracrine IL-1, leading to decreased anti-tumor cytotoxicity and interferon-gamma secretion by liver sinusoidal lymphocyte. ICAM-1-induced tumor COX-2 activity led to the inhibition of regional anti-tumor immunity during hepatic colon carcinoma metastasis via IL-1-dependent endothelial ManR.

ManR may constitute a common mediator for prometastatic effects induced by IL-1, COX-2 and ICAM-1. The finding that antitumor hepatic immune response activation occurs via ManR deactivation may account for antimetastatic effects of IL-1, COX-2 and ICAM-1 inhibitors.

INNATE IMUNITY IN ALCOHOLIC LIVER DISEASE: ROLE OF KUPFFER CELLS

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Alcoholic liver disease (ALD) develops in approximately 20% of all alcoholics and is a complex process involving both parenchymal and non-parenchymal cells resident in the liver, as well as the recruitment of other cell types to the liver in response to damage and inflammation. Components of the innate immune response, including NK and NKT cells, Kupffer cells (resident hepatic macrophages) and the complement system, as well as T-cells and antibody-dependent adaptive immune responses, contribute to the pathophysiology of ALD.

Activation of the innate immune response in the liver during chronic ethanol exposure is associated with increased production of pro-inflammatory cytokines and chemokines, as well as reactive oxygen species (ROS). Kupffer cells are critical to the onset of ethanol-induced liver injury. Ablation of Kupffer cells prevents the development of fatty liver and inflammation, early stages in the progression of ethanol-induced liver damage, in rats chronically exposed to ethanol. Endotoxin/ lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, is an important activator of Kupffer cells. LPS concentration is increased in the blood of alcoholics and animals exposed to ethanol, probably due to impaired barrier function of the intestinal mucosa.

In addition to increasing the exposure of Kupffer cells to LPS, chronic ethanol also sensitizes Kupffer cells to activation by LPS, leading to increased production of ROS and inflammatory cytokines, including tumor necrosis factor α (TNFα). The mechanisms for this ethanol-induced sensitization of Kupffer cells is not well understood, but recent data suggest that decreased expression of adiponectin, an adipokine with anti-inflammatory properties, during ethanol exposure contributes to a more robust inflammatory response by Kupffer cells. Studies in our laboratory have investigated the molecular mechanisms by which ethanol and adiponectin interact to modulate Kupffer cell sensitivity to LPS. LPS rapidly increases ROS production in Kupffer cells, with ROS production 2.5-fold greater in ethanol-fed compared to pair-fed. Pre-treatment with diphenyleneiodonium (DPI), which inhibits NADPH oxidase, normalizes ROS production in Kupffer cells from ethanol-fed rats. LPS rapidly increases the quantity of GTP bound-Rac1 and $p67^{phox}$ translocation to the plasma membrane in Kupffer cells from pair-fed rats; these responses are enhanced in Kupffer cells from ethanol-fed rats. These results demonstrate that chronic ethanol feeding increases LPS-stimulated NADPH oxidase activity in Kupffer cells. Treatment of Kupffer cells with adiponectin restores LPS-stimulated TNFα production to control values; this normalization is associated with decreased GTP bound-Rac1, as well as decreased LPS-stimulated ROS production. Adiponectin also normalizes the expression of $p47^{phox}$ mRNA and protein after ethanol feeding. Taken together, these data suggest that increased LPS-stimulated NADPH oxidase activity contributes to the sensitization of Kupffer cells to LPS and that adiponectin, a critical anti-inflammatory modulator, can specifically ameliorate these ethanol-induced changes in the TLR4 signaling cascade. Supported by NIH grants AA11876 and AA13868.

KINETICS AND REPLACEMENT OF KUPFFER CELLS AFTER BONE MARROW TRANSPLANTATION IN MICE AND RATS

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Abstract

Kupffer cells were thought to be derived from bone marrow precursors via blood monocytes. In this present study, we examined replacement of Kupffer cells after bone marrow transplantation (BMT) using CD45 congenic mice, and GFP rats. Until 6 months after BMT, numbers of Kupffer cells did not change in the liver of mice after BMT. In rats, replacement of ED1-positive Kupffer cells occurred gradually until 6 months after BMT as previously reported, and the percentage of GFP-positive Kupffer cells was not more than 40% even 6 months after BMT. However, ED1-positive Kupffer cells gradually decreased in the liver of rats after BMT. In order to clarify the difference of numbers of Kupffer cells between rats and mice, we applied liposome encapsulated dichloromethylene diphosphonate (lipo-MDP) to the mice just before BMT. Repopulation of Kupffer cells was completed by 2 weeks in mice after lipo-MDP injection. In mice after simultaneous treatment with BMT and lipo-MDP, Kupffer cells repopulated within 4 weeks after BMT, however, the numbers of Kupffer cells gradually decreased until 6 months after BMT. Furthermore, long term observation of lipo-MDP injected mice without BMT revealed that Kupffer cells decreased gradually after repopulation. These results suggested that Kupffer cells were surely replaced by blood precursors, however, they could not be maintained only by the influx of bone marrow precursors, and they were supposed to be maintained by self proliferation. The difference of numbers of Kupffer cells between mice and rats after BMT might be dependent on their survival time.

HIGH FAT FEEDING INDUCES HEPATIC INSULIN RESISTANCE AND PREFERENTIAL DISTRIBUTION OF KUPFFER CELLS AROUND LIPID LOADED HEPATOCYTES

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Introduction:

Inflammation and macrophage infiltration of the adipose tissue have been causally associated with the development of insulin resistance.

We formulate the hypothesis that Kupffer cells, the hepatic resident macrophages, are implicated in the pathogenesis of hepatic insulin resistance. The aim of the study is to analyse the Kupffer cell distribution in a model of hepatic insulin resistance and steatosis induced by high fat feeding.

Materials and methods:

Male C57BL6 mice of 5 weeks of age (n=24) were fed a high fat diet (60% energy as fat, principally saturated) or a regular chow (10% energy as fat) for 3 days preceded or not by a 24h fasting period. This short term exposure to the high fat diet was chosen to preferentially induce hepatic insulin resistance. The fasting period was applied to enhance hepatic de novo lipogenesis and hepatic fat accumulation.

Body weight gain, liver weight, blood plasma glucose and insulin levels were studied. A separate group of mice was used to assess the hepatic insulin signaling cascade. Hepatic steatosis was evaluated on hepatic lipid content and liver histology. Kupffer cell distribution was studied by immunohistochemistry using F4/80, a cell surface glycoprotein expressed on a wide range of mature tissue macrophages. Real-time PCR was used to examine mRNA expression of TNF-alpha and phosphoenolpyruvate carboxykinase (PEPCK).

Results and conclusions:

Mice fasted for 24h and refed a high fat diet exhibit the most impressive changes. Compared with mice refed the control diet, mice refed the high fat diet for 3 days gain more weight (p=0.005), have increased liver weight (p<0.001) and hepatic lipid content (56.2 ± 11.5 versus 11.9 ± 3.6 mg/100 mg liver, p<0.001).

Fat accumulates as lipid vacuoles predominantly in the hepatocytes of the intermediate and the periportal lobular zones. The majority of F4/80 positive cells is found in these zones in close proximity of fat loaded hepatocytes contrasting with the diffuse panlobular distribution in controls. A trend in upregulation of TNF-alpha is observed.

In this model, fat accumulation is associated with insulin resistance. Plasma insulin levels (2.9 ± 1.5 versus 1.1 ± 0.4 µg/l, p=0.02) and glucose (187.0 ± 17.5 versus 121.7 ± 22.2 mg/dl, p<0.001) are significantly higher than in controls. Moreover mRNA levels of phosphoenolpyruvate carboxykinase shows an increased gluconeogenesis in the high fat group and Western blot analysis of liver proteins demonstrates a decreased expression of insulin receptors (p=0.002) as well as an impaired insulin-stimulated receptor phosphorylation.

Our data provide evidence that the fast/high fat refed model induces steatosis and insulin resistance. Cytokines or other soluble mediators released by Kupffer cells in the vicinity of hepatocytes may play a role in the development of steatosis and hepatic insulin resistance. This relationship is being further examined in Kupffer cell depletion experiments.

ARACHIDONATE 5-LIPOXYGENASE MODULATES HEPATOCYTE FATTY ACID UPTAKE AND MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN ACTIVITY.

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The 5-lipoxygenase (5-LO) pathway generates lipid mediators from arachidonic acid with potent pro-inflammatory and pro-fibrogenic properties. Recent studies have found that the 5-LO pathway is overexpressed during experimental liver injury and that among the different liver sinusoidal cells, Kupffer cells are the only cell type endowed with the complete enzymatic machinery for the biosynthesis of 5-LO products (i.e. leukotrienes (LTs)). These Kupffer cell-derived eicosanoids display potent pro-fibrogenic properties by activating hepatic stellate cells in a paracrine fashion. In fact, a remarkable protection against carbon tetrachloride (CCl_4)-induced necro-inflammation and fibrosis is observed *in vivo* following the blockade of the 5-LO pathway with either a selective 5-LO inhibitor (CJ-13, 610) or a potent 5-LO-activating protein (FLAP) inhibitor (Bay-X-1005). In light of recent findings postulating an emerging role for the 5-LO pathway in obesity and insulin resistance, we hypothesized that this pathway may also be implicated in the pathogenesis of hepatic steatosis. In the current study, we provide evidence that the hepatic formation of 5-LO products is increased in *ob/ob* mice, an experimental model of obesity-related steatohepatitis. In this model, inhibition of 5-LO pathway results in down-regulation of genes involved in hepatocyte fatty acid uptake (i.e. L-FABP and FAT/CD36) and normalization of PPARα and acyl-CoA oxidase expression. No changes in the lipogenic genes, FASN and SREBP-1c, were observed. In addition, 5-LO inhibition restored the hepatic activity of microsomal triglyceride (TAG) transfer protein (MTP). Consistent with these findings, direct addition of 5-LO products such as LTB_4 and LTD_4 to hepatocytes in culture triggered cytosolic TAG accumulation by reducing MTP activity and TAG secretion. The functional consequence of 5-LO inhibition was a remarkable protection from hepatic steatosis as revealed by decreased oil red-O staining and hepatic TAG concentrations. Interestingly, 5-LO-deficient mice exhibited a protection against liver injury and a differential expression of a number of lipid metabolism-related genes including C/EBPbeta, SCD2, Acly and Elovl3/6. Taken together, these findings identify a novel role for arachidonate 5-LO in the regulation of fatty acid uptake and TAG transport in hepatocytes.

CROSS TALK BETWEEN PGE_2- AND IL-6-MEDIATED SIGNALLING PATHWAYS IN CULTURED RAT KUPFFER CELLS

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Introduction: Kupffer cells (KC) are tissue specific resident macrophages of the liver. During local and systemic inflammation KC release inflammatory mediators like cytokines (e.g. IL-6), prostaglandins (e.g. PGE_2) and nitric oxide (NO). These mediators can act on nearby hepatocytes in a parakrine mode to elicit an acute phase response, but they also can act back on KC in an autokrine loop. IL-6 operates via a tyrosine kinase associated receptor that phosphorylates and activates a transcription factor "signal transducer and activator of transcription 3" (STAT3). Among its target genes is the inducible form of NO synthase and the inducible PGH synthase (COX2) resulting in enhanced NO and PGE_2 production. PGE_2 acts on G_s-protein coupled EP-receptors (EP2-R/EP4-R) that increase the intracellular cAMP-concentration. Since IL-6 and PGE_2 are released concomitantly from KC, it was the purpose of the study to analyze whether a signal cross talk exists between IL-6- and PGE_2 -pathways and if, therefore, PGE_2 could modulate the IL-6 dependent iNOS and COX-2 induction?

Material and methods: primary KC from male Wistar rats were cultured for 72 h. At the end of this culture period they were stimulated with IL-6, PGE_2, forskolin (direct activator of adenylate cyclase) and RPcAMPs (direct inhibitor of protein kinase A) for 2 h. Cell were lyzed and lysates were used for Western Blots with antibodies against phosphorylated and total STAT3, COX-2 and iNOS. cAMP was determined by RIA. The KC-supernatant was collected to measure NO by Griess assay and IL-6 by rat IL-6-ELISA.

Results and discussion: In Kupffer cells, a combination of PGE_2 and IL-6 strongly increased NO-formation while either stimulus alone increased NO formation only slightly or not at all, respectively. In accordance with this observation, a combination of PGE_2 and IL-6 induced the inducible form of NO synthase 4-fold, while PGE_2 alone cause a 2-fold induction and IL-6 alone merely a 1,5-fold induction. A similar synergism between PGE_2 and IL-6 was observed with the induction of COX2. Both genes are regulated by the transcription factor STAT3, which is a downstream target of the IL-6 receptor signal chain that gets activated through phosphorylation. As expected, the ratio between phosphorylated STAT3 (pSTAT) and total STAT3 was increased about 2-fold in IL-6-treated Kupffer cells. Surprisingly, PGE_2 alone also increased STAT phosphorylation about 1,5-fold. IL-6 and PGE_2 synergistically increased STAT3 phosphorylation about 4-fold. PGE_2 induced IL-6 production in Kupffer cells, however, the amount produced was 3 orders of magnitude lower than the exogenously added concentration and thus most likely did not contribute to the synergism observed. Experiments with receptor subtype specific agonists indicated that the Gs-coupled EP2 receptor mediated the PGE_2-dependent STAT3 phosphorylation, however, this effect appeared to be independent of the EP2-mediated increase in cAMP formation because neither could direct activation of adenylate cyclase with forskolin mimic the PGE_2-dependent STAT3 phosphorylation nor could inhibition of adenylate cyclase with RPcAMPs abolish it. In conclusion, simultaneous release of PGE_2 and IL-6 from Kupffer cells in the course of local or systemic inflammation may synergistically enhance the release of proinflammatory mediators from Kupffer cells through a STAT3-dependent, cAMP-independent signaling pathway.

THE INFLAMMATORY RESPONSE DURING DRUG-INDUCED LIVER INJURY: TOXICITY VERSUS REGENERATION

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Acetaminophen (APAP), a widely used analgesic, is generally considered a safe drug at therapeutic levels but can induce centrilobular necrosis and even liver failure at higher doses. Although most studies still focus mainly on the intracellular signaling mechanisms of cell death in hepatocytes, there is an increasing interest in the role of hepatic nonparenchymal cells (Kupffer cells, NK- and NKT cells) and infiltrating leukocytes (neutrophils, monocytes) in the pathophysiology. For each cell type there is experimental evidence in the literature suggesting a contribution to the mechanism of toxicity. However, the validity of several of these conclusions has been questioned recently. The focus of our investigations is to elucidate the role of neutrophils in APAP-induced liver injury and regeneration.

Treatment of fasted C57Bl/6 mice with 300 mg/kg APAP resulted in substantial liver injury as indicated by the increase of plasma ALT activities between 3 and 12 h, and the development of extensive centrilobular necrosis. Plasma ALT activities declined to baseline levels within 48 h and the injury was completely repaired by removal of necrotic cells and replacement by newly regenerated hepatocytes by 96 h. Neutrophil progressively accumulated in the liver after the initiation of cell injury (ALT release). Most of the leukocytes were present in sinusoids throughout the liver lobes and only a smaller fraction was found extravasated into the parenchyma of the necrotic area during the first 24 h. However, the neutrophils in sinusoids disappeared and a massive accumulation of neutrophils and also macrophages was observed in the necrotic areas by 48 -72 h. The area of necrosis disappeared together with neutrophils and macrophages in the liver by 96 h. A number of interventions against neutrophils and macrophages (phox91 or ICAM-1 knock-out mice; NADPH oxidase inhibitors DPI and apocyanin) showed no protective effect against APAP-induced liver injury, oxidant stress or DNA fragmentation. On the other hand, pretreatment with the neutropenia-inducing antibody Gr-1 for 24 h before APAP caused protection. However, this effect was not due to the absence of neutrophils but was caused by upregulation of inflammatory and cytoprotective genes, e.g. metallothionein (MT), heme oxygenase-1 (HO-1), triggered by Kupffer cell activation during the phagocytosis of antibody-tagged neutrophils in sinusoids. Selective induction of metallothionein gene expression by $ZnCl_2$ treatment confirmed the protective effect of MT induction in this model. *Conclusions:* Neutrophils are systemically activated during APAP-induced necrosis and accumulate first in sinusoids and later, together with liver macrophages, in the area of necrosis. There is no reliable evidence to suggest that neutrophils (or macrophages) contribute to APAP-induced liver injury. However, neutrophils and macrophages are responsible for removal of necrotic tissue. This inflammatory response creates the space for regenerating hepatocytes to restore the original liver mass.
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CECAL LIGATION AND PUNCTURE SEPSIS IS ASSOCIATED WITH A SHIFT IN ADENYLYL CYCLASE EXPRESSION IN SINUSOIDAL LIVER CELLS

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Introduction: One reason for the fatal inflammatory response causing organ injury and death in sepsis is the loss of immune modulatory mechanisms. The intracellular signaling mediator cyclic adenosine monophosphate (cAMP) is a potent modulator of innate immune cell activation, and elevation of cAMP by phosphodiesterase inhibitor treatment can increase survival in endotoxemia. We have previously demonstrated that expression of cAMP-producing adenylyl cyclases (AC) are attenuated by bacterial lipopolysaccharide (LPS) *in vivo* and in Kupffer cell cultures. Here we explore AC gene expression and regulation in a rat model of cecal ligation and puncture (CLP) sepsis and in liver cells isolated from this model.

Methods: Wistar rats were subjected to CLP procedure or sham operated, and after 10 or 18 hours organs were removed. Hepatocytes, liver sinusoidal endothelial cells (LSEC) and Kupffer cells were isolated 18 hours after CLP/sham-operation. RNA was isolated and analysed for AC mRNA expression.

Results: We found significantly attenuated liver expression of AC6 ($P \leq 0.0336$) and AC9 ($P \leq 0.0144$) mRNAs in CLP animals compared to sham, an attenuation also found in Kupffer cells (AC6 $P \leq 0.0134$; AC9 $P \leq 0.0623$) isolated from the model. AC9 was also attenuated in spleen ($P \leq 0.0154$) and kidney ($P \leq 0.0091$) after 18 hours. AC7 mRNA was only attenuated in spleen ($P \leq 0.0183$, 18h), and appeared to increase in kidney, liver and LSEC.

Conclusion: CLP sepsis is associated with reduced AC mRNA expression in spleen, kidney, liver and Kupffer cells, which may reduce cAMP production and impair cAMP-mediated immune modulation.

SPLENCHNIC VITAMIN A-STORING CELL SYSTEM AND ORGAN FIBROGENESIS

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Organ fibrogenesis is a complex and dynamic pathological process that is controlled by the synthesis and degradation of extracellular matrix materials including fibril-forming collagens and by the proliferation and apoptosis of fibroblastic cell populations. It has been proven that hepatic stellate cells (HSCs) that comprise the hepatic sinusoidal wall play multiple roles in the hepatic fibrotic process. Transformation of HSCs from the vitamin A-storing phenotype to the "myofibroblastic" phenotype closely correlates with hepatic fibrogenesis during chronic liver trauma. Recent reports indicate that vitamin A-storing cells are not liver-specific and exist in all splanchnic organs as stroma-constituent cells. Pancreatic stellate cells are one of them, which are now evident to contribute to fibrotic process in chronic pancreatitis. The molecular analyses of the phenotypic change of these stellate cell lineages, so called "activation", have made great progress, in particular, in the field of intracellular signal transduction of transforming growth factor-beta (TGF-beta) and platelet-derived growth factor-BB (PDGF-BB) and collagen gene expression. Accumulation of information on stellate cell activation would shed light on the establishment of a novel therapeutic strategy against organ fibrosis.

In order to promote a better understanding of molecular dynamics associated with stellate cell activation, we performed proteomics approach that compares comprehensive protein expression pattern between vitamin A-storing phenotype of HSCs and activated myofibroblasts. This approach has clarified the induction of actin-binding proteins, several types of collagens, neural cell adhesion molecule and galectins in the course of cell activation process. We additionally discovered cytoglobin (Cygb) that has amino acid homology with vertebrate myoglobin (Mb), hemoglobin (Hb), and neuroglobin (Ngb), a globin expressed in neuronal cells.

Unlike Hb, Mb and Ngb, Cygb is uniquely localized in fibroblast-like cells in splanchnic organs, namely vitamin A-storing cell lineage, but neither in epithelial cells, endothelial cells, muscle cells, blood cells, macrophages, nor dermal fibroblasts. Cygb-positive cells in the extrahepatic organs include pancreatic stellate cell, reticular cells in the spleen, and mesangial cells in the kidney. That is, Cygb is a useful molecular marker of splancnic vitamin A–storing cells. Expression of Cygb is upregulated in fibrotic lesions of pancreas and kidney in which activated fibroblast-like cells or myofibroblasts increase in number. Cygb is co-localized with HSP47, a collagen-specific molecular chaperon.

The X-ray crystal structure of recombinant human Cygb is dimerized through two intermolecular disulfide bonds between Cys38 (B2) and Cys83 (E9). The overall backbone structure of the Cygb monomer exhibits a traditional globin fold with a three-over-three helical sandwich. The His81(E7) imidazole directly coordinates to the heme iron as a sixth axial ligand to form a hexacoordinate heme, like Ngb and rice Hb. Cygb may be involved in currently unknown physiological actions through a mechanism that is linked to conformational changes in the protein upon O_2 binding.

Molecular function of Cygb and, in particular, its role in organ fibrogenesis will be discussed in the lecture.

MODULATION OF HUMAN HEPATIC STELLATE CELLS CHEMOTAXIS BY INTRACELLULAR ROS GENERATION AND c-JUN N-TERMINAL KINASE ACTIVATION

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Background & Aims. Liver fibrogenesis, occurring in conditions of chronic liver diseases of different aetiology, is modulated and sustained by myofibroblasts-like cells that may originate from activated hepatic stellate cells or HSC/MFs, portal (myo)fibroblasts and, as proposed in recent years, from bone-marrow derived mesenchymal stem cells or MSC. Since activated HSC/MFs play a major role in fibrogenesis and migrate in response to several stimuli, here we investigated whether PDGF-BB and other polypeptide factors able to stimulate migration/chemotaxis of human HSC/MFs may act with a common signaling pathway involving intracellular generation of reactive oxygen species (ROS) and activation of c-Jun N-terminal kinase isoforms (JNKs).
Methods. Experiments were performed on activated human HSC/MFs displaying antigen repertoire of interface myofibroblasts, and on HSC cell extracts obtained from liver of rat sacrificed after acute CCl_4 administration. *In vitro* and *in vivo* signal transduction were evaluated by performing both cell and molecular biology techniques; non-oriented migration and chemotaxis by using the wound healing assay and the modified Boyden's chamber assay, respectively.
Results. Exposure of human HSC/MFs to PDGF-BB, MCP-1, AT-II, VEGF and extracellular superoxide anion induced both chemokinesis and chemotaxis. These phenotypic responses were abolished by pre-treating cells with SP-600125 or diphenylene-iodonium (DPI), pharmacological inhibitors of JNK-1/2 and NADPH-oxidase, respectively, as well as by employing siRNAs able to silence JNK1/2. The involvement of JNKs and intracellular ROS generation in modulating HSC/MFs migration was further confirmed by: 1) activation of JNK1/2 and up-regulation of heme oxygenase I in extracts obtained from HSCs isolated from rat livers 24 and 48 hrs after acute CCl_4 administration; this was concomitant with increased *in vivo* generation of ROS; 2) only some stimuli such as extracellular superoxide and PDGF-BB (used as positive stimuli) but not other, like TGFbeta1 (ineffective on migration) were able to activate JNK-1/2; 3) intracellular generation of superoxide or hydrogen peroxide by using menadione (MEN) or dimethyl-naphtoquinone (DMNQ), respectively, was sufficient to induce JNK activation and migration; 4) migration observed after PDGF-BB, MEN and DMNQ treatment also involved activation of ASK-1, an oxidative-stress sensitive upstream kinase for JNK1/2 and was prevented by either DPI and pegylated – superoxide dismutase (PEG-SOD, a cell permeable form of SOD).
Conclusions. Migration/chemotaxis of human HSC/MFs critically requires activation of JNK isoforms and is positively modulated by NADPH oxidase - dependent intracellular generation of ROS.

CYSTEINE CATHEPSINS IN HEPATIC STELLATE CELLS

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Acute or chronic injury to the liver leads to fibrosis and subsequently to cirrhosis or hepatocarcinoma. Activation of hepatic stellate cells (HSCs) during liver fibrosis is a major event associated with an increase in extracellular matrix deposition. Not only the amount of secreted ECM molecules, but also the composition of the collagens is changed during HSC activation. Consequently, the increase in ECM stiffness impairs the normal function of the hepatocytes. HSCs are recognized as one of the major targets in the process of matrix deposition, although recent findings suggest the participation of other cell types.
The role and function of cysteine cathepsins in HSCs have so far not yet been studied. Cysteine cathepsins are lysosomal cysteine proteases, whose main function is the terminal degradation of proteins in the lysosomes. Some are ubiquitously expressed (B, L and F), while others (cathepsin S, K and W) are tissue- or cell type-specific. Cathepsins are implicated in several diseases for example pycnodysostosis, rheumatoid arthritis and different forms of cancer. In addition, the involvements of cathepsin K in lung fibrosis and dermal scar formation, and the association of cathepsin B with liver fibrosis have been demonstrated. Thus far, we have studied the cathepsins S and F in HSCs.
HSCs have been shown to be professional antigen presenting cells.Treatment with IFN-γ leads to an upregulation of surface molecules essential for the process of antigen presentation. On the molecular level, we show that the class II transactivator (CIITA), the invariant chain (CD74), the MHC class II molecules (RT1-B, RT1-D), as well as cathepsin S, molecules which are responsible for the initial steps of successful antigen presentation, are expressed in activated rat HSCs. We determine that IFN-γ upregulates the mRNA and the protein expression of CIITA, CD74, MHC class II molecules and cathepsin S in activated HSCs. More importantly, IFN-γ increases cathepsin S activity, suggesting a possible involvement of this protease in CD74 processing.
In another study, we have identified a nuclear cysteine protease activity partially attributed to cathepsin F, which co-localizes with nuclear speckles. Treatment with retinol/palmitic acid for 48 hours leads to a decrease in nuclear cathepsin F activity, which is coupled to an increase in cystatin B and C transcripts. The inhibition of the cathepsin F activity by a cysteine protease inhibitor and siRNA results in a differential regulation of smooth muscle alpha actin (SMAA) and collagen type I transcripts. These findings indicate a possible link between nuclear cathepsin F activity and the transcriptional regulation of HSC activation markers.

CANONICAL WNT SIGNALING AND HEPATIC STELLATE CELLS

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Our research has demonstrated the importance of adipogenic transcriptional regulation in the maintenance of hepatic stellate cell (HSC) quiescence (*J Biol Chem* 279:11392, 2004; 280:4959, 2005; 280:40650, 2005). This concept has an eminent implication because molecules that render anti-adipogenic effects may now need to be considered as pro-fibrogenic mediators. Indeed, many known inhibitors of adipogenesis such as leptin, TGFβ, TNFα, and β-adrenergic agonists, activate HSCs. One notable class of anti-adipogenic mediators is the Wnt family of proteins. Wnts bind to their Frizzled (Fz) receptor-LRP co-receptor complexes to transduce canonical Wnt signaling which culminates to β-catenin stabilization and nuclear translocation, and β-catenin-Tcf dependent transcriptional activation. In this study, we tested the roles of Wnt signaling in HSC activation *in vitro* and *in vivo*. Real-time PCR screening demonstrates 3~12 fold induction of canonical (Wnt3a and 10b) and non-canonical (Wnt4 and 5) Wnts, their receptors (Frz-1 and 2) and co-receptors (LRP6 and Ryk) in rat primary HSCs activated *in vitro* on plastic or *in vivo* by cholestatic liver fibrosis induced by common bile duct ligation. Activated canonical Wnt signaling is demonstrated by increased nuclear β-catenin level, enhanced Tcf binding to DNA, and heightened Tcf-promoter activity which is suppressed by ectopic expression of the nuclear β-catenin antagonist Chibby or the Wnt co-receptor antagonist Dkk-1. Adenoviral expression of Dkk-1 in culture-activated HSCs, causes a reduction in nuclear β-catenin, a morphologic reversal to the quiescent phenotype including increased intracellular lipids, represses HSC activation markers such as PCNA and α1 (I) procollagen, and more importantly up-regulates two key adipogenic transcription factors, PPARγ and C/EBPα. Finally, this viral mode of Dkk-1 transduction in the liver via splenic injection significantly attenuates the development of cholestatic liver fibrosis in mice. In summary, these results demonstrate that activated canonical Wnt signaling underlies at least in part, the anti-adipogenic mode of HSC activation and serves as a potential molecular target for anti-fibrotic modality. (Supported by NIH grants P50AA11999, R24AA12885 and Department of Veterans Affairs).

PROLIFERATION OF HEPATIC STELLATE CELLS IN RATS WITH ACUTE HEPATITIS IS DEPENDENT ON VAGAL INNERVATION VIA MUSCARINIC ACETYLCHOLINE RECEPTOR SUBTYPE 2

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Background: In galactosamine induced hepatitis, hepatocytes lose their replicative capacity and hepatic progenitor cells (HPC) and hepatic stellate cells (HSC) become the driving forces behind liver tissue regeneration. The liver receives parasympathetic innervation through the hepatic branch of the vagus nerve, whose main efferent neurotransmitters are acetylcholine (ACh) and vasoactive intestinal peptide (VIP). We have shown that HPC express M3 and VIP2 receptors and mRNA for several ACh receptors. Moreover, we showed that hepatic branch vagotomy (HV) inhibits the proliferation of HPC. To examine whether HSC proliferation could also be neurally modulated, we determined the neurotransmitter receptor expression profile of HSC and their proliferation in acute hepatitis after surgical and pharmacological denervation.
Methods: RT-PCR with receptor-specific primers was performed on total RNA from isolated HSC. Total rat brain RNA was used as a positive control. Identification of amplification products was achieved by sequencing.
Male Wistar rats were subjected to HV (n=20) or sham operation (n=19). A second group of rats received IP injections of atropine (ATR, n=11), mecamylamine (MEC, n=16) or saline (Control, n=20). In all rats, acute hepatitis was induced by a single intravenous injection of galactosamine (500mg/kg) and lipopolysaccharides (1μg/kg). Rats were sacrificed 12 and 48 hours later, the time points with maximal necrosis and maximal numbers of HSC, respectively. Frozen liver tissue sections were stained for HSC using polyclonal antibodies against desmin (for periportal HSC) and GFAP (for pericentral HSC). Numbers of HSC were quantified using image analysis software (Olympus).
Results: We found that HSC express mRNA for muscarinic receptor subtype 2, for nicotinic receptor subtype α1, α5 and β1, and for VIP1 receptor. HV caused significantly less desmin-positive HSC than sham operation at 12 and 48h (p=0,008 and p=0,01 respectively), and significantly less GFAP-positive HSC at 48h (p=0,03). Administration of ATR (and thus blocking of muscarinic receptors) also resulted in less desmin- and GFAP-positive HSC at both 12h ($p= 2{,}7.E^{-19}$ and p=0,008) and 48h (p=0,03 and p=0,02), as compared to Control. Blocking of nicotinic receptors by administration of MEC caused less desmin-positive HSC at 12h (p=0,009) but not at 48h and for GFAP-positive HSC no differences were found between MEC and Control. These results show that inhibition of vagal neurotransmission significantly decreases the proliferation of HSC in acute hepatitis. The stimulatory effect of the vagus nerve on HSC might occur via the direct action of vagal neurotransmitters on the receptors expressed on these cells. Moreover, since the effect of ATR administration seems to be more pronounced than the effect of MEC, the vagus nerve might exert its effect on HSC mainly through muscarinic receptors.
Conclusion: As we previously demonstrated for HPC, intact vagal innervation also appears necessary to provoke a proliferative stimulus for HSC, to ensure repair, repopulation and regeneration of liver tissue. The latter effect is most likely exerted through direct binding of ACh on muscarinic receptor subtype 2, expressed by HSC.

EPIGENETIC REGULATION OF HEPATIC STELLATE CELLS

Jelena Mann

Epigenetics literally means “above the genetics” but is now generally used as a term to describe changes in genes expression that occur without the need for changes in DNA sequence. Epigenetic variation involves chemical modifications of DNA and chromatin of which the most extensively studied are DNA methylation and post-translational modifications of histone lysine and arginine tails. Other epigenetic mechanisms include the actions of microRNAs and higher order structural organisation of chromatin within the nucleus. At the cellular level, modulation of these “epigenetic marks” significantly influences the differentiation status and functions of the cell. However, the impact of epigenetics extends much further than the cellular level. A major outcome of human and animal genome mapping projects was the realisation that the information encoded in DNA is insufficient to explain the gross phenotypic differences between species, let alone the more subtle differences seen between individuals within the same species. It is now emerging that so-called “epigenetic factors” fine tune the genome to shape body form and physiology. More remarkably, we are learning that epigenetic marks can become stable phenotypic instructions that not only survive mitosis but are also stable throughout meiosis, zygote formation and animal development. Subtle changes to these stable epigenetic marks can generate an “epigenetic memory” of past events such as exposure to toxins, alterations in dietary conditions and even stress, which are passed on through subsequent generations. The consequences can be profound for human physiology resulting in altered susceptibility of the individual to the development of heart disease, hypertension, diabetes, alcoholism etc.

The progression of chronic liver disease is not a predictable uniform process, but instead the development of cirrhosis and cancer differs enormously between individuals even where the primary cause of liver injury is identical. Genetic factors have been described which have the potential to impinge on susceptibility to disease progression. However, genetic polymorphisms are alone insufficient to explain for example, why only 25% of alcoholics develop cirrhosis. Our group in Newcastle are working on the hypotheses that epigenetic factors impinge at the cellular level to regulate fibrogenesis but may also operate at a higher level via epigenetic memory to help dictate susceptibility to disease progression.

In this lecture, I will explain the basic principles of epigenetic control of gene expression, describe how DNA methylation and histone structure influence gene transcription and introduce the some of the key epigenetic regulators of these modifications (e.g. methyl-DNA binding proteins, histone methyltransferases and chromatin remodelling enzymes). I will place these regulators into physiological context using my own work to explain how they control the activity of fibrogenic hepatic stellate cells. Finally, I will provide examples to explain the concept of epigenetic inheritance of physiological states and disease susceptibility.

RAT HEPATIC STELLATE CELLS ACQUIRE RETINOID RESPONSIVENESS AFTER ACTIVATION IN VITRO

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Introduction: Activation of hepatic stellate cells (HSCs) is a key process in liver fibrogenesis and retinoid loss is a remarkable feature of activated HSCs. However, relevance of retinoid signaling in liver fibrogenesis is still in controversy. For example, while several studies reported decreased retinoid signaling in activated HSCs both in vivo and in vitro, others reported that HSCs could respond to retinoids after activation in vitro. We first questioned why cellular retinol-binding protein I, one of the well characterized RAR target genes containing RARE, was up-regulated during HSC activation, in spite of the reported diminished retinoid signaling in activated HSC. We show here that retinoid responsiveness is increased during rat HSC activation in vitro, presumably through post-transcriptional up-regulation of RARalpha gene expression.
Materials and methods: Male Wistar rat livers were perfused with bacterial collagenase solution, and dispersed cells were centrifuged F and pelleted cells were further purified by density gradient centrifugation with Percoll/RediGrad. The layer containing HSCs was collected, and cells were seeded on uncoated plastic dishes or glass bottom dishes. Protocols for animal experimentation were approved by the Animal Research Committee, Akita University School of Medicine. All animal experiments adhered to the "Guidelines for Animal Experimentation" of the University.
Results: By using an RARE-driven reporter assay, we show that retinoid responsiveness is increased during rat HSC activation in vitro. Am580, a selective RARalpha agonist, increased the reporter response, indicating that the transcriptional activity in activated HSCs was mediated, at least in part, by RARalpha subtype. Quantitative RT-PCR of RARalpha, beta and gamma transcripts and Western blotting of RARalpha, beta and gamma proteins during HSC activation in vitro showed that the increased retinoid responsiveness is ascribed to the post-transcriptional up-regulation of RARalpha, and to some extent, beta gene expression. Immunocytochemistry showed that RARalpha proteins were mainly distributed as many spots in cytosol throughout the activation period. Addition of ATRA during HSC activation lowered cytosolic RARalpha protein levels. Treatment of retinoid antagonist LE540 alone did not affect both RARalpha protein quantity and localization, but attenuated the effect of ATRA.
Conclusions: Retinoid responsiveness is increased during rat HSC activation in vitro, through post-transcriptional up-regulation of RARalpha gene expression. This result can explain previous paradoxical observations of reduced mRNA expressions of RARs and remaining retinoid responsiveness in activated HSCs.

INVOLVEMENT OF ADRP AND TIP47 IN LIPID DROPLETS FORMATION OF HEPATIC STELLATE CELLS

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Hepatic stellate cells (HSCs) lie in the perisinusoidal space and play central role in production and degradation of extracellular matrix (ECM) components in the liver. The cells also store about 80% of total body vitamin A as retinyl esters (RE) in their lipid droplets (LDs) and play key roles in hepatic uptake and release of retinoids. Under pathological conditions such as liver fibrosis, the cells proliferate rapidly, produce a large amount of ECM components, and lose their LDs and RE. Although the existence of the LDs rich in RE is the utmost character of stellate cells, the mechanisms of formation and disappearance of them are not well understood. It is reported that LDs in adipocyte harbor PAT proteins around them, which were named after perilipin, adipocyte differentiation-related protein (ADRP) and TIP47. Although the function of PAT proteins is unclear, it is suggested that they are involved in LDs formation and lipid metabolism. In this study, we show that ADRP and TIP47 were localized around LDs of HSCs.

HSCs were separated from male Wistar rat livers with collagenase perfusion followed by Percoll density gradient centrifugation. The freshly separated cells were seeded on glass bottom dishes for analyses next day (A) or seeded on plastic dishes for activation by subculture. The activated cells were seeded on glass bottom dishes and administrated with retinol to induce LDs containing RE (B). LDs were detected with BODIPY 493/503 and vitamin A-autofluorescence. ADRP and TIP47 were detected with immunofluorescence. ADRP localized around LDs emitting vitamin A-autofluorescence in both A and B. TIP47 also localized around the LDs in B. However, it did not localized around the LDs but diffusely localized in cytosol in A. The manner of involvement of the two proteins in LDs formation of HSC will be discussed.

HEPATIC STELLATE CELLS EXPRESS TIM-2: A POSSIBLE MEDIATOR OF FERRITIN-INDUCED NFκB ACTIVATION AND PROINFLAMMATORY GENE EXPRESSION.

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Introduction ~ Ferritin is comprised of 24 individual proteins of either heavy (H) or light (L) subtypes. The main function of ferritin is the storage or iron in a non-toxic but biologically available form, however, during times of inflammation linked to or independent of iron status, serum ferritin is significantly elevated. The exact reason for this elevation is unclear but the expression of H and L ferritins is known to be regulated by inflammatory mediators such as TNFα and IL-1α. The recent discovery that the regulator of Th2 dependent immune responses, T-cell immunoglobulin and mucin domain-2 (Tim-2) is a receptor for H ferritin endocytosis further suggests that ferritin may have physiological roles other than iron storage. Hepatic Stellate Cells (HSC) express a receptor for ferritin that mediates internalisation and we have recently shown that ferritin upregulates a number of proinflammatory fibrogenic mediators in HSC, including IL-1β, RANTES, ICAM1 and iNOS.
Aims ~ This study was designed to (i) examine the kinetics of Tim-2 expression with HSC culture-induced activation, (ii) further characterise the signaling cascade activated in response to ferritin, and (iii) define the role of Tim-2 in mediating the this proinflammatory signaling event.
Methods ~ HSC were isolated from rat liver and cultured on plastic culture dishes for up to 6 days. Whole cell protein and total RNA were isolated at regular 24 hour time points up to day 6. Activated HSC (day 5) were treated with tissue ferritin (iron replete), or the iron-free recombinant human H ferritin (rHF) and L ferritin (rLF) molecules, in the absence or presence of various inhibitors targeted at specific signaling proteins. Protein expression and phosphorylation status were assessed by western blotting and immunoprecipitation assay, and mRNA expression was investigated using quantitative real time PCR.
Results ~ Real time PCR analysis of total RNA isolated from activating HSC demonstrated that Tim-2 mRNA is present 24 hours post isolation and is slightly but significantly elevated at day 5 to 2.2-fold versus day 1. Tim-2 protein was also present in HSC from quiescence to activation, however, there was no significant change in expression over the differentiation period. Western blotting showed marked phosphorylation of the PI3-kinase target motif YXXM, as well as PKCz, p42/p44 (MAPK) and IKK, in a time-dependent manner following HSC incubation with 10 nM tissue ferritin, rHF or rLF. Tissue ferritin induced a 48-fold increase in the expression of the NFκB-regulated gene IL-1β. Pharmacological inhibition of PI3-kinase, PKCz, MAPK and IKK was able to significantly inhibit (80-100%) the tissue ferritin-induced expression of IL-1β. Immunoprecipitation of proteins containing a phosphorylated YXXM PI3-kinase target motif induced following tissue ferritin, rHF and rLF incubation was performed. Precipitated proteins were then blotted for the presence of Tim-2. Data demonstrated no observable change in the levels of Tim-2 YXXM phosphorylation following HSC incubation with ferritin.
Conclusions ~ This study demonstrates for the first time the expression of Tim-2 by rat HSC. Our data implicates a number of signaling intermediaries induced by tissue ferritin in HSC. As iron-free rHF and rLF were able to elicit similar cellular responses to tissue ferritin, this suggests that the effects of ferritin on HSC may be independent of ferritin-bound iron. These results provide further support for a role for ferritin as a mediator of inflammation in addition to its role as an iron storage protein. Our results also suggest that if Tim-2 is mediating the effects of ferritin on HSC it is not through the internal YXXM PI3-kinase phosphorylation motif.

CELLULAR DISTRIBUTION OF LECITHIN: RETINOL ACYLTRANSFERASE (LRAT) AND CELLULAR RETINOL BINDING PROTEIN-1 (CRBP-1) IN HUMAN NORMAL AND PATHOLOGICAL LIVERS

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Background/Aims: In a normal liver, hepatic stellate cells (HSC) store 80-90% of hepatic retinoid in characteristic lipid droplets as ***fat-storing cells***. HSC are activated and change their phenotype under conditions of liver damage, often losing their lipid droplets, promoting liver fibrosis. To examine how much HSC contributes to liver fibrosis, it is necessary to develop a structural and functional HSC marker for *in situ* studies. Although several HSC markers have been reported, those are hardly associated with particular HSC functions. Lecithin: retinol acyltransferase (LRAT) is a physiological retinol esterification enzyme in a liver and its activation is occurred especially in HSC. Cellular retinol-binding protein-1 (CRBP-1) also mediates retinoid metabolism and retinol bound CRBP-1 is a substrate of LRAT. Therefore interaction between LRAT and CRBP-1 seems to contribute the retinol esterification of HSC as ***fat-storing cells***. The present study is to reveal whether co-expression of LRAT and CRBP-1 could be a potential and relevant tissue marker for HSC. To do this we detected the expression of LRAT in HSC during fibrotic/cirrhotic liver disease through correlation with CRBP-1.

Materials and Methods: An antibody specific to human LRAT was generated on the basis of their amino acid sequence. To examine the distribution of LRAT in detail, other antibodies against CRBP-1 and α-smooth muscle actin were also prepared. These antibodies were used for immunohistochemical examination in normal and diseased human liver including chronic hepatitis, alcoholic liver damage, non-alcoholic steatohepatitis, and liver cirrhosis.

Results: In a normal liver, quiescent HSC in sinusoid co-expressed both LRAT and CRBP-1, while portal fibroblasts did not. In a fibrotic and a cirrhotic liver, HSC in the space of Disse co-expressed both LRAT and CRBP-1 and a part of portal and septal myofibroblasts co-expressed both LRAT and CRBP-1. Portal and septal fibrotic fascicles strongly expressed α-smooth muscle actin, whereas they did not expressed LRAT.

Conclusions: Our study demonstrated that LRAT could be an excellent alternative marker to identify quiescent HSC as well as CRBP-1 in a normal liver. In a fibrotic/cirrhotic liver, the different pattern of expression for LRAT and α-smooth muscle actin enabled to distinguish between various subsets of fibroblastic cells involving fibrogenesis. However, we suggest that small amount of cells co-expressing LRAT and CRBP-1 in septa in fibrotic liver still maintain original function of quiescent HSC with respect to vitamin A metabolism.

EXPRESSION OF THE TRANSCRIPTION FACTOR, EARLY GROWTH RESPONSE (EGR)-1, IS A CRITICAL REGULATOR OF CARBON TETRACHLORIDE-INDUCED FIBROSIS IN MICE.

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Hepatic fibrosis is a common outcome associated with chronic liver disease, regardless of etiology, and is characterized by excessive extracellular matrix deposition. The transcription factor, early growth response (Egr)-1 is an immediate early gene that is both rapidly and transiently induced in response to a variety of stressors. Egr-1 has been described as a master regulator of inflammatory disease in animal models as it regulates a cadre of genes characteristic of this disease class, such as TNFα, MIP2, and ICAM1. Expression of genes involved in the wound healing response, including bFGF, VEGF, TGFβ, PDGF, is also regulated by Egr-1. Because Egr-1 modulates the expression of inflammatory genes and those important for matrix turnover, we hypothesized that Egr-1 would play important roles in both the development and resolution of fibrosis.

To determine the contribution of Egr-1 to CCl_4-induced hepatic fibrosis, we performed our studies in wild type (C57BL/6nTac) and *egr-1-/-* mice. In wild type mice, Egr-1 is induced rapidly after a single CCl_4 exposure peaking at 2h and returning to baseline by 4h post CCl_4. After 5 weeks of biweekly CCl_4 injections to wild type and *egr-1-/-* mice, Sirius red staining of liver sections demonstrated hepatic fibrosis. In wild type mice, fibrosis was intermittently bridging whereas in *egr-1-/-* mice fibrosis was completely bridging. In addition, distinct fibrotic septa were present in livers of wild type mice whereas collagen deposition was diffuse in the livers of *egr-1-/-* mice. The altered distribution of collagen deposition in *egr-1-/-* mice was associated with enhanced and prolonged expression of a smooth muscle actin (SMA), a marker of hepatic stellate cell activation, in the liver of *egr-1-/-* mice compared to wild type controls. Gelatin zymography demonstrated that matrix metalloproteinase (MMP) activity was greater in the livers of *egr-1-/-* mice 7d after cessation of chronic CCl_4 exposure and was associated with enhanced MMP2 and MMP14 expression measured by real time PCR. To investigate early events in CCl_4-induced fibrogenesis, we gave mice a single dose of CCl_4 and examined hepatic injury. ALT activity was 2-fold and 4-fold greater in the plasma of *egr-1-/-* mice compared to wild type mice 18h and 72h after CCl_4 exposure, respectively. Seventy-two hours after CCl_4 exposure, there was a greater amount of TUNEL positive staining in the livers of *egr-1-/-* mice compared to wild type mice. Collectively, these data suggest that Egr-1 plays a protective role in acute liver injury and fibrosis induced by CCl_4. This work was supported by grants to M.T.P AA015833 and L.E.N AA13868 from the NIAAA.

VITAMIN E SUPPRESSED THE PROLIFERATION AND INDUCED APOPTOSIS OF "ACTIVATED" HEPATIC STELLATE CELLS.

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Hepatic stellate cells are localized in perisinusoidal space of liver. In pathological conditions, such as liver fibrosis or liver cirrhosis, they lose lipid droplets, morphologically change to myofibloblast-like phenotype and acquire increased proliferation activity. They also become synthesizing relatively large amount of matrix components including fibrillar collagens, what is called the "activated" state. Vitamin E is composed of eight different forms: alpha-, beta-, gamma- and delta-tocopherols and alpha-, beta-, gamma- and delta-tocotrienols. Tocol is lacking methyl groups attached to the chromanol ring and utilized for a comparative substance. All vitamin E molecules are well known as antioxidants, however, recent research developments demonstrated that they possess powerful cholesterol lowering, platelet adhesion inhibition and anti-cancer properties.

We have investigated the treatment for liver fibrosis based on the concept of targeting "activated" hepatic stellate cells by introducing "non-activated" condition. In this study, four tocopherols and tocol were applied to the "activated" hepatic stellate cells and examined the effects on proliferation activity of stellate cells. Rat hepatic stellate cells were prepared by collagenase perfusion and the "activated" state was induced by culture in vitro and passaged once. Among four tocopherols and tocol, relatively high proliferation inhibition effects were detected in delta-tocopherol and tocol. In addition to proliferation inhibition, cell detachment and apoptosis were observed in delta-tocopherol treated and tocol treated cells in a dose response manner. These data suggest that vitamin E is effective for the treatment of hepatic fibrosis and liver cirrhosis.

BLOOD PLATELETS IN CHOLESTATIC LIVER DISEASE

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Introduction: After the red blood cell, platelets are the most abundant cells of the hepatic sinusoid. These cells are increasingly recognized as active players, not only participating in haemostasis but also in various disease processes. In a recent study, platelets were shown to contribute to cholestatic liver disease by deteriorating microperfusion and assisting in leucocyte recruitment. Blockage of platelet activation or platelet depletion could both prevent this, resulting in significantly less liver damage (lower AST and ALT).

In cholestatic liver diseases, platelet function is impaired as evidenced by platelet aggregometry (i.e. the gold standard test of platelet function). However, other assays demonstrated a platelet hyperactivity (thromboelastography, sonoclot analysis) and intracellular calcium release (the final common pathway to aggregation) in platelets is increased in bile duct ligated (BDL) rats. Our aim is to characterize platelet function in BDL rats and to compare with thio-acetamide (TAA) induced liver fibrosis.

Materials and methods: 6 BDL and 6 sham-operated rats at day 14 post-surgery (group1) and 6 TAA and 6 controls at week 12 of intoxication (i.e. fibrotic stage; group2), were sacrificed, for liver tests, complete blood count, whole blood platelet-aggregometry (stimulation with collagen and ADP), platelet electron microscopy, thrombin-antithrombin complexes and liver histology (image analysis of percentage sirius red stained surface). A crossover aggregometry experiment with BDL platelets in Sham plasma and vice versa was also performed, to distinguish platelet-related from plasma-related effects.

Results: In group 1: BDL rats versus sham-operated rats showed clearly elevated liver tests (alkaline phosphatase, AST, ALT, bilirubin). There was a prominent ductular reaction on histology and liver fibrosis (6.57±2.98% sirius red stain versus 0.65±0.36, p=0.002). On whole blood count there was no difference in platelet count, but a decreased platelet volume (6.33fl versus 7.85fl, p=0.002) and a marked inflammatory state. There was a decreased whole blood aggregability in response to ADP and collagen (stimulation with ADP 0.8μM: 50.4±27.2% versus 93.4±7.8% aggregation, p=0.004; stimulation with ADP 1.7μM: 93.0±13.1% versus 100±0% aggregation p=0.015; stimulation with collagen 1.25 ng/ml: 60.0±32.1% versus 94.3±12.9% aggregation, p= 0.015). Thrombin-antithrombin complexes were identical and platelet electron microscopy was normal.

In group 2: TAA rats showed also less abnormal liver tests but equal degree of fibrosis (6.23±2.28%) compared to BDL and normal platelet counts and platelet volume compared to controls. Compared to controls there was a normal aggregation in response to ADP (stimulation with ADP 0.8μM: 87.8±21.9% versus 97.2±3.9% aggregation, p=NS; stimulation with ADP 1.7μM: 99.35±1.6% versus 100±0% aggregation, p=NS) and a decreased response to stimulation with collagen 1.25 ng/ml: (63.8±29.4% versus 99.7±0.5% aggregation, p= 0.026). The aggregations to ADP were significantly more preserved than in the BDL model. Thrombin-antithrombin complexes were not elevated.

Cross-over experiments using optical platelet aggregometry, showed normal platelet function of BDL platelets when resuspended in sham plasma and decreased sham platelet function when resuspended in BDL plasma.

Conclusions: Blood platelets in cholestatic liver disease (BDL) demonstrate normal ultrastructure and normal function when resuspended in sham plasma. However, in BDL whole blood aggregation testing, the platelet function is clearly inhibited and more so than in other models of liver cirrhosis (TAA). This is most pronounced in stimulation with ADP, which is suggestive of a role of the cholestasis-induced presence of 5'-nucleotidase, a well-known marker of cholestasis.

Since intrinsic platelet function is normal, platelets could contribute to development of cholestatic liver disease.

STUDY ON A RABBIT MODEL OF STEATOHEPATITIS THAT DEVELOPS LIVER CIRRHOSIS

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Introduction: We previously reported a model of non-alcoholic steatohepatitis (NASH) that was produced by feeding rabbits with 1.25% cholesterol diet for 8 weeks. The liver of this model exhibited steatosis, oxidative stress, inflammation, iron deposition in hepatocytes and macrophages, and fibrosis whose characteristics are very similar to human NASH liver. In this study, we have tried to improve this model, resulting in the production of an advanced model having cirrhosis of the liver, a life-threatening stage of chronic liver disease.

Materials and Methods: Japanese white rabbits were fed 0.75% cholesterol diet including 10% corn oil for 9 months. After the sacrifice, the rabbit livers and spleens were removed and used for histological evaluations of steatosis, oxidative stress, inflammation, and fibrosis by using H&E staining, Sirius red staining, and Sudan III staining. The remaining liver tissues were utilized for evaluating mRNA expressions of interleukins 1 and 6, TNF alpha, MCP-1, TGF-beta, collagen 1A1, TIMPs, and MMPs by real-time PCR. The level of lipid peroxide and 4-hydroxynonenal in the liver evaluated oxidative stress by western blotting and immunohistochemistry.

Results: The livers looked whitish and the surface exhibited irregularity. Steatosis of hepatocytes was evident. Staining showed that inflammatory cells including RAM-11-positive macrophages accumulated in the sinusoids and the formation of fibrotic septum composed of collagens and activated stellate cells crossed over central-central and central-portal veins, indicating the reconstruction of parenchyma. Triglyceride, lipid peroxide, and total cholesterol in sera of model rabbits were increased 1.9-, 38-, and 1277–fold, respectively, compared to the control. Real-time PCR revealed the increase of mRNA levels of collagen 1A1 (32-fold), MMP-2 (11-fold) and MMP-9 (170-fold), TIMP-1 (20-fold) and TIMP-2 (21-fold) and HO-1 (32-fold). The spleens exhibited splenomegaly and the accumulation of foamy macrophages.

Conclusions: The livers of this model showed liver fibrosis of stage 3 (bridging fibrosis) or 4 (cirrhosis) by Brunt's staging score. Thus, this rabbit model is useful to study the pathogenesis and molecular analysis of human NASH.

C-REACTIVE PROTEIN MINIMIZES EXPERIMENTAL HEPATIC FIBROSIS BY INHIBITING TRANSACTIVATION OF HEPATIC STELLATE CELLS

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Background and aims: C-reactive protein (CRP) is an acute phase reactant that participates in inflammatory responses involving complement activation. Recently, minimal increment in serum CRP levels has been recognized as a sensitive marker of micro-inflammation in atherosclerosis and NASH. In the present study, we investigated the direct effect of CRP in transactivation of isolated hepatic stellate cells (HSCs), and experimental hepatic fibrogenesis. **Methods:** HSCs isolated from male Wistar rats were cultured in DMEM supplemented with 10% heat-inactivated FBS up to 7 days. Cell proliferation was evaluated by BrdU incorporation, and phosphorylation of ERK1/2 and Akt was detected by Western blotting. For experiments in vivo, rats were given repeated injections of thioacetamide (TAA, 200 mg/kg, i.p. 3 times/week) for 6 weeks, simultaneously treated with purified rat CRP (1 mg/kg, i.p.) starting from the third week for 4 weeks. Hepatic fibrosis was assessed by picro-sirius red staining and smooth muscle α-actin (αSMA) immunohistochemistry. Further, mRNA levels for α1(I)procollagen (COL1A1) and TGF-β were detected by Northern blotting. **Results:** Human CRP blunted PDGF-induced uptake of BrdU in 3 day-cultured HSCs in a dose-dependent manner, reaching 57% inhibition at a concentration of 1 mg/dl ($p<0.05$, vs. PDGF alone). The similar tendency was observed in 7 day-cultured, activated HSCs. This anti-proliferative effect was also observed in rat CRP, which lacks complement activation capability. PDGF-induced phosphorylation of ERK1/2 was also inhibited by CRP; however, phosphorylation of Akt was not decreased by addition of CRP. On the other hand, TAA-induced hepatic fibrosis was ameliorated markedly when purified rat CRP was given simultaneously for 4 weeks. Indeed, induction of αSMA in the liver was prevented remarkably, indicating that CRP inhibited transactivation of HSCs in vivo. TAA-induced increases in hepatic COL1A1 and TGF-β mRNA levels were also blunted significantly. **Conclusions:** These findings clearly indicated that CRP has an anti-fibrogenic property involving direct inhibitory effect on HSC transactivation. The anti-proliferative effect of CRP on HSCs appears to be independent of complement activation, and most likely involves inhibition of the MAP kinase pathway. It is therefore postulated that CRP produced from hepatocytes plays a regulatory role in progression of hepatic fibrosis.

STUDIES ON DETECTION AND PREVENTION OF HEPATIC FIBROSIS TARGETING TGF- ß ACTIVATION REACTION

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Transforming growth factor (TGF)-ß is the most fibrogenic cytokine that plays a pivotal role in the pathogenesis of liver diseases. It is produced as a high molecular weight latent form, and thus must be activated before exerting its biological activities. TGF-ß activation is the reaction, by which 25 kD active TGF-ß molecule is released from the latent complex. Using animal models, we showed that TGF-ß is activated by proteases such as plasmin (PLN) and plasma kallikrein (PLK) during pathogenesis of liver fibrosis and impaired liver regeneration, respectively, and that blockage of these activation reactions with low molecular weight protease inhibitors prevented the development of the diseases.

To confirm that a similar activation reaction occurs in human liver disease, we determined that PLN and PLK cleaved between K56-L57 and R58-L59 in LAP portion of human latent TGF-ß, respectively, and produced antibodies that specifically recognize the neo-epitopes formed by protease degradation, namely the cut ends of each cleavage site. The antibodies against the PLK-cut ends, namely anti-R58 and anti-L59 antibodies recognized the degradation products of PLK-cleaved latent TGF-ß produced during PLK-mediated activation of latent TGF-ß, but did not recognize uncleaved latent TGF-ß and only weakly recognized PLN-cleaved latent TGF-ß degradation products. A similar result was obtained with the antibodies against the PLN-cut ends, namely anti-K56 and anti-L57 antibodies.

We have established a novel sandwich ELISA (sELISA) using a combination of the antibodies recognizing LAP and the L^{59} cut ends containing LAP fragments produced during the process of the activation of TGF-ß by PLK. Using this sELISA and TGF-ß1 Emax ImmunoAssay system, we measured parallel generation of LAP degradates and active TGF-ß1 in proportion to the activation of the hepatic stellate cells (HSCs). Furthermore, using this sELISA we measured the concentration of LAP degradates released in the serum in rat fibrosis models including bile duct ligation model, and compared with hepatic contents of hydroxyproline (HDP). Serum levels of LAP degradates in the fibrotic animals were 3-fold higher than those in the control animals (no treatment or sham operation). The increase showed a good correlation with hepatic HDP levels. We are now applying this ELISA for patients.

Next, we produced peptides containing these cleavage sites as well as their decoy peptides in which cleavage site amino acids were all mutated to either A or Q, and examined the effect of these peptides both *in vitro* and *in vivo*. Western blotting with anti LAP antibody showed inhibition of LAP cleavage reaction by these peptides. TGF-ß ELISA and PCR showed reduced generation of active TGF-ß and suppression of HSC activation with these peptides. Namely, these peptides efficiently suppressed the TGF-ß activation reaction and prevented the activation of HSCs in culture. Furthermore, these peptides were effective to prevent TGF-ß-dependent impaired liver regeneration observed in LPS-pretreated partially hepatectomized mice. Histochemical staining with anti-proliferating cell nuclear antigen (PCNA) showed the improvement of impaired liver regeneration in animals administered with these peptides at 10 mg/kg/day.

These results suggest that the LAP degradates produced during the TGF-ß activation reaction may be a promising novel biomarker for hepatic fibrogenesis, and decoy peptides might be novel new agents against liver diseases targeting TGF-ß activation reaction.

EXTRACELLULAR MATRIX IN SINUSOIDAL WALL OF FROZEN MAMMOTHS

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To examine characteristics of extracellular matrix (ECM) components supporting sinusoidal wall (scaffolding function) of the liver, we analyzed livers of 2 frozen baby mammoths died about 40,000 years ago and buried in permafrost in Siberia.

We observed the livers of the 2 mammoths (kept in fixatives in Russian Academy of Sciences in St. Petersburg) by light and electron microscopy, scrutinized localization of ECM components by immunofluorescence, and analyzed amino acid contents.

The livers were preserved at gross anatomical and histological levels. Sinusoidal walls of the liver were kept. Ultrastructure of ECM components, namely fibrillar structure having characteristic pattern of cross striation and basement membrane structure, were clearly demonstrated by transmission (TEM) and scanning electron microscopy (SEM). Type I and type IV collagens were shown in ECM components by immunofluorescence.

These results indicate that ECM components including collagen were stable and preserved in the livers of these frozen mammoths for 40,000 years. These findings suggest that three-dimensional structure of ECM is important for maintaining gross and histological morphology of the sinusoidal wall in the liver.

A STUDY OF MORPHOLOGICAL CHANGES IN LAMPREY LIVER DURING THE COURSE OF METAMORPHOSIS WITH SPECIAL REFERENCE TO HEPATIC STELLATE CELLS

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We have reported that a lamprey (*Lampetra japonica*), the most primitive vertebrate, has hepatic stellate cells (HSCs) storing an abundance of vitamin A in their cytoplasm. Similar cells storing vitamin A were present in the intestine, kidney, gill, and heart (Wold, H. L., et al.: Vitamin A distribution and content in tissues of the lamprey (Lampetra japonica). Anat Rec., 276A, 134-142, 2004). Lamprey eggs hatch in a river, the larva transform into the adult and then migrates to the sea. The adult lamprey becomes a parasite on a big sea fish and obtains nutrients from the body fluid of the fish. When the lamprey enters the spawning migration stage, it leaves the fish and goes up a river without taking food. The bile ducts of larval lamprey degenerate and disappear during metamorphosis, so that no bile duct is observed in the adult liver. While the highly regulated nature of lamprey biliary atresia offeres a valuable model for studyng liver pathgenesis of human biliary atresia, jaundice and a hepatic dysfunction aren't observed in the adult lamprey.

We performed microstructural analyses of bile duct degeneration of lamprey in larval and spawning stages by transmission electron microscopy. Furthermore, to estimate the contribution of apoptosis in bile duct degenerating process, we examined tissue sections by TUNEL method and immunofluoresent staining of activated caspase 3 using confocal laser scanning microscopy. In larval lamprey, bile canaliculi, intra- and extra-hepatic bile ducts, and gall bladder were clearly observed. The apoptotic cells were detected in the epithelium of extra-hepatic bile ducts by the TUNEL method in the latter stage of larva. The convoluted bile ducts were surrounded by fibrous deposits of extra-cellular matrix components, where sinusoids were abundant. The HSCs in perisinusoidal space stored lipid droplets and a several liver parenchymal cells constructed bile canaliculi. In the adult lamprey, the entire biliary system and thick periductal fibrosis disappeared. HSCs with large quantities of vitamin A and hepatic parenchymal cells with large amount of lipid droplets were observed, however, hepatic fibrosis and cirrhosis have not been accompanied.

These results strongly suggested that degeneration and disappearance of bile ducts in lamprey were caused by apoptosis of bile duct epithelium during metamorphosis when the larva transformed into the adult. HSCs were probably responsible for fibrosis which accompanies the degeneration of bile ducts.

CRYOPRESERVATION FOR LONG-TERM STORAGE OF HUMAN PRIMARY CULTURED HEPATIC STELLATE CELLS

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BACKGROUND AND AIMS: Primary cultured cells are very useful for examining the morphologic and functional characteristics of the target cells. However, such cells cannot be used repeatedly. This study examined whether cryopreservation can be recommended for long-term storage of human primary cultured hepatic stellate cells (HSCs).

METHODS: We used liver tissues obtained by surgical resection from patients with metastatic liver cancer or hepatocellular carcinoma. The study protocol was approved by the Institutional Review Board of The Kurume University Hospital. HSCs were isolated from the tissues by pronase/collagenase digestion, followed by density gradient centrifugation using OPTIPREP. The cells were then seeded in DMEM containing 20% fetal calf serum, and cultured. After confluence, half of the cells were frozen, and the other half remained in culture at 37° for 1 week. The procedure was repeated once a week for 3 weeks (the non-frozen HSC group). For the passage of cryopreserved HSCs, the cells were thawed, cultured and harvested using 0.05% EDTA trypsin, and seeded for the next passage and cultured for 1 week. The procedure was repeated once a week for 3 weeks (the frozen HSC group). We examined the passaged (passage 0-3) and cryopreserved HSCs (passage 0-3). 1) Identification of the HSC by morphological observations using a light microscope, and immunostaining for αSMA and desmin expressions 2) Cells proliferation assay (growth curve, MTT assay, PI staining for cell cycle), 3) Western blot and Real Time PCR for receptors of PDGF and TGFβ and αSMA expressions.

RESULTS: 1) The purity of HSCs was over 90% in all passages, and the HSC cultures consistently yielded a homogeneous cell population growing to confluence in each passage. The shape of the cells was spindle type and fat droplets were visible in the cytoplasm. αSMA positive HSCs were gradually increased as passage progressed, and the positive cell rate was almost the same in the two groups. 2) The rate of increase in cell number by counting and MTT assay in the frozen HSC group was similar to that in the non-frozen HSC group. The ratio of G1, S, and G2 cell cycle stages was almost the same in the two groups. 3) PDGF receptorα, TGFβ receptor and αSMA mRNA expressions in HSCs were similar among each passage in the two HSC groups.

CONCLUSIONS: From these results, the establishment of a protocol for cryopreservation of human primary cultured HSCs is possible, and the cryopreserved/thawed HSCs in each passage may be useful for examining the function and characteristics of the target cells.

IMMUNOBIOLOGY OF LIVER SINUSOIDAL ENDOTHELIAL CELLS

Percy A. Knolle, MD

The liver is known for its capacity to induce T cell tolerance rather than immunity. We are interested in a particular liver-resident cell population, liver sinusoidal endothelial cells (LSEC) that combine extraordinary scavenger activity with the capacity to present exogenous antigens to CD4 and CD8 T cells. Recently, we have reported that LSEC cross-present circulating antigens to naïve CD8 T cells mediating T cell tolerance. Tolerance-induction by LSEC was shown to be important in oral tolerance and in the development of tolerance towards tumor cells metastasizing to the liver via the hematogenous route thus establishing tumor immune escape. Here, I will discuss the molecular mechanisms that enable LSEC to induce tolerance in naïve CD8 T cells through delivery of co-inhibitory signals via the B7-H1/PD1 receptor ligand interaction. We have discovered that tolerance induction by LSEC involves instructional programming of naïve CD8 T cells that is overcome by exogenous IL-2. Furthermore, control of IL-2 expression in LSEC-stimulated T cells is critical to assure the correct functional outcome of tolerogenic programming. Finally, the initial mechanisms underlying establishment of physical interaction of cross-presenting LSEC with circulating naïve CD8 T cells and their relevance for subsequent tolerance induction will be discussed.

THE PROMETASTATIC ENVIRONMENT OF THE LIVER

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The liver is a major metastasis-susceptible site and majority of patients with hepatic metastasis die from the disease in the absence of efficient treatments. The intrahepatic circulation and microvascular arrest of cancer cells trigger a local inflammatory reaction leading to cancer cell apoptosis and cytotoxicity via oxidative stress mediators (mainly nitric oxide and hydrogen peroxide) and hepatic natural killer cell-derived factors. However, certain cancer cells that resist or even deactivate these anti-tumoral defense mechanisms still can adhere to endothelial cells of the hepatic microvasculature through proinflammatory cytokine-mediated mechanisms. During their temporary residence, some of these cancer cells ignore growth-inhibitory factors while respond to proliferation-stimulating factors released from tumor-activated hepatocytes and sinusoidal cells, which leads to avascular micrometastasis generation in periportal areas of hepatic lobules. Both hepatocytes and myofibroblasts derived from portal tracts and sinusoids (activated hepatic stellate cells) are next recruited into some of these avascular micrometastases, creating a private microenvironment that supports their development through the specific release of both proangiogenic factors and cancer cell invasion- and proliferation-stimulating factors. Moreover, soluble factors from tumor-activated hepatocytes and myofibroblasts also contribute to the regulation of metastatic cancer cell genes. Therefore, the liver offers a prometastatic microenvironment to circulating cancer cells that supports metastasis development. The ability to resist anti-tumor hepatic defense and to take advantage of hepatic cell-derived factors are key phenotypic properties of liver-metastasizing cancer cells. Knowledge on hepatic metastasis regulation by microenvironment opens multiple opportunities for metastasis inhibition at both subclinical and advanced stages of the process. In addition, together with metastasis-related gene profiles revealing the existence of liver metastasis potential in primary tumors, new biomarkers on the prometastatic microenvironment of the liver may be helpful for the individual assessment of hepatic metastasis risk in cancer patients.

NERVE GROWTH FACTOR EXPRESSION BY HEPATIC PARENCHYMAL AND NON-PARENCHYMAL CELLS DURING METASTATIC COLORECTAL DEVELOPMENT IN HUMAN AND MURINE LIVER

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In addition to its contribution to the differentiation and survival of neuronal cells, nerve growth factor (NGF) also plays a role in tumor progression and metastasis. In the liver, expression of NGF is increased during tissue regeneration and hepatocellular carcinoma development, but its role during hepatic metastasis is not well understood.

We investigated NGF and neurotrophin receptor p75 (p75-NTR) expression by cancer and host cells in the hepatic metastasis microenvironment. A tissue-array collection of paraffin-embedded colorectal carcinoma metastasis from 24 patients who had undergone hepatic excision of moderately and poorly differentiated colorectal adenocarcinoma metastases was used. In vivo, the concentration of NGF was determined by ELISA in the hepatic blood from mice affected by hepatic metastases, on the 12th day after intrasplenic injection of C26 colorectal carcinoma cells. Anti-NGF monoclonal antibodies were used to study in situ NGF expression by cancer and stromal cells in hepatic C26 colorectal carcinoma metastases and tumor-unaffected hepatic areas. Finally, primary cultured murine hepatocytes and hepatic stellate cells (HSCs) were incubated in the presence of conditioned medium from murine C26 cancer cells and the production and effects of recombinant murine and endogenous NGF were studied.

NGF immunostaining of metastatic colon cancer cells only occurred in 2 out of 24 patients with hepatic metastases, while around 80% of studied patients had hepatic metastases with NGF-expressing stromal cells. Not statistically significant correlation was demonstrated between NGF immunostaining of tumor-infiltrated stromal cells and cancer cell immunostaining with anti-ki67 antibodies, suggesting that NGF was not involved in the regulation of metastatic cancer cell proliferation. Hepatocytes and hepatic sinusoidal cells showed weak NGF immunostaining, while cholangiocytes had a high immunostaining in the hepatic tissue unaffected by cancer development. Staining with alpha-smooth muscle actin (alpha-SMA) confirmed that sinusoidal immunoreactivity was unequivocally localized to HSCs, but not to other sinusoidal lining cells. Hepatic C26 colorectal carcinoma metastases had an intense NGF immunostaining in those hepatocytes and alpha-SMA-expressing stromal cells located at the invasion front of metastases. NGF-expressing hepatocytes were specifically located among cancer cells and had phenotypic features suggesting epithelial-to-mesenchymal transition. C26 cancer cells did neither express in situ nor secrete in vitro NFG. p75-NTR had a low expression level in normal hepatic tissue, but it significantly increased in hepatocytes and HSCs located around and within hepatic metastases, while C26 cancer cells were negative. Consistent with these in situ findings, NGF significantly increased in the hepatic blood obtained from livers affected by hepatic metastasis development of C26 colorectal carcinoma. NGF concentration was 7 times higher in the supernatants from primary cultured HSCs than in those from hepatocytes. In addition, it significantly increased in the supernatant of HSCs given C26 cancer cell-conditioned medium, and in cultured hepatocytes given tumor-activated HSC-conditioned medium. Finally, recombinant murine NGF dose-dependent increased chemotactic migration, but not proliferation, of HSCs in vitro. Moreover, HSC migration-stimulating activity from tumor-activated hepatocyte supernatant was 50% NGF-mediated in vitro. Our results demonstrate for first time that hepatocytes and sinusoidal stellate cells express neutrotrophin receptor p75 and secrete NGF in response to specific stimulating factors released by cancer cell in the hepatic metastasis microenvironment of human and rodent colorectal carcinoma. Thus, NGF may contribute to metastasis progression through the specific activation of intratumor stromal cell migration.

TUMOR-ASSOCIATED MACROPHAGES IN EXPERIMENTAL MURINE TUMORS

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Introduction. The proteolytic enzymes are essential for tumor angiogenesis, invasion and metastases, the major defining feature of malignancy (Atkinson et al., 2008). Tumor development is characterized by participation of cysteine, aspartic, serine and metallo-proteases, playing the important role both in tumor growth and metastazing. In tumor tissue interaction occurs between tumor cells and macrophages (Mphs). Tumor-associated Mphs are still poor characterized, and their role in tumor development is controversial. Activated Mphs play the important role in the degradation of component of extracellular matrix (ECM) due to production and secretion of metalloproteases (MMPs). Mphs regulate activity of MMPs , especially MMP-2 and MMP-9 (gelatinases), MMP-12 (metalloelastase) and MMP-7 (matrilisin) and in certain conditions – they induce the release of collagenases MMP-1 and MMP-13. TIMPs are the main regulator of MMPs fuctions.
The aim of the study – to investigate tumor-associated Mphs phagocytic function and MMPs / TIMP-1 ratio during tumor growth and metastazing.
Materials and Methods. Experiments were performed in male CBA mice (Institute of Cytology and Genetics RAS, Novosibirsk, Russia). Lewis lung adenocarcinoma, metastazing into lung was used in our study. Intravenous injection of gadolinium (Gd) chloride (10 mg/kg, single) was used as a model of selective liver Mph depression, whereas Gd in the doses of 14 and 28 mg/kg induced depression of liver and lung Mph pools. Uptake and intralysosomal accumulation of Gd by tumor, lung and liver was assayed in tissues by adsorptive spectroscopy method. Gd concentration was measured on a JI-70 spectrometer, Jober Ivon (Catalist, Novosibirsk). MMP activity was determined against fluorogenic subsrate MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Nagase et al., 1994). TIMP-1 concentration in liver, lung and tumor homogenates of mice was measured with help of commercial ELISA kits for mice (Ray Biotech, USA), MMP-2 concentration –by ELISA kits for human/rat/mice (R&D, USA).
Results and conclusions. Uptake of lysosomotropic Gd by liver cells, mainly by Mphs (up to 70% of the injected dose) of intact mice was shown at 12- 24 hrs after injection, followed by long-term accumulation of Gd in liver cells up to 60 days and increased secretion of lysosomal enzymes – β-D-glucuronidase and β-D-galactosidase (at days 2-5, when liver Mph repopulation was noted). Gd (14, 28 mg/kg) significantly decreased the number of metastases into lung when it was administered to mice at dissemination stage (at 8^{th} day after tumor transplantation). In tumor bearing mice uptake of Gd was shown by liver, lung and tumor (less than 10%) tissue Mphs. During tumor growth the total uptake of Gd by tumor increased (as well as tumor weight), however the concentration of Gd in tumor even had a tendency to decrease. Gd uptake by liver cells of intact mice did not influence on serum TIMP-1 concentration and MMP activity in liver; TIMP-1 concentration increased in liver tissue. Total MMP activity was highest in lung (lung>liver>tumor tissue). Tumor growth was followed by increased serum TIMP-1 concentration, but in less degree as compare to mice with tumor pretreated by Gd chloride. Metastases development induced decreasing of lung MMP activity, and Gd pretreatment had no effect on lung MMP activity in tumor bearing mice.
One can *conclude*, that Gd–induced depression of lung macrophages had protective effect during dissemination phase of metastases into lung. Tumor-associated Mphs were able to phagocytose Gd during all period of tumor growth. Increased serum TIMP-1 concentration in tumor bearing mice reflected the disturbances of ECM turnover.

Abstracts: Poster presentations

THE EFFECTS OF RESVERATROL ON HEPATIC PSEUDOCAPILLARIZATION IN AGING MICE FED A HIGH FAT DIET

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Age-related changes in the hepatic microcirculation called pseudocapillarization include reduction in the number and diameter of pores in the liver sinusoidal endothelial cells called fenestrations (1, 2, 3). Reduction of the porosity of fenestrations causes impaired lipoprotein uptake by the liver and potentially contributes to age-related atherosclerosis (4). The sirtuin agonist, resveratrol, increases longevity in mice fed high fat diets, in association with improved lipid profiles and reduction in vascular disease (5). Here we studied the effects of high and low dose resveratrol in high fat-fed mice on aging changes in the hepatic microcirculation using electron microscopy in fixed livers from mice aged 22 months. Resveratrol was associated and greater diameter of fenestrations (61±3 nm high dose resveratrol; 57±3nm low dose resveratrol; 49±2 nm controls, P=0.008, approx 1000 fenestrations and 3 mice per group). There was a non-significant trend towards increased porosity (1.4±0.1% high dose resveratrol; 1.3±0.2 low dose resveratrol; 1.1±0.1% controls). However there was no effect of resveratrol on other changes in the hepatic sinusoid commonly seen in old age such as fenestration frequency, endothelial thickness, basal lamina formation, collagen deposition and inactive fat-filled stellate cells. On electron microscopy, the resveratrol-treated animals had less hepatocyte steatosis, confirming previous light microscopy findings. In summary, resveratrol was associated with increased diameter of fenestrations in fat fed old mice, which provides one plausible mechanism for the improved lipid profiles and vascular health seen with resveratrol that is independent of its widespread effects on ageing and bioenergetics seen in other cell types.

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STABILIN-1 AND -2 RECOGNIZE AND MEDIATE ENDOCYTOSIS OF OXIDIZED LDL

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Background and aim of study - Oxidized low density lipoproteins (Ox-LDL) are found in increased amounts in atherosclerotic plaques, and are implicated in the pathogenesis of the disease. Liver sinusoidal endothelial cells (LSEC) mediate the clearance of vast array of waste macromolecules including Ox-LDL from blood via scavenger receptors (SR) mediated endocytosis.
Stabilin-1 and -2 receptors are major members of the SR family expressed in LSEC. Stabilin-1 and -2 recognize most of known SR ligands including acetylated LDL (Ac-LDL). However this form of Ac-LDL represents only a research model for modified LDL and is not formed *in vivo*. The ability of stabilin-1 and -2 to recognize and mediate endocytosis of OxLDL still remains controversial.
The purpose of this study was therefore to determine the role of Stabilin receptors in the recognition of oxLDL.
Methods **-** LDL was isolated from fresh plasma by density gradient ultracentrifugation. Ox-LDL with different degrees of modification was prepared by incubation with copper sulfate for different lengths of time: 3 h (3hOxLDL) 6 h (6hOxLDL) and 24 h (24hOxLDL). Ligand competition studies were carried out in mouse stabilin-1 transfected HEK 293 cells (mS1-HEK). The uptake of radiolabelled formaldehyde treated serum albumin (^{125}I-FSA) (a known ligand for stabilin-1) by mS1-HEK was conducted in the presence or absence of excess amounts of native LDL and OxLDLs with different degrees of oxidation.
Results - The uptake of ^{125}I-FSA by mS1-HEK was inhibited by the 3 forms of OxLDL: 3hOx-LDL, 6hOx-LDL and 24hOx-LDL (50,6%, 58,5%, and 87% respectively). Inhibition by native LDL was not observed.

Conclusion - We conclude that stabilin-1 recognizes OxLDL. The ability of stabilin 1 to recognize OxLDL oxidized as briefly as 3h may suggest that this receptor may mediate uptake of in vivo formed OxLDL as well.

CLEARANCE OF TFPI WITH AND WITHOUT HEPARIN ADMINISTRATION

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Introduction: Tissue factor pathway inhibitor (TFPI) is the only endogenous serine protease inhibitor of tissue factor (TF)-induced blood coagulation. It exerts its action by neutralizing the catalytic activity of factor Xa (FXa) by forming a TFPI-FXa complex, and by feedback inhibition of the factor VIIa-TF complex in the presence of FXa. Infusions of bacterial TFPI was shown to be taken up by the liver and kidneys in animal studies. Prolonged administration of unfractionated heparin (UFH) is known to cause depletion of intravascular TFPI, even though UFH increase synthesis and release of TFPI from endothelial cells. Experimental studies have shown that bacterial TFPI binds to the LRP-receptor on hepatocytes with subsequent endocytosis.
Aim of the study: To investigate the effect of heparin treatment on the clearance of recombinant bacterial TFPI (r-TFPI) from *E.coli* and mammalian TFPI (rg-TFPI) from BHK cells.
Materials and Methods: rg-TFPI and r-TFPI radiolabeled with ^{125}I was used for *in vivo* and *in vitro* studies in Sprague-Dawly rats.
Results: Both rg-TFPI and r-TFPI showed a biphasic elimination curve with a fast t½ α-phase. Almost 50% of the injected radiolabelled TFPI was eliminated during the α-phase and the clearance was significantly faster for r-TFPI than for rg-TFPI ($p<0.001$). Administration of 100 IU/kg UFH reduced significantly the circulatory survival of rg-TFPI ($p<0.001$) without affecting survival of r-TFPI. Anatomical distribution of radiolabelled-ligand revealed that r-TFPI was mainly found in the liver, whereas rg-TFPI was equally found in liver and kidneys. Fluorescence microscopy on liver sections showed accumulation of FITC-labelled rg-TFPI in both parenchymal cells and in cells lining the liver sinusoids. In kidneys, the fluorescence was observed mainly in the cells of the proximal convoluted tubules. Hepatocellular distribution of radiolabelled TFPI revealed that parenchymal cells were the main cells responsible for the uptake of both types of TFPI. *In vivo* administration of 100 IU/kg UFH switched the uptake of r-TFPI from parenchymal cells towards the non-parenchymal cells, without affecting the cellular distribution of rg-TFPI. *In vitro* studies revealed that liver sinusoidal endothelial cells (LSECs), besides parenchymal cells, are involved in the uptake and degradation of both types of TFPI.
Conclusions: Parenchymal cells play a pivotal role for clearance of both mammalian and bacterial TFPI, but LSECs also contribute significantly to the TFPI clearance. Heparin treatment increased the elimination rate for rg-TFPI and switched the uptake of r-TFPI towards non-parenchymal cells, probably due to heparin-TFPI complexes. These observations may represent the underlying mechanism for the selective depletion of TFPI during heparin treatment and may indicate different receptors for endocytosis.

INVESTIGATION OF CLEARANCE OF HUMAN FACTOR VIIA AND A PEGYLATED VARIANT IN A PERFUSED RAT LIVER MODEL

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The metabolism of human recombinant activated coagulation factor VII (rFVIIa, NovoSeven®) remains largely unknown. However, tissue distribution studies (1,2) suggest that the liver may play a role. Consequently, rFVIIa metabolism was explored in perfused rat livers.

Proteins were produced at Novo Nordisk. Rat livers were cannulated via the portal vein and vena cava to generate an ex vivo recirculating liver perfusion model. Perfusions were performed between 1- 2 hours and samples from the perfusate and bile were collected at several timepoints and analysed for FVIIa activity and antigen concentration.

The perfusion studies showed that rFVIIa uptake appears to be biphasic in the absence of plasma proteins. Approximately 35% of rFVIIa activity is cleared by the liver within the first 30 min, while only an additional 5-10% is cleared during the remaining 95 min. A small percentage of rFVIIa was also seen in the bile lending support to the existence of an active protein uptake mechanism. Previously, studies to understand the biphasic uptake of rFVIIa suggested a subpopulation of rFVIIa was being cleared (3). It was of interest to see if this subpopulation existed for a GlycoPEGylated variant of rFVIIa (FVIIa-10K). All liver perfusions were performed at a concentration of 10nM. The clearance for pegylated rFVIIa was significantly reduced in comparison to rFVIIa. Since clearance was drastically reduced. This reduction in uptake from the perfusate was also mirrored in a reduced amount of pegylated protein secreted in the bile.

Together, these studies showed that in the absence of plasma proteins, the liver is active in the uptake of rFVIIa in a physiologically relevant model. Data also showed that the pegylated rFVIIa derivative was cleared by the liver to lesser extent than that observed with rFVIIa. The perfused liver model could be useful for mechanistic studies of the clearance of proteins and their analogues. The type of liver cells involved and the exact mechanism of liver clearance of rFVIIa and the 10K-pegylated variant is still to be investigated, as well as, the relevance of the liver in total clearance of rFVIIa in vivo.

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PROTECTIVE EFFECT OF NITRIC OXIDE (NO) INDUCED BY ISCHEMIC PRECONDITIONING (IP) ON COLD ISCHEMIC REPERFUSION INJURY OF RAT LIVER GRAFT: AN EXPERIMENTAL STUDY

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Background: To investigate the protective effect of nitric oxide (NO) induced by ischemic preconditioning (IP) on ischemic reperfusion injury (IRI) of rat liver graft.
Methods: Male Sprague Dawley rats were used as donors and recipients of orthotopic liver transplantation. One hundred and twenty-eight rats were randomly divided into 4 groups (n=32): 1) administering heparin before ischemic reperfusion (IR) (control group), 2) IPC with 10 min ischemia and 10 min reperfusion before IR (IP group), 3) administering adenosine before IR (Ade group), 4) L-NAME (Nω-nitro-L-arginine methyl ester)+IP before IR (NAME group). One half of each group were used to investigate one week survival rate of recipients, and another half of each group were used to take sample of blood and hepatic tissue after 2 h reperfusion. Differences between the groups were analyzed by q-test, log-rank test was performed for the survival study.
Results: One week survival rate, bile production, serum NO and activity of anti-oxidase levels were higher in IP group and Ade group than those in control group ($P<0.05$): meanwhile, serum ALT, TNF-α and superoxide levels in hepatic tissue were lower in IP group and Ade group than those in control group ($P<0.05$). Sinusoidal endothelial cells were the principal target of reperfusion injury and their deaths were caused by apoptosis. Sinusoidal endothelial cells in IP group and Ade group showed less injury than those in control group. All findings in NAME group showed similar results to those in control group ($P>0.05$).
Conclusion: Through the stimulation of endogenous NO, IP can protect sinusoidal endothelial cells from injury during the early phase of reperfusion and improve the microcirculation and function of rat liver graft.
Key words: Nitric oxide; Ischemic preconditioning; Rat; Liver graft; Ischemic reperfusion injury

PROTECTIVE ROLE OF SHENFU INJECTION IN ISCHEMIA-REPERFUSION INJURY OF RAT LIVER GRAFT

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Aims: To investigate the protective role of Shenfu injection (SF) in ischemia-reperfusion injury (IRI) of rat liver graft.
Methods: Ninety-six male Sprague Dawley (SD) rats were used as donors(48 rats) and recipients(48 rats) of orthotopic liver transplantation, and randomly divided into two groups: in control group, donor livers were injected saline immediately after hepatic blood recovering through portal vein; in SF group, donor livers were injected shenfu instead of saline. Each group was further divided into 3 groups equally to take sample of blood and hepatic tissue at 2h, 4h and 6h after reperfusion**.**
Results: At each phase in SF group, bile production was significantly higher ($P<0.05$) and serum levels of ALT and TNF-α, NF-κB expression in hepatic tissue were obviously lower ($P<0.05$) than those in control group. In SF group, hepatic tissues showed less injury compared with control Group
Conclusion: Shenfu injection can protect hepatocyte from injury during the early phase of reperfusion and improve the function of rat liver graft.
[Key words] SF injection; Liver transplantation; Ischemia-reperfusion injury

EHD3: FUNCTIONAL ANALYSIS OF A LIVER SINUSOIDAL ENDOTHELIAL CELL SPECIFIC PROTEIN INVOLVED IN ENDOCYTOSIS

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Endothelial cells are heterogeneously differentiated along the different segments of the vascular tree as well as in the vascular beds of different organs. Vis à vis normal continuous blood vascular endothelial cells, non-continuous endothelial cells such as lymphatic endothelial cells, liver or lymph node sinusoidal endothelial cells and tumor endothelial cells serve specialized functions; they are involved in scavenging processes for macromolecules such as hyaluronan, they exert an active sieve function for cellular debris and microorganisms, and they mediate tumor metastasis as well as antigen presentation and induction of immune tolerance.

To identify genes specifically expressed by non-continuous liver sinusoidal endothelial cells (LSEC) we established a method to purify rat liver sinusoidal endothelial cells by using monoclonal antibodies against LSEC specific surface molecules (Stabilin-2, SE-1) followed by magnetic activated cell sorting (MACS). By gene profiling (Affymetrix Rat genome 230 2.0 DNA chips) of LSEC against CD31 sorted rat lung microvascular endothelial cells (LMEC), we identified over-expression of approx. 300 genes in diverse functional groups. Among them, we found well known LSEC specific genes like Stabilin-1, Stabilin-2, and LYVE-1. By gene ontology (GO)-analysis, a major group of differentially expressed genes is involved in processes referred to endocytosis and trafficking. The protein EHD3 was significantly over expressed in LSEC as compared to LMEC. EHD3 belongs to the eps15 homology domain (EHD) containing protein family. It was described to be localized close to endocytic vesicles indicating that EHD3 shuttles within the endocytic recycling compartment. To verify the gene profiling data of EHD3 in LSECs, we first confirmed the differential expression on the mRNA (RT-PCR) as well as protein (Western blot) level. To address the question of endocytic activity, we showed co-localization of EHD3 with endocytosed ligands as well as other components localized to the endocytic machinery (e.g. EEA-1, Stabilin-1, and Stabilin-2). By confocal microscopy we showed that Stabilin-1, but not Stabilin-2 co-localise with EHD3 suggesting that these two receptors follow different pathways after internalisation. The functional relevance of EHD3 on endocytosis of different ligands (acetylated LDL, hyaluronan, AHGG, Transferrin, EGF, and AGE-BSA) of LSEC-associated scavenger receptors is currently studied using quantitative endocytosis assays and si-RNA to modulate EHD3 expression.

INVOLVEMENT OF NITRIC OXIDE IN THE CONTROL OF THE HEPATIC MICROCIRCULATION OF THE STEATOTIC LIVER OF THE OBESE ZUCKER RAT

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Nitric oxide (NO) is known to play an important role in the maintenance of hepatic haemodynamics in normal liver. Its role in the control of the haemodynamics of steatotic (fatty) liver is less well understood. This current study was designed to evaluate the involvement of NO in the control of the hepatic microcirculation of the steatotic liver of the obese Zucker rat.

Obese (n=13, 724±27g) and lean male Zucker rats (n=13, 464±5g) were anaesthetised with sodium pentobarbital (50mg/kg i.p.). Seven animals in each group were administered the nitric oxide synthase (NOS) inhibitor, L-NAME (10mg/kg, i.a.), 6 animals received saline. The hepatic microcirculation was investigated using intravital fluorescence microscopy (IVFM). IVFM and haemodynamic parameters were measured before and after administration of L-NAME.

L-NAME caused a significant rise in MAP in obese and lean rats. Portal pressure fell to the same extent in both groups after NOS inhibition. Basal portal venous blood flow (PVBF) was significantly lower in obese animals. NOS inhibition caused a fall in PVBF in both groups. Hepatic arterial blood flow rose in the lean but not the obese rats. Basal hepatic microcirculatory perfusion was higher in non-steatotic liver but fell to the same extent as in steatotic liver (obese animal) after NOS inhibition. L-NAME caused a significant drop in lobular perfusion index in the steatotic liver (obese animal). Basal sinusoidal erythrocyte velocity ~250 mm/sec in both groups with similar velocity profiles. NOS caused a significant drop in velocity and a left-hand shift in velocity profile in both groups. Likewise NOS inhibition led to a significant fall in sinusoidal diameter in both non-steatotic and steatotic livers.

We conclude from our results that (i) the hepatic haemodynamics are adversely affected by steatosis (ii) NO is involved in the maintenance of hepatic haemodynamics in both the non-steatotic and steatotic liver of the rat.

Study support by the Royal Society of New Zealand Marsden Fund and was performed with the approval of the Otago University Animal Ethics Committee

INHIBITORY EFFECTS ON THE ACTIVATION OF KUPFFER CELLS BY LXR α THROUGH DOWN-REGULATION OF IRF3-GRIP1

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Abstract – Objective: To explore the possible mechanism of LXRα for inhibition of lipopolysaccharide (LPS) induced Kupffer cells (KCs) activation.
Methods: The KCs were isolated from 40 KM mice and divided into four groups: the LPS group, the LPS-T0901317 (LXRα agonist) group, the T0901317 group, the control group. The LPS group was treated with 1μg/ml LPS for 6 h, in the LPS-T0901317 group, T0901317 (10μmol/L) was given 24 h before, and the T0901317 group treated with T0901317 for 24h. The mRNA levels and protein expression of LXRα, IRF3 and GRIP1 were determined by Real Time-PCR and Western blot respectively, and IFNγ levels were detected by enzyme 1inked immunosorbent assay (ELISA).
Results: The levels of IRF3 and GRIP1 gene and protein were higher in LPS group than in another three groups, and also the IFNγ levels ($P<0.05$).
Conclusion: Treatment with LXRα agonist on KCs could effectively repress the activation of KCs induced by LPS. And one of the reasons for this repression is the expression of IRF3 and GRIP1 could be inhibited by LXRα.
Key words: Kupffer cells; Endotoxins; LXR; IRF3; GRIP1

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EXPRESSION AND FUNCTION OF FASCIN, AN ACTIN BUNDLING PROTEIN, IN HUMAN HEPATIC STELLATE CELLS.

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(Introduction) Fascin is abundant in tissues such as brain and spleen. At cellular level, it is expressed in specific types of cells such as neuronal cells, microcapillary endothelial cells, antigen-presenting dendritic cells and several kinds of cancer cells. Fascin is a member of actin-bundling proteins and causes F-actin to aggregate side-by-side into bundles and is localized in structures containing actin bundles including filopodia and stress fibers of cultured cells. In some cancer cells, fascin is reported to be involved in the proliferation and invasion. Expression and function of fascin in the liver cells has not been investigated yet.

(Materials and Methods) In order to investigate the presence of fascin in the liver, immunohistochemistry was performed in human normal liver and fibrotic liver tissues. To reveal the cells expressing fascin, serial sections were stained with fascin and HSP47 (a marker of fibroblasts) or alpha-SMA. Expression of fascin in LX-2 cells, human hepatic stellate cell line, was examined by reverse transcriptase polymerase chain reaction (RT-PCR), western blotting, and immunocytochemistry. To reduce the expression of fascin in LX-2 cells, two kinds of siRNAs for fascin were transfected. As a control, non-targetting siRNAs were transfected. At 72h after transfection, cell morphology cell number cell and cell migration were examined. Cell morphorogy was microscopically evaluated, cell number were assayed by CyQuant assay kit, and Cell migration was assayed with modified Boyden Chambers consisting of Transwell -precoated Matrigel membrane filter inserts with 8 μm pores in 24-well tissue culture plates.

(Results) In normal liver as well as fibrotic liver, fascin immunoreactivity was found at sinusoid cells. Serial staining with HSP47 or alpha-SMA indicate that HSCs in sinusoids express fascin. However, fibroblasts in periportal region and fibrotic septa, were fascin negative. In LX-2 cells, expression of fascin was detected by RT-PCR, western blot and immunocytochemistry. At 72h after transfection of siRNA into LX-2 cells, shape of cells were changed to be more spindle or round, extension area of cell body were decreased and attached cell number was significantly lower than control (65% and 71%). Migration assay showed that suppression of fascin significantly reduce the migration rate of LX-2 cells.

(Conclusion) Fascin is expressed in human hepatic stellate cells, but not in fibroblasts at portal area or fibrotic septa. Fascin expression may distinguish hepatic stellate cells from fibroblasts at portal area or fibrotic septa. In addition, fascin may be involved in the proliferation and migration of LX-2 cells.

HEPATOCYTES, HEPATIC STELLATE CELLS AND KUPFFER CELLS: AN UNBIASED ESTIMATION OF THEIR NUMBER USING DESIGN-BASED STEREOLOGICAL METHODS

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A solid quantitative background of the normal liver is relevant in several circumstances, such as when monitoring the evolution of inflammatory and fibrotic conditions. Even though advances in cell isolation, molecular biology and other have contributed extensively to our current understanding of the role of different cells in hepatic injury, morphological studies, by providing visual assessments of the *in vivo* status, continue to be powerful ways by which one can investigate cellular interplays. In recent years we have been using modern stereology, like the optical smooth fractionator, in the rat liver. This technique combines the optical disector, a 3D probe that samples cells in proportion to their number, with the (smooth) fractionator principle, in which a known fraction of the organ is systematically sampled, using random starts for selecting the first unit. Our aim herein was to apply a strategy to unbiasedly estimate the total number (N), number per gram (N/g) and % cell proportions for hepatocytes, HEP (mono- and binucleated), hepatic stellate cells (HSC) and Kupffer cells (KC).

We used 3-month old male Wistar rats (n=5), being their liver fixed by perfusion, using 10% buffered formalin, and submitted to a systematic random sampled (SRS) cascade with a random start, according to the smooth fractionator rules, and providing final sample made of 5 fragments, on average. These were processed for paraffin embedding and exhaustively sectioned (30 μm thick) in a motorized rotary microtome. In every 30 sections, 4 consecutive ones were SRS and used for immunohistochemistry against: 1) glial fibrillary acidic protein, for HSC; 2) carcinoembryonic antigen, to differentiate mononucleated hepatocytes (MnHEP) from binucleated ones (BnHEP), and 3) ED2 for KC. We counted from 445 to 1577 cells per rat, respectively for KC and MnHEP estimations. This allowed us to estimate the N, N/g and the cell number proportions. The coefficient of error (CE) of the N estimate was calculated, using statistics for systematic sampling. A correlation analysis studied the relation between those cells types and the liver and body weight.

Data are given in Table 1. As to HEP, 26% were BnHEP. A correlation existed between the N of HEP and liver weight, the N of HSC and liver weight, and the N of BnHEP and body weight.

Table 1 – Total number (N) and number per gram of liver (N/g) of all the cells (*i.e.*, stromal and parenchymal), mononucleated (MnHEP) and binucleated hepatocytes (BnHEP), hepatic stellate cells (HSC) and Kupffer cells (KC). Data given as: mean (CV). CE: coefficient of error.

CELL TYPE	N	N/g	CE (N)	% OF ALL CELLS
All liver cells	3.25×10^9 (0.22)	216.8×10^6 (0.14)	0.02	100
MnHEP	1.44×10^9 (0.23)	96.2×10^6 (0.17)	0.03	44.5
BnHEP	520×10^6 (0.31)	33.0×10^6 (0.24)	0.05	15.2
HSC	207×10^6 (0.17)	13.9×10^6 (0.14)	0.04	6.4
KC	283×10^6 (0.18)	19.4×10^6 (0.25)	0.05	9.0

The latest generation of stereology techniques for number estimation could be implemented in the rat liver, providing sound baseline data. The techniques relied on sampling designs (rather than on unrealistic geometrical models, as in the past), granting unbiased and precise data, meaning that: 1) the estimations were inherently close to the real number; 2) the CE was low, demonstrating the precision; 3) neither assumptions on cell shape, irregularity, size, and distribution, nor on the proportion of BnHEP were made. By its evident discriminative power, the strategy seems valuable for detecting regenerative and fibrotic responses in the rat liver models, from its very early onset.

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STUDY INTO THE EXPRESSION OF ADAM17, ADAMTS-1, -4, -5 AND TIMP3 BY HEPATIC STELLATE CELL LINE LX-2 AND HEPATOMA CELL LINE HEPG2

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Introduction: In the liver, activation of growth factors is required during tumour growth and invasion and regeneration to enable cell proliferation and remodelling of the liver extracellular matrix (ECM). ADAM17 (**a** **d**isintegrin **a**nd **m**etalloproteinase) is a membrane-bound sheddase, which can release membrane-bound proteins including growth factors, such as the epidermal growth factor family of ligands. Release of growth factors from normal or transformed liver cells may contribute to tissue regeneration or tumour growth. ADAMTS (**a** **d**isintegrin **a**nd **m**etalloproteinase with **t**hrombo**s**pondin motifs) -1, -4 and -5 are secreted enzymes, which degrade ECM components chondroitin sulphate proteoglycans. They have not been extensively studied in the liver, but may participate in remodelling liver ECM during invasive or regenerative processes. TIMP3 (**t**issue **i**nhibitor of **m**etallo**p**roteinases) is a major, naturally occurring inhibitor of ADAM17, ADAMTS-1, -4 and -5.
We have studied the expression of ADAM17, ADAMTS-1, -4, -5 and TIMP3 in human hepatic stellate cell line LX-2 and human hepatoma cell line HepG2 at the mRNA and protein level, and their modulation by cytokines IL-1β, IL-6 or TNF-α. This work will enable a better evaluation of the potential role of ADAM17, ADAMTS-1, -4, -5 and TIMP3 in tissue remodelling and/or invasion.

Methods: ADAM17, ADAMTS-1, -4, -5 and TIMP3 were studied at the mRNA level by real time RT-PCR in the LX-2 and HepG2 cell lines. Cells were treated with 0, 1, 10 or 100ng/mL IL-1β, IL-6 or TNF-α (Peprotech) for 24 hours in serum-free media. RNA was extracted with Tri-Reagent™ (Sigma), reverse transcribed and cDNA was amplified by real-time PCR, using the SYBR green detection method. Housekeeping gene (β-actin and HPRT) transcript levels were used to normalise gene expression. ADAM17 protein expression in LX-2 and HepG2 cells was performed by western blotting. Cells were cultured for 48 hours, and protein extracted with CelLytic™-M (Sigma) with 10% protease inhibitor cocktail (Sigma). Proteins (6mg/lane) were fractionated under reducing conditions on 10% NuPage gels (Invitrogen) using the SDS-PAGE Laemmli system, and then transferred to Hybond-C nitrocellulose membrane (Amersham). ADAM17 was visualised using rabbit polyclonal anti-human ADAM17 antibody (Chemicon) and HRP conjugated goat anti-rabbit IgG (Sigma).

Results: Untreated LX-2 cells were shown to express mRNA for ADAM17, ADAMTS-1, -4, -5 and TIMP3, whereas HepG2 cells expressed mRNA for ADAM17, ADAMTS-4 and TIMP3. Treatment of cells with 1, 10 or 100ng/mL of either IL-1β or IL-6 had no effect on expression of ADAM17, ADAMTS-1, -4, -5 or TIMP3. Stimulation of HepG2 cells with TNF-α also did not result in significant changes in gene expression. However, TNF-α treatment of LX-2 cells resulted in a decrease of ADAM17 (1, 10 & 100ng/mL) and TIMP3 (1 & 100ng/mL), but ADAMTS-1, -4 and -5 were unaffected. Western blot data indicates that ADAM17 protein is present in untreated LX-2 and HepG2 cells.

Conclusions: There is differential mRNA expression of ADAM17, ADAMTS-1, -4, -5 and TIMP3 in LX-2 and HepG2 cells. There was very little modulation of gene expression with the cytokines investigated. However, in LX-2 cells, TNF-α caused decreased mRNA levels of ADAM17 and TIMP3. ADAM17 protein was detected in LX-2 and HepG2 cells, in agreement with the mRNA studies. Regulation of ADAM17 by TNF-α in hepatic stellate cells may facilitate the ECM remodelling phase of hepatic regeneration and also the growth of colorectal liver metastases into the liver parenchyma.

APOPTOSIS INDUCED IN THE MOUSE LIVER INTOXICATED WITH LIPOPOLYSACCHARIDE AND D-GALACTOSAMINE AND ITS INHIBITION BY GLYCYRRHIZIN

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Background/Aims: Glycyrrhizin (GL) has been used as a clinical treatment for chronic hepatitis, but its effects on acute hepatic injury have been unclear. Our objective is to evaluate the effect of GL on the mouse liver injury induced by an injection of lipopolysaccharide (LPS)/D-galactosamine (D-GalN).
Methods: Male Balb/c mice were treated with LPS/D-GalN. Serum transaminase, cytokines such as tumor necrosis factor-α and interleukin-18 (IL-18), and caspase-1, -3 and -8 were evaluated. We submitted tissue sections to immunohistochemistry for *in situ* immunohistochemical labeling of nuclear DNA fragmentation (TUNEL), IL-18, or F4/80. Hepatic lobules were divided into pericentral and periportal zones, and the cell number/mm^2 of $F4/80^+$ or $TUNEL^+$ cells was quantified. Furthermore, cell apoptosis was confirmed using microdissection-system and oligonucleosome-bound DNA ELISA.
Results: The degree of hepatic injury was associated with a substantial number of hepatocytes undergoing apoptosis in the centrilobular area. GL inhibited the elevation in serum levels of transaminase and IL-18 but not caspase activities. GL significantly reduced the number of TUNEL-labeled cells in acute hepatitis induced with LPS/D-GalN-treatment. Furthermore, DNA-ladder was clearly documented on agarose gel electrophoresis and confirmed with oligonucleosome-bound DNA ELISA. The number of F4/80-immunoreactive macrophages was significantly increased in the liver injury. Increase in the number of macrophages after LPS/D-GalN-treatment was relatively reduced by intraperitoneal administration of GL, but not significantly.
Conclusion: These findings demonstrate that the protective role of GL in LPS/D-GalN-induced liver injury is performed through the inhibition of hepatic apoptosis. These effects might be accompanied by inhibition of caspase-independent apoptotic DNA degradation through IL-18 signalling pathways.

HGF UP-REGULATES SDF-1 ACTIVITY IN FIBROTIC LIVER SUPPRESSING DPPIV EXPRESSION AND ACCELERATS RECRUITMENT OF BONE MARROW-DERIVED CELLS

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Anti-fibrogenic effect of hepatocyte growth factor (HGF) has been well documented, while HGF has a variety of biological effects, such as mitogenic, motogenic, anti-apoptotic, angiogenic activities. Meanwhile, recruitment of bone marrow-derived cells (BMC) into injured organs and differentiation of these cells into organ-specific cells has been reported. In the present study, we investigated how BMC is recruited in a remodeling process of carbon tetrachloride (CCl_4)-induced liver fibrosis following HGF gene transfer. **Methods:** C57BL/6 J mice were treated with CCl_4 for 10 weeks. At week six, the mice underwent whole body irradiation (900cGy) and transplantation with bone marrow cells ($5x10^6$) from syngenic LacZ-transgenic mice. After the transplantation, gene transfer of HGF (HVJ liposomes containing 200 mg of human HGF expression vector) into skeletal muscles was performed once a week for four weeks. In the control group, sterile saline was injected. **Results:** HGF gene transfer ameliorated the CCl_4-induced liver fibrosis, accelerating recruitment of LacZ-expressing cells into the liver. This phenomenon was accompanied by increased gelatinase activity in the fibrous area. A large number of the LacZ-positive cells expressed markers of vascular endothelial cells, while some of them had a marker of macrophages. HGF up-regulated the expression of stromal cell-derived factor (SDF)-1 in the liver around the injured central veins, recruiting chemokine (C-X-C motif) receptor (CXCR) 4, a receptor for SDF-1, -positive cells in this area. The double fluorescent staining showed a close relation between SDF-1 and CXCR-4 around the central veins. Expression of dipeptidyl peptidase (DPP) IV (CD26), an enzyme inactivating SDF-1, was markedly suppressed around the central veins by HGF gene transfer, up-regulating SDF-1 activity in this area. **Conclusion:** Transplanted BMC participate in the HGF-induced remodeling process of liver fibrosis. The roles of HGF in this process include the recruitment of BMC, possibly through increased activity of SDF-1, as well as anti-apoptotic, mitogenic and antifibrotic activities on liver cells.

PREVENTION OF HEPATIC DISEASES USING TGF-β ACTIVATION INHIBITORY DECOY PEPTIDES

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TGF-β, the most fibrogenic cytokine, is constitutively synthesized and secreted in a biologically latent form and thus must be activated before exerting its biological activities. TGF-β activation is the reaction, by which 25kD active TGF-β molecule is released from the latent complex. In the liver, TGF-β is produced by hepatic stellate cells (HSCs) and activated on their surface. The resultant active TGF-β induces hepatic fibrosis and impaired hepatic regeneration. Thus, HSCs play a central role in the pathogenesis of hepatic fibrosis by virtue of their ability to undergo a process termed activation under the stimuli of TGF-β. In the liver, TGF-β is known to be activated by proteases such as plasmin (PLN) and plasma kallikrein (PLK). Blockage of these activation reactions with low molecular weight protease inhibitors prevented the development of disease.

We determined that PLN and PLK cleaved between K56-L57 and R58-L59 in latency associated protein (LAP) portion of human latent TGF-β molecule. We produced peptides containing these cleavage sites as well as their decoy peptides in which cleavage site amino acids were all mutated to either A or Q, and examined the effect of these peptides on TGF-β activation and associated phenomenon both *in vitro* and *in vivo*. Western blotting with anti LAP antibody shows inhibition of LAP cleavage reaction by these peptides. TGF-β ELISA and PCR shows reduced generation of active TGF-β and suppression of HSC activation with these peptides. Namely, these peptides efficiently suppressed the TGF-β activation reaction and prevented the activation of HSCs in culture. Furthermore, these peptides were effective to prevent impaired liver regeneration observed in LPS-pretreated partially hepatectomized mice. Histochemical staining with anti proliferating cell nuclear antigen (PCNA) shows the improvement of impaired liver regeneration by these peptides.

These results suggest that these peptides might be novel new agents against liver diseases targeting TGF-β activation.

SANDWICH ELISA OF LAP DEGRADATES FOR MEASUREMENT OF HEPATIC FIBROGENESIS.

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Although hepatic fibrosis is an important pathology common in many hepatic diseases, there is no good biomarker reflecting fibrogenesis in the liver, which limits producing a good diagnostic method and development of new drugs. We have established a novel sandwich ELISA based on our recent findings that fibrogenesis accompanies the activation of transforming growth factor (TGF)-ß, the most fibrogenic cytokine playing a pivotal role in the pathogenesis of the liver fibrosis. TGF-ß is produced as a high molecular weight latent form, and thus must be activated, namely released from the latent complex, before exerting its biological activities.

Using animal models, we showed that TGF-ß is activated by proteases such as plasmin (PLN) and plasma kallikrein (PLK). We determined that PLN and PLK cleaved between K^{56}-L^{57} and R^{58}-L^{59}, respectively, within latency associated protein (LAP) portion of human latent TGF-ß1, produced antibodies that specifically recognize the neoepitopes formed by protease degradation, namely the cut ends of each cleavage site, and made sandwich ELISA (sELISA) using a combination of the antibodies recognizing LAP and the L^{59} cut end-containing LAP fragments produced during the process of the activation of TGF-ß by PLK.

In Western Blotting, the antibodies against the PLK-cut ends, namely anti-R^{58} and anti-L^{59} antibodies recognized the degraded fragments of latent TGF-ß, but not uncleaved latent TGF-ß. Next, using this sELISA and TGF-ß1 Emax ImmunoAssay system, we measured parallel generation of LAP degradates and active TGF-ß1 in proportion to the activation of the hepatic stellate cells (HSCs). Furthermore, using this sELISA we measured the concentration of LAP degradates released in the serum in rat fibrosis models including bile duct ligation model, and compared with hepatic contents of hydroxyproline (HDP). Serum levels of LAP degradates in the fibrotic animals were 3-fold higher than those in the control animals (no treatment or sham operation). The increase showed a good correlation with hepatic HDP levels.

These results suggest that the LAP degradates produced during the TGF-ß activation reaction may be a promising novel biomarker for hepatic fibrogenesis.

LECITHIN: RETINOL ACYLTRANSFERASE (LRAT) AND CELLULAR RETINOL BINDING PROTEIN-1 (CRBP-1) ARE STRONGLY CO-EXPRESSED IN THE LIVER OF POLAR BEARS

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Background/Aims: Polar bears, which exist on the top of food chain in Arctic animals, are the valuable mammals for studying retinoid metabolism. The reasons of their decreasing population are suggested due to not only melting the sea ice by global warming but also accumulation of some environmental pollutants to their bodies, particularly dioxins. Polar bear is known to have an unique retinoid accumulation system in the liver, since hepatic stellate cells (HSC) in polar bear store 20-100 times high level of vitamin A compare to human or rat. Lecithin: retinol acyltransferase (LRAT) is a physiological retinol esterification enzyme in the liver and its activation is occurred especially in HSC. Retinol bound cellular retinol-binding protein-1 (CRBP-1) is a substrate of LRAT. In the present study, we would like to examine if LRAT has anything to do with CRBP-1 in terms of their expression and localization in HSC. We determined the expression level of LRAT in HSC among different weight of polar bears through correlation with CRBP-1.
Materials and Methods: An antibody specific to LRAT was generated on the basis of human LRAT amino acid sequence. To examine the distribution of LRAT in detail, other antibodies, CRBP-1 and α-smooth muscle actin, were also prepared. Immunohistochemical study on HSC was performed in the liver of four different polar bears ranging from 100kg to 500kg.
Results: Enlargement of lipid droplets in HSC was observed correlatively in the order of the bears' weights. Nucleus and cytosol were pushed aside by the large lipid droplets, especially for heavy polar bears. Quiescent HSC with lipid droplets in the space of Disse strongly expressed both LRAT and CRBP-1 proteins in many fields. The strength of immunostaining for LRAT was also increased correlating with a body weight. In portal area, portal fibroblasts strongly expressed α-smooth muscle actin, whereas they did not express LRAT. The co-expression of LRAT and CRBP-1 in polar bear liver was apparent compared to that in human liver.
Conclusions: We speculated that interaction between LRAT and CRBP-1 could play an important role in unique vitamin A storage function in HSC. The cells expressing both LRAT and CRBP-1 were recognized as the functional quiescent HSC concerning vitamin A metabolism.

HEPATIC STELLATE CELL ACTIVATION AND FIBROGENESIS ARE INHIBITED BY SOLUBLE FACTORS FROM PARASITE EGGS

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Introduction. Adult worms of the parasite *Schistosoma mansoni* reside in the human gut mesenteries where females produce significant numbers of eggs. More than 50% of eggs are carried to the liver by the portal circulation where they become trapped in the liver sinusoids and generate a host immune response often resulting in pathology and fibrosis. The aim of this study was to investigate the fibrogenic nature of the host-parasite interaction, focusing specifically on that between parasite eggs and hepatic stellate cells (HSC). The immortalised human HSC-line (LX-2) is a recently-available resource for liver fibrogenesis studies. LX-2 cells retain many primary HSC characteristics but display a progressively more activated, myofibroblast-like phenotype after being introduced onto tissue culture plastic. Since liver fibrosis requires transdifferentiation of HSC from a quiescent to an active myofibroblastic phenotype, we have developed a model in which LX-2 is manipulated to yield either phenotype, prior to co-culture with isolated *S.mansoni* eggs or a homogenised soluble egg antigen (SEA) extract.

Methods. HSC were investigated for biomarkers of their activation; by phase contrast imaging for morphology and staining with oil-red O for lipids; by immunofluorescence techniques for α-smooth muscle actin (αSMA) and F-actin (FA); by realtime PCR techniques for gene expression of pro-fibrogenic collagen type I (col1A1), connective tissue growth factor (CTGF), αSMA and anti-fibrogenic peroxisome proliferator-activated receptor (PPAR)γ. HSC were cultured for 7days either in DMEM + FBS on cell culture plastic for activation or in DMEM+ FBS + adipogenic factors on cell culture plastic for quiescence. HSC were then co-cultured for up to 7 days in the presence of eggs (0-1,500 per well) or SEA (0-15μg/ml per well).

Results. Quiescent HSC were able to respond to a positive control, transforming growth factor (TGF)-β, and became activated as expected. HSC exposed either directly to viable parasite eggs or indirectly to SEA, adopted a quiescent phenotype according to morphological activation markers. In the absence of parasite material, HSC displayed a flat, spread morphology with cells abundantly expressing αSMA and FA in a stress fibre-like pattern and showed little evidence of lipid accumulation. In contrast, in the presence of eggs or SEA, HSC were reduced in size, exhibited fine cellular processes, reduced their expression of actins that were not linked to stress fibres and showed abundant lipid staining. HSC exposed either directly to viable parasite eggs or indirectly to SEA, adopted a quiescent phenotype according to their expression of fibrogenic genes. Thus, expression of αSMA, CTGF and col1A1 genes were all highly downregulated and simultaneously expression of PPARγ was upregulated.

Conclusions. In conclusion, our data indicate that human HSC are directly regulated by *S.mansoni* eggs. HSC exposed directly to viable eggs or to SEA adopted a quiescent phenotype. The identification and isolation of soluble factor(s) in parasite eggs with anti-fibrotic activity responsible for this effect may have benefits against liver fibrosis.

HEPATIC PROGENITOR CELLS AND FIBROSIS IN THE CHOLINE DEFICIENT/ETHIONINE INDUCED MODEL OF CHRONIC LIVER INJURY.

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Background and aims: when severe liver injury coincides with incapacity of the left-over hepatocytes to proliferate, activation of dormant rescue cells occurs. These liver progenitor cells (LPCs) are believed to repopulate the damaged liver with, at least, hepatocytes and bile duct cells. For this LPC-aided regeneration, a well orchestrated symphony of secreted cytokines and growth factors, and both cell-cell and cell-matrix interactions are necessary. We developed a particular interest in the relationship between LPCs and hepatic stellate cells (HSCs), specialized extracellular matrix (ECM) producing cells.

Methods: A choline deficient diet, supplemented in ethionin (CDE diet) was given to five- week-old C57Bl6 mice. This diet fulfills the two conditions necessitated for the activation of the progenitor compartment (i.e. inducing severe liver damage and proliferative incapacity of the remaining hepatocytes). After 2 to 3 weeks of administration of the diet, liver histology is investigated and LPC number is determined by immunostaining with CK19. Sirius red (SR) staining, to point out excess of ECM was also carried out. Morphometrical analysis of the (immuno)histochemistry was obtained and the results were confirmed by RT-PCR analysis of mRNA expression of CK19 and several ECM components.

Results: We observed in these CDE models a LPC proliferative response, which, in some cases, was massive. The morphometrical analysis of CK19 and SR staining demonstrates a parallelism between the increasing number of LPCs and growing excess of ECM. Even more, the available data show a co-localisation, with the LPCs embedded in the ECM. In a time course experiment (CDE diet for 0 to 14 days), RT-PCR reveals as soon as day 3, an increase of 7 times the normal value of α-SMA, marker of the myofibroblasts. An increased expression of ECM components collagen 1 and 3, and a minor increase of laminin were also noticed.

Conclusion: These preliminary results support the hypothesis that LPCs need a support matrix such as fibrotic tissue for migration and anchor in order to differentiate for restitution of the damaged liver. We show with our data that already at day 3, the liver is put in a high state of readiness for the upcoming proliferation of the LPCs, with first the production of ECM and secondly the appearance of LPCs.

THE TUMOR SUPPRESSOR PROTEIN PTEN INHIBITS RAT HEPATIC STELLATE CELL ACTIVATION

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Introduction: Following a fibrogenic stimulus, the hepatic stellate cell (HSC) transforms from a quiescent to an activated cell type associated with numerous morphological and phenotypic changes including increased proliferation, excess collagen synthesis, and expression of smooth muscle β-actin (αSMA). PTEN (phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor phosphatase, has been shown to play a role in several nonmalignant diseases. We investigated the role of PTEN in HSC activation.
Materials & Methods: HSCs were isolated from SD rats and cultured for 2 days and 14 days, used as quiescent HSCs and activated HSCs, respectively. HSCs were transduced with adenoviruses including WT-PTEN which overexpresses PTEN. Morphological changes and associated HSC activation was investigated with phase-contrast microscopy. Apoptosis was evaluated by Caspase3/7 assay. The phosphorylation status and the expression of proteins related to HSC activation and cell cycle were investigated by Western blot analysis.
Result: In quiescent HSCs, expression of WT-PTEN inhibited morphological changes and αSMA expression by 66% compared to transduced control cells, both associated with HSC activation. Overexpression of PTEN blocked HSC proliferation with reduced PCNA and cyclin D1 expression by 96% and 93% compared to transduced control cells, respectively. WT-PTEN also induced apoptosis with increased activity of caspase 3/7 4.4-fold in quiescent HSCs compared to the control cells. The phospholyration of Akt, p70S6K, and Erk, related to HSC activation was inhibited by WT-PTEN, suggesting that these findings were caused by inactivation of these pathways. However, these effects were not observed with overexpression of PTEN in activated HSCs.
Conclusion: PTEN represents an important regulator for the activation of HSCs, suggesting that PTEN is one of the important therapeutic target genes for the prevention and the treatment in early stage of liver fibrosis.

THE CONTRIBUTION OF ACTIVATED LIVER MYOFIBROBLASTS AND HEPATIC STELLATE CELLS IN THE RECRUITMENT AND POSITIONING OF LYMPHOCYTES WITHIN THE LIVER.

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Introduction: The recruitment of lymphocytes to inflamed tissues via the endothelium has been extensively studied but less is known about the signals that direct migration of cells within tissues. Hepatic stellate cells (HSC) possess several immunological properties including the production of cytokines, the expression of adhesion molecules and are positioned below the sinusoidal endothelium through which activated lymphocytes are recruited to the damaged liver. Moreover, in chronic liver disease, activated liver myofibroblasts (aLMF) are associated with infiltrating lymphocytes at sites of damage. Here we investigate whether the myofibroblast population in the liver can direct the migration of lymphocytes within the inflamed liver.

Methods: The ability of aLMF isolated from diseased human liver and HSC isolated from normal tissue and activated in vitro to promote lymphocyte chemotaxis, adhesion and migration was studied using chemotaxis, adhesion and transmigration assays.

Results and Conclusions: In vitro, aLMF secreted a similar profile of cytokines to that observed in HSCs, characterised by high levels of IL-6, IL-12, HGF, VEGF and the chemokines CCL2 and CXCL8. In response to proinflammatory cytokine treatment CXCL9, CXCL10, CCL5 and CCL3 were secreted. Following treatment with TNFα and IFNγ we also report secretion of IL-17 by both aHSC and aLMF. Cell-conditioned supernatants promoted a rapid chemotactic response in blood lymphocytes which was increased further upon stimulation with pro-inflammatory cytokines. This response was partially inhibited by pertussis toxin and neutralisation of IL-6, HGF and VEGF suggesting both GPCR-dependent as well as independent mechanisms of lymphocyte chemotaxis. Lymphocytes bound to aLMF in using ICAM-1 and VCAM-1 and a proportion of the adherent cells migrated on and through the fibroblast monolayer. This migration was only partially inhibited by pertussis and was again dependent on HGF and VEGF. Human in vitro activated aHSC and in vivo activated aLMF support lymphocyte adhesion and promote lymphocyte migration in-vitro in both GPCR-dependent and independent mechanisms. These properties may allow the regulation of the recruitment and positioning of lymphocytes in chronic inflammatory liver disease.

PRETRANSPLANT VIABILITY TESTING OF EXTENDED CRITERIA DONOR LIVERS DURING HYPOTHERMIC MACHINE PERFUSION

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Background: Using extended criteria donor livers (steatotic, very old, donation after cardiac death donors) offer the most immediate promise to decrease the critical organ shortage. The growing urge to use such livers makes it necessary to improve current preservation techniques and to develop objective criteria to assess graft quality prior to transplantation (Tx). Hypothermic Machine Perfusion (HMP) has the potential to fulfill these goals. The aim of this study was therefore to test during HMP of extended criteria donor liver grafts, certain biochemical parameters that may reflect viability prior to Tx.

Methods: HMP was conducted on human livers (n=10) initially accepted and allocated for Tx in our centre but discarded due to unexpected *in vivo* findings {>50% steatosis (n=5); other reasons (n=4); or failed rescue allocation in case the recipient was found not transplantable (n=1)}. Following a period of cold storage of 13h30min ± 5h42min, livers were HMP preserved for 24h with non-oxygenated 4-6°C KPS-1™ . Release of pH, lactate, glucose, and Aspartate Aminotransferase (AST), was determined in KPS-1™ samples obtained during HMP (30min, 1, 6, and 24h). Retrospectively, these parameters were compared in 2 different groups: a first group comparing > or <50% steatosis (n=5 in each group), a second group comparing livers regarded -in retrospect- as absolutely not (n=7) or potentially well transplantable (n=3).

Results: No differences between the various groups were observed when determining pH, lactate and glucose levels. During HMP of >50% steatotic livers, AST release was higher ($p<0.05$) compared to <50% steatotic livers (4672±2049 *vs* 287±224, 4843±2171 *vs* 330±255, 7101±3207 *vs* 455±318, 8802±5070 *vs* 791±382 U/L, at 30min, and 1, 6, 24h). AST was higher ($p<0.05$) when comparing livers regarded as absolutely not- *vs* potentially transplantable, (4672±2049 *vs* 287±224, 4843±2171 *vs* 330±255, 7101±3207 *vs* 455±318, 6618±5572 *vs* 547±261 U/L, at 30min, and 1, 6, 24h, respectively).

In conclusion, this study indicates that AST in the perfusate during HMP is a simple/reliable marker to assess the quality of liver grafts prior to Tx and to discriminate transplantable *vs* non transplantable livers, thereby allowing wider and safer use of extended criteria donor livers.

EARLY PROINFLAMMATORY CYTOKINE RESPONSE DURING LIVER REGENERATION IN NORMAL, TLR4-MUTANT AND TLR4-DEFICIENT MICE.

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Introduction: The liver has a remarkable capacity to regenerate following any insult or injury. To date, tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are two important cytokines that have been identified as being responsible for initiating or triggering this event. The signal that triggers cytokine secretion is believed to come from gut-derived lipopolysaccharide (LPS), which is a ligand for toll like receptor 4 (TLR4). Liver regeneration is delayed in TLR4-mutant C3H/HeJ mice, indicating that LPS and/or TLR4 is involved during liver regeneration. We therefore investigated the proinflammatory cytokine response following partial hepatectomy (PH) in normal LPS-sensitive (C3H/HeN, C57BL/6), TLR4-mutant (C3H/HeJ) and TLR4-deficient (TLR4-/-) mice. **Methods**: Male, 8-10 weeks old C3H/HeN, C3H/HeJ, C57BL/6 and TLR4-/- mice were used in this study. A 70% PH or sham operation was performed in these mice under sodium pentobarbital anaesthesia (50 mg/kg). Bloods were collected by axillary exsanguinations and mice were sacrificed at 0 (control), 3, 6, 12 hours, 1, 2, 3 and 7 days after PH. Serum cytokines (TNF-α, IL-6) were measured using the Bio-plex kit. Liver regeneration was assessed by calculating the liver weight-to-body-weight ratio. **Results:** Restoration of normal liver weight-to-body-weight ratio after PH was delayed in the C3H/HeJ but not in the TLR4-/- mice when compared to control (C3H/HeN and C57BL6, respectively). Our data showed that the production of TNF-α peaked at 3 hours after PH in C3H/HeN mice. This was followed by the peak IL-6 levels at 6 hours after PH. In C3H/HeJ mice, in which the liver regeneration is delayed, the peaked production of TNF-α and IL-6 occurred at later time points (2 days and 12 hours after PH, respectively). TNF-α secretion after PH in TLR4-/- mice was depressed, while the peak level of IL-6 production occurred at 3 hours after PH. **Conclusion**: In conclusion, proinflammatory cytokines TNF-α and IL-6 were upregulated at the early phase of liver regeneration in normal, C3H/HeN mice. Delayed cytokine production in TLR4-mutant mice might have been the cause of the delay in liver regeneration in these mice. Normal restoration of liver mass after PH in the TLR4-deficient mice indicates that IL-6, rather than TNF-α is more important during liver regeneration.

TOLL-LIKE RECEPTOR 4 IS INVOLVED IN THE MECHANISM OF CONCANAVALIN A INDUCED HEPATITIS IN MICE

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Background: Concanavalin A (Con A) administration in mice leads to T-cell mediated hepatitis, which involves cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The lipopolysaccharide (LPS)/TLR4 signaling pathway has been shown to be involved in the underlying mechanism in a number of liver pathologies (I/R-induced injury, alcohol-induced hepatitis). Involvement of TLR4 in T-cell mediated hepatitis has not been elucidated. In the current study the impact of TLR4 gene deletion (TLR4-/- mice) and a defective TLR4 (C3H/HeJ mice) was investigated. **Methods:** Mice with normal TLR4 (C3H/HeN and C57BL/6), C3H/HeJ and TLR4-/- were injected with Con A (20 mg/kg). Additional groups were treated with anti-TLR4 mAb (MTS510;100 mg/mouse iv). Plasma ALT was measured to assess liver injury. Plasma TNF-α and IFN-γ were measured by Bio-Plex suspension array system. **Results:** Following Con A administration, C3H/HeN and C57BL/6 mice developed severe liver injury indicated by elevated plasma ALT. However, no ALT increase was seen in C3H/HeJ, TLR4-/-, or anti-TLR4 treated C3H/HeN mice. Histological examination confirmed signs of necrosis following Con A in the wild-type animals that was absent in the C3H/HeJ, TLR4-/- or anti-TLR4 mAb treated C3H/HeN mice. TNF-α was significantly elevated at 2 hours post-Con A in C3H/HeN (1448 ± 715 pg/mL) and C57BL/6 mice (7302 ± 3500 pg/mL) but not in C3H/HeJ mice (347 ± 199 pg/mL). IFN-γ levels were significantly higher in C3H/HeN (770 ± 160 pg/mL) and C57BL/6 mice (1113 ± pg/mL) compared to C3H/HeJ mice (330 ± 30 pg/mL). **Conclusions:** Our results show that Con A causes liver injury and proinflammatory cytokine production in mice with functional TLR4. In the absence of the TLR4 signaling pathway, cytokine production is attenuated and injury is prevented (C3H/HeJ and TLR4-/- mice). Thus we have shown for the first time the involvement LPS/TLR4 signaling may play a role in Con A induced hepatitis, probably mediated by proinflammatory cytokines produced by T cells and Kupffer cells.

SINUSOIDAL DILATATION AND PELIOSIS IN INFLAMMATORY HEPATOCELLULAR ADENOMAS

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Hepatocellular adenomes [HCAs] are monoclonal tumors, which have been divided up into three subtypes of tumors depending on the molecular alteration detected in the tumors: an acute inflammatory response in the tumor (40-50 %), an HNF1α inactivation (30 -35%), α β-catenin activation (20% -10% being also inflammatory). These molecular features are closely related to clinical and pathological characteristics, and one of the most critical correlations is the higher risk of malignant transformation for β-catenin activated HCA cases.
Inflammatory HCAs are characterized by the presence of inflammatory infiltrates. These nodules exhibited additional features such as sinusoidal dilatation found at the periphery of the nodule and peliotic cavity more centrally located, dystrophic artries and ductular reaction, and included most of the previously described so-called ‘‘telangiectatic focal nodular hyperplasia’’ cases. In these tumors, we detected elevated expression of members of the acute phase inflammatory response (serum amyloid A protein, and C-reactive protein) at both the mRNA and protein levels.
The aim of this study was to correlate pathological findings with MRI features.
We analyzed MRI data from twenty-three inflammatory HCA. It showed: 1) hyposignal or isosignal on T1W images, 2) an absence or only focal signal dropout on chemical shift sequence, 3) marked hypersignal on T2W sequences, with a stronger signal in the outer part of the lesions, 4) strong arterial enhancement, with persistent enhancement in the portal venous and delayed phases. Marked hypersignal on T2W sequences associated with delayed persistent enhancement had a positive predictive value of 88.5%, a negative predictive value of 84%, a sensitivity of 85.2% and a specificity of 87.5% for the diagnosis of inflammatory telangiectatic HCA. A target-like feature was observed for a large proportion of these lesions on T2 W images, with a very high-intensity signal forming a rim in the outer part of the lesion. A strong correlation was found between these rims and the peripheral sinusoidal dilatation.
For comparison we analysed 15 HNF1α mutated HCA a histologically homogeneous group of tumours, characterized by marked steatosis, no inflammatory infiltrate and no abnormal arteries, sinusoidal dilatation or peliotic cavities. The corresponding lesions showed 1) hyposignal or isosignal on T1W images, with diffuse signal dropout on chemical shift sequence, 2) isosignal or slight hypersignal on T2W images, 3) moderate enhancement in the arterial phase, with no persistent enhancement in the portal venous and delayed phases. For the diagnosis of HNF1α-inactivated HCA the positive predictive value of homogeneous signal dropout on chemical shift images was 100%, the negative predictive value was 94.7%, the sensitivity was 86.7% and the specificity was 100%.
In conclusion, inflammatory HCA the most numerous subgroup of HCA identified on molecular criteria present distinct radiological criteria which allows their identification prior resection. The link between these highly vascularized tumors with dystroplic arteries, sinusoidal dilatation and peliotic cavities and the expression of acute inflammatory proteins by tumoral hepatocytes needs further evaluation

EXPRESSION OF NUCLEAR RECEPTORS IN HUMAN AND MURINE LIVER

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Introduction: Nuclear receptors (NRs) are transcriptional regulators expressed in a variety of tissues and play important roles in embryonic development, organogenesis and metabolic homeostasis. In order to investigate the tissue-specific expression pattern of NRs, we generated mouse anti-human monoclonal antibodies against various NRs.

Materials and Methods: Paraffin sections of human and murine liver were immunostained using mouse anti-human monoclonal antibodies against NRs (Perseus Proteomics Inc., Tokyo, Japan). Specificities of the antibodies were confirmed by immunoblotting using overexpressed proteins. Immunoblotting was performed using human and murine tissues and anti-nuclear receptor antibodies.

Results: Among 32 immunohistochemically examined antibodies against NRs, we observed the expression of 14 NRs in hepatocytes of human liver specimens. Different combinations of six NRs were expressed in Kupffer cells, endothelial cells, and hepatic stellate cells, while 13 NRs were expressed in bile ducts. In the murine liver, immunohistochemical examination indicated that 14 NRs are expressed in hepatocytes, 7 NRs in Kupffer cells and hepatic stellate cells, 6 NRs in endothelial cells, and 13 NRs in bile ducts, respectively.

HNF4α (P1) was expressed in hepatocytes, while HNF4α (P2) was displayed in bile ducts. COUP-TFII expression was observed only in the endothelial cells and bile ducts. RXRα was expressed both in Kupffer cells and hepatic stellate cells, while FXR was expressed in hepatocytes and hepatic stellate cells and LXRα expression was observed in Kupffer cells. Immunoblotting using these antibodies revealed similar corresponding bands both in human and mouse liver.

Conclusions: These monoclonal antibodies may serve as powerful tools to detect tissue-specific localization of NRs and provide a platform for future studies of NRs in human and murine tissues.

GLYCOGEN, α-SMA AND E-CADHERIN IN MARGINAL HUMAN LIVER GRAFTS DISCARDED FOR CLINICAL USE AND PRESERVED BY HYPOTHERMIC MACHINE PERFUSION

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Data on Hypothermic Machine Perfusion (HMP) of human marginal livers are virtually inexistent. Superiority of HMP of marginal human livers must be first proven *ex vivo* before this is applied clinically. In this study we aimed to visualize markers for glycogen, the stellate cell activation and the intercellular connections. METHODS: Six marginal human livers discarded for clinical transplantation were recovered and cold stored in UW during 14±4h. Four livers had >30% steatotis; two had 20-30% steatotis and other co-morbidity. These livers were perfused by HMP during 4h via hepatic artery and portal vein with oxygenated, 4-6 C KPS-1 using an ORS-HMP-prototype. The livers were then rewarmed (36°C) with RS-I and red blood cells (2h). Tissue samples were taken just before HMP (0'), after 1h, and 4h of HMP, at the start of warm reperfusion, and after 1h and 2h of warm reperfusion. The tissues were stained with PAS-stain to stain glycogen, for α-smooth muscle actin (α-SMA) (a marker for stellate cell activation) and for E-cadherin (a protein in hepatocytes tight junctions). RESULTS: PAS-stain revealed a decrease in the glycogen content of the hepatocytes 4h after HMP; this decrease was even aggravated after 2h of warm reperfusion. In the pericentral areas the glycogen had totally disappeared and at the periportal areas the glycogen was still present but much less than at the start of HMP. α-SMA decreased when comparing 0' and 2h of reperfusion. The timing of decrease followed 2 distinct patterns: either after 1h of HMP or during the rewarming. E-cadherin decreased in the HMP preservation period either after 3h or 4h HMP. At that time point a discontinuity was observed in the tight junctions of the hepatocytes. This decrease was even more pronounced during the warm reperfusion. CONCLUSIONS: HMP of marginal livers is associated to a substantial reduction of glycogen primarily in the pericentral areas. α-SMA decreases during HMP or during the reperfusion, suggesting that stellate cells become less activated. That E-cadherin decreases after HMP is intriguing and suggests that the intercellular connections are damaged.

EVIDENCE FOR NO RELEVANCE OF ANTI-MICA ANTIBODIES IN LIVER TRANSPLANTATION

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Introduction Little is known regarding the specificity of antibodies directed to tissue-/organ-specific antigens in patients receiving liver allografts. Recently, the polymorphic MHC class I-related chain A (MICA) is increasingly recognized as a potential target molecule for immune cells during allograft rejections. Here, we studied whether MICA is a target antigen for antibodies in liver transplanted (LTx) patients.

Material and Methods Eighty-four patients were investigated for the presence of MICA reactive antibodies pre and post-LTx, using MICA transfected cells and flow cytometry. MICA typing was performed by PCR. Expression of MICA in liver cells was determined by RT-PCR and Western Blotting. Liver biopsy specimens from patients after transplantation were examined for MICA expression.

Results We found a total of 22/84 (26%) pts with MICA abs either pre and/or post-tx. 8/84 (9.5%) patients had MICA antibodies in the pre- and 14/84 (17%) in the post-tx period. Overall 43/84(51%) patients had rejections. No correlation between rejection frequencies (14/22, 63%) or other clinical parameters was observed in patients with MICA Ab as compared to those without MICA Abs (29/62, 47% p=ns). We found weak mRNA expression for MICA in liver cells, but no protein or cell surface expression by western blots or flow cytometry. In addition, no MICA expression in liver biopsy sections was observed from LTx patients at any time point including rejections.

In conclusion we found no causal relationship between the presence of MICA antibodies and liver allograft rejections. Thus, MICA may not be an important target antigen during liver allograft rejections.

IMPLICATION OF DISCOIDIN DOMAIN RECEPTORS IN THE ACTIVATION OF HEPATIC STELLATE CELLS DURING LIVER METASTASIS

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Discoidin Domain Receptors DDR1 and DDR2 are two novel tyrosine kinase receptors for collagen previously described in aggressive epithelial tumors and their associated stroma. In the metastasized liver, stromal areas are populated by tumor-activated stellate cells of myofibroblastic phenotype releasing tumor cell proliferation and migration factors. The aim of our study was to elucidate the role of DDRs in tumor-activated stellate cells during liver metastasis.

Results: Immunohistochemical analysis of human livers metastasized by colon carcinoma demonstrated co-expression of DDR1, DDR2 and the myofibroblast marker alpha-smooth muscle actin in stromal hepatic stellate cells (HSC). *In vitro*, freshly isolated human HScs cultured in the presence of media from HT29 human colon carcinoma cells showed enhanced DDR1, DDR2 and alpha smooth actin expression than HSCs cultured in basal media. Experimental liver metastases generated by intrasplenic injection of MC38 colon carcinoma cells in DDR2-deficient mice developed faster than in wild type ones. Immunohistochemical co-localization of desmin and alpha-smooth muscle actin was 3-fold higher in livers from DDR2-deficient mice than in the wild type, indicating a higher rate of tumor-associated activated HScs. *In vitro*, supernatant from primary cultures of DDR2-deficient HScs exposed to MC38-conditioned media exerted a 2-fold higher MMP2 activity than wild type ones. Also, MC38 cell proliferation and migration was maximal when cultured in the presence of supernatants from DDR2-deficient HSCs. Furthermore, real time quantitative RT-PCR analysis showed a 10-fold increase in DDR1 mRNA expression of DDR2-deficient HSCs, compared to wild type ones. DDR2-deficient HSCs transfected with a vector encoding a DDR1-interference RNA showed diminished proliferative, adhesive and migratory rates, compared to cells treated with a control vector encoding an irrelevant RNA or wild type ones. In vivo silencing of DDR1mRNA by daily intrasplenic administration of the appropriate vector resulted in a dramatic reduction in the development of experimental liver metastasis by MC38 cells compared to wild type mice. Conclusion: These results demonstrate for the first time a direct role of both DDR receptors in the functional behaviour of stromal hepatic stellate cells during liver metastasis. Ongoing cDNA microarray analysis will reveal how gene regulation in DDR2 deficient mice modulates this response.

NERVE GROWTH FACTOR EXPRESSION BY HEPATIC PARENCHYMAL AND NON-PARENCHYMAL CELLS DURING METASTATIC COLORECTAL DEVELOPMENT IN HUMAN AND MURINE LIVER

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In addition to its contribution to the differentiation and survival of neuronal cells, nerve growth factor (NGF) also plays a role in tumor progression and metastasis. In the liver, expression of NGF is increased during tissue regeneration and hepatocellular carcinoma development, but its role during hepatic metastasis is not well understood.

We investigated NGF and neurotrophin receptor p75 (p75-NTR) expression by cancer and host cells in the hepatic metastasis microenvironment. A tissue-array collection of paraffin-embedded colorectal carcinoma metastasis from 24 patients who had undergone hepatic excision of moderately and poorly differentiated colorectal adenocarcinoma metastases was used. In vivo, the concentration of NGF was determined by ELISA in the hepatic blood from mice affected by hepatic metastases, on the 12th day after intrasplenic injection of C26 colorectal carcinoma cells. Anti-NGF monoclonal antibodies were used to study in situ NGF expression by cancer and stromal cells in hepatic C26 colorectal carcinoma metastases and tumor-unaffected hepatic areas. Finally, primary cultured murine hepatocytes and hepatic stellate cells (HSCs) were incubated in the presence of conditioned medium from murine C26 cancer cells and the production and effects of recombinant murine and endogenous NGF were studied.

NGF immunostaining of metastatic colon cancer cells only occurred in 2 out of 24 patients with hepatic metastases, while around 80% of studied patients had hepatic metastases with NGF-expressing stromal cells. Not statistically significant correlation was demonstrated between NGF immunostaining of tumor-infiltrated stromal cells and cancer cell immunostaining with anti-ki67 antibodies, suggesting that NGF was not involved in the regulation of metastatic cancer cell proliferation. Hepatocytes and hepatic sinusoidal cells showed weak NGF immunostaining, while cholangiocytes had a high immunostaining in the hepatic tissue unaffected by cancer development. Staining with alpha-smooth muscle actin (alpha-SMA) confirmed that sinusoidal immunoreactivity was unequivocally localized to HSCs, but not to other sinusoidal lining cells. Hepatic C26 colorectal carcinoma metastases had an intense NGF immunostaining in those hepatocytes and alpha-SMA-expressing stromal cells located at the invasion front of metastases. NGF-expressing hepatocytes were specifically located among cancer cells and had phenotypic features suggesting epithelial-to-mesenchymal transition. C26 cancer cells did neither express in situ nor secrete in vitro NFG. p75-NTR had a low expression level in normal hepatic tissue, but it significantly increased in hepatocytes and HSCs located around and within hepatic metastases, while C26 cancer cells were negative. Consistent with these in situ findings, NGF significantly increased in the hepatic blood obtained from livers affected by hepatic metastasis development of C26 colorectal carcinoma. NGF concentration was 7 times higher in the supernatants from primary cultured HSCs than in those from hepatocytes. In addition, it significantly increased in the supernatant of HSCs given C26 cancer cell-conditioned medium, and in cultured hepatocytes given tumor-activated HSC-conditioned medium. Finally, recombinant murine NGF dose-dependent increased chemotactic migration, but not proliferation, of HSCs in vitro. Moreover, HSC migration-stimulating activity from tumor-activated hepatocyte supernatant was 50% NGF-mediated in vitro. Our results demonstrate for first time that hepatocytes and sinusoidal stellate cells express neutrotrophin receptor p75 and secrete NGF in response to specific stimulating factors released by cancer cell in the hepatic metastasis microenvironment of human and rodent colorectal carcinoma. Thus, NGF may contribute to metastasis progression through the specific activation of intratumor stromal cell migration.

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