ENDOTOXINS AND CYTOKINES IN THE PATHOGENESIS OF NASH

Anna Mae Diehl, Johns Hopkins University, Baltimore, MD, USA

The pathogenesis of nonalcoholic fatty liver disease (NAFLD) is unknown. NAFLD is strongly associated with obesity and type-2 diabetes, suggesting that some of the metabolic and/or hormonal alterations that occur in these conditions might promote NAFLD. To evaluate this possibility, our laboratory has been studying genetically obese, leptin-deficient ob/ob mice which develop hyperinsulinemia, insulin-resistance and fatty livers spontaneously. Similar to obese humans, these mice are known to over-express TNFα in white adipose tissue. Moreover, TNFα contributes to ob/ob insulin-resistance because the latter phenotype disappears when ob/ob mice are inter-bred with TNF receptor-1 null mice. Ob/ob mice with fatty livers are exquisitely sensitive to lipopolysaccharide endotoxin (LPS)-induced liver injury, a process that is also mediated by TNFα. Treatment with recombinant leptin decreases ob/ob vulnerability to LPS-liver damage, suggesting that leptin deficiency potentiates TNFα hepatotoxicity. Leptin therapy also reduces hyperinsulinemia and insulin-resistance in leptin-deficient mice. Taken together, these observations suggest that leptin inhibits the expression and/or activity of TNFα.

On going work in our laboratory indicates the mRNA levels of TNFα, TNF receptor-1, and several Th-1 (pro-inflammatory) cytokines are increased constitutively in the fatty livers of ob/ob mice. These changes in the hepatic cytokine milieu are associated with a selective reduction of the hepatic CD4 (+) NK T cell population and consequent deficiency of IL-4, a cytokine that these cells produce. Intrahepatic deficiency of IL-4 (the cytokine that promotes the expansion of lymphocytes that produce Th-2 anti-inflammatory cytokine), is accompanied by a relative deficiency of IL-10, an anti-inflammatory cytokine. Thus, the innate immune system in the fatty livers of ob/ob mice has been altered to favor the over-production of TNFα, IL-6, and pro-inflammatory cytokines, such as IL-12. Study of Kupffer cells isolated from the livers of ob/ob mice demonstrates that these cells over-express inflammatory cytokines in vitro. Moreover, recombinant leptin results in a dose-dependent inhibition of inflammatory cytokine production by cultured ob/ob Kupffer cells. Hence, leptin deficiency contributes directly to the localized inflammatory state within ob/ob livers. To determine if it is this localized inflammatory state that causes hepatic insulin-resistance and NAFLD, leptin-deficient ob/ob mice were treated with metformin, a biguanide that improves hepatic insulin-resistance. Metformin therapy cured the NAFLD in ob/ob mice, reversing hepatomegaly, eliminating hepatic steatosis, and normalizing serum liver enzyme values. The benefits of metformin occurred despite persistent over-expression of TNFα mRNA in white adipose tissue. However, they were associated with the normalization of hepatic TNFα gene expression and the down-regulation of several TNF-inducible, pathogenically-relevant, gene products (e.g., mitochondrial uncoupling protein-2, sterol-regulatory element binding protein-1, and fatty acid synthase) to levels that occur in normal livers. Taken together, these results suggest that leptin deficiency potentiates a localized inflammatory state within the liver which, in turn, causes insulin-resistance and NAFLD.

Cyclooxygenase-2: A Potential Target for the Prevention and Treatment of Alcoholic Liver Disease

Andrew J. Dannenberg, M01 and Amin A. Nanji, MD2

1Department of Medicine, Weill Medical College of Cornell University and Strang Cancer Prevention Center, New York, N.Y. 10021 USA
2Department of Pathology, The University of Hong Kong, Hong Kong

Cyclooxygenase-2 (COX-2), the inducible form of COX, catalyzes the synthesis of proinflammatory eicosanoids from arachidonic acid. The potential importance of COX-2 in liver injury was demonstrated by the finding that COX-2 deficient mice are resistant to endotoxin-induced liver injury (Nature 378:406409, 1995). In the rat intragastric feeding model for alcoholic liver disease (ALD), we demonstrated that up-regulation of COX-2 and eicosanoid synthesis occurred in the presence of increased levels of endotoxin, TNF-a and lipid peroxidation (Gastroenterology 112:943-951, 1997). Subsequently, studies were done in
established ALD to determine whether dietary saturated fatty acids could reverse pathologic liver injury and decrease endotoxemia, lipid peroxidation and the expression of COX-2 and TNF-a (Hepatology 26:1538-1545, 1997). Male Wistar rats were fed fish oil, a lipid enriched in omega-3 fatty acids, and ethanol (FE) to induce severe liver injury. After six weeks of the FE diet, rats were switched to diets containing dextrose and either fish oil or saturated fatty acids (SFA) (medium chain triglycerides or palm oil). Treatment with SFA for two weeks led to near normalization of histologic changes including fatty liver, necrosis, inflammation and fibrosis. The improvement in histopathology in the SFA fed groups was associated with reduced endotoxemia and lipid peroxidation as well as decreased amounts of COX-2 and TNF-a. In contrast, minimal improvement was observed in rats that continued to be fed fish oil. Taken together, these findings raise the possibility that therapy which reduces the amount or activity of COX-2 will be useful in the management of ALD. The availability of newly developed selective COX-2 inhibitors will permit this hypothesis to be tested.

**Inducible Cytochromes P450 And Lipid Peroxidation In NASH**

GC Farrell, Leclercq, G Robertson, M Weltman, C Liddle. Storr Liver Unit, Westmead Millennium Institute, University of Sydney at Westmead Hospital, NSW 2145, Australia.

The two hepatic microsomal cytochromes P450 (CYP) most involved with lipid oxidation are CYP2E1 and CYP4A. CYP2E1 is induced by ethanol, but also by the factors most often associated with NASH in humans - diabetes and obesity associated with insulin resistance. CYP2E1 has a relatively low affinity for oxygen, allowing "leakage" of electrons that reduce O2 to form reactive oxygen species (ROS) that initiate lipid peroxidation (1). CYP2E1 is usually present in a rim 2-3 hepatocytes thick in perivenular zone 3. In alcoholic hepatitis, there is extensive immunostaining of CYP2E1 in the liver (2). Using immunohistochemistry, we have also demonstrated increased expression of CYP2E1 protein in livers of patients with NASH. In all 31 livers studied, there was extensive staining for CYP2E1 throughout the hepatic lobule, or nodule in cases with cirrhosis (3).

To pursue the potential mechanistic relevance of CYP2E1 in NASH, we have studied rodent models of obesity and steatohepatitis. Leptin or leptin receptor deficient rodents (eg ob/ob mice) develop diabetes and profound hepatic steatosis but not NASH. Levels of CYP2E1 are reduced or normal in these animals (4), and there is no accumulation of lipid peroxides. Rats or mice fed a lipid-rich diet deficient in methionine and choline (MCD diet) develop extensive steatosis, focal inflammatory lesions and fibrosis, at 10 weeks in mice, 16-24 weeks in rats (5,6). This model is associated with induction of CYP2E1, shown by immunohistochemistry and apparently following the distribution of steatosis, with two-4fold or greater increases of CYP2E1 mRNA, microsomal 2E1 protein and related enzyme activities.

Livers from C57BL6/J (wildtype) mice fed the MCD for 10 weeks contained massive amounts of lipid peroxides (6). Microsomal NADPH-dependent lipid peroxidases contributed to the formation of these lipid peroxides, a process completely abrogated by chemical blockade or immunoinhibition of CYP2E1. Thus CYP2E1 is a plausible source of pro-oxidants as part of an oxidative stress mechanism of liver injury in NASH, as has also been proposed for alcoholic hepatitis (2).

In order to establish whether CYP2E1 over-expression is essential for pathogenesis of NASH, we fed Cyp2el 4- nullizygous mice (collaboration with Frank Gonzales, NCI) the MCD diet. Cyp 2el 4- mice developed florid NASH and hepatic accumulation of lipid peroxides, as for their heterozygous littermates and wildtype mice. Livers from MCD4ed Cyp2el 4- mice expressed no Cyp2el but the two major murine CYP4As, 4a10 and 4a14 were upregulated (6). Further, hepatic microsomes from these mice generated NADPH-dependent lipid peroxides that could not be inhibited by CYP2E1 inhibitors, but which was substantially inhibited by anti-mouse Cyp4al 0 antibody. Other evidence that CYP2E1 is not a unique source of pro-oxidants in NASH comes from mice lacking acyl CoA oxidase (AOX). These animals develop florid NASH related to over-expression of PPAR-a, with peroxisomal proliferation, increased hepatic production of H2O2 and over expression of other PPAR-dependent proteins involved with hepatic lipid metabolism, including CYP4A (7).

In summary, experimental NASH is strongly associated with hepatic microsomal lipid peroxidation. CYP2E1 appears to be the main inducible cytochrome involved with this process in humans, rats and wildtype mice, but this enzyme is not unique among P450 proteins in catalyzing peroxidation of endogenous lipids, and may not be the only biochemical pathway leading to oxidative stress. We have now identified CYP4A enzymes as alternative initiators of oxidative stress in NASH, although the mechanism for coordinate regulation of CYP2E1 and 4A remain unclear. Interventional studies are now required to prove the essential link between over-expression of microsomal lipid oxidase pathways, steatosis and oxidative stress in NASH, and the relevance of these biochemical changes to liver injury, inflammation and hepatic fibrogenesis.

Session III

Pathogenic Mechanisms of NASH and ASH (Part II)

Mitochondrial Dysfunction in Hepatic Steatosis

D. Pessayre, INSERM U481, Hopital Beaujon, 92118 Clichy, France

Mitochondria play a major role in hepatic fatty acid oxidation, ATP formation, and the basal formation of reactive oxygen species (ROS) in the cell. Mitochondrial dysfunction may be both the cause and the consequence of steatosis.

From mitochondrial dysfunction to steatosis

Ethanol, copper, drugs, cytokines or sex hormones may impair the mitochondrial oxidation of fatty acids and cause steatosis by 1) oxidatively damaging mtDNA (e.g., ethanol, copper), 2) impairing mitochondrial DNA (mtDNA) replication (dideoxynucleosides, fialuridine), 3) impairing mtDNA transcription (IFN-a), 4) directly impairing mitochondrial respiration (amiodarone, perhexiline, tamoxifen, buprenorphine, TNF-a, endotoxin, NO), 5) sequestering CoA, a cofactor involved in the initial activation of fatty acids (salicylic acid, valproic acid), 6) directly impairing II-oxidation (amiodarone, perhexiline, arylpropionate NSAIDs, aminoptine and tianeptine, tetracyclines, valproate), or 7) causing ultrastructural and functional mitochondrial lesions (female sex hormones).

From steatosis to ROS

Primary NASH. NASH patients exhibit ultrastructural mitochondrial lesions, decreased activity of respiratory chain complexes and impaired in vivo ATP regeneration after a fructose challenge. The basal mitochondrial ROS formation oxidizes fat deposits causing lipid peroxidation, which releases 4-hydroxynonenal that impairs mitochondrial respiration. Furthermore, adipocytes are an important source of TN F-a, which also impairs mitochondrial respiration. Whenever the flow of electrons is interrupted at some step of the respiratory chain, the respiratory chain components which are located upstream are overly reduced and directly transfer their electrons to oxygen forming the superoxide anion and other ROS. These ROS in turn oxidatively damage mtDNA, causing more impairment of respiration and more ROS formation in a vicious circle. Some aggravating factors may further increase ROS formation in patients with primary NASH, including 1) CYP2E1 induction and increased hepatic iron stores (two other sources of ROS), and 2) lipid peroxidation-mediated consumption of GSH, anti-oxidant vitamins and perhaps anti-oxidant enzymes (thus decreasing cell defences against ROS). Vitamin E may be decreased and vitamin E supplements may improve liver tests in children with NASH.

Secondary NASH. In secondary NASH, the situation is even worse as the causative disease itself also increases ROS formation. Ethanol and copper directly increase ROS formation. Amiodarone, perhexiline and tamoxifen first impair respiration, which secondarily increases ROS formation. Total parenteral nutrition and jejunohoral bypass may cause bacterial proliferation in the unused/excluded intestine, thus releasing cytokines, NO and endotoxin, which all impair respiration.

From ROS to steatohepatitis

Secondary hit. When ROS formation is increased, this "second hit" may cause more lipid peroxidation and more cytokine release by Kupifer cells and hepatocytes themselves. Both lipid peroxidation and cytokines may cause 1) hepatocyte death, 2) crosslinking of cytokeatins and Mallory body formation, 3) activation of Ito cells and fibrogenesis and 4) recruitment of polymorphonuclear cells.

UCP2. This uncoupling protein is induced in steatosis. It may decrease the mitochondrial membrane potential and sensitize cells to various causes of cell death.

The Role of Iron in the Pathogenesis of Alcoholic and Non-Alcoholic Fatty Liver

Yves Deugnier, Michel Mendler, Romain Moirand, Anne Guillygomarc'h and Pierre Brissot
Clinique des Maladies du Foie and INSERM U522, H–pital Pontchaillou, 35033
Rennes, France
Abnormalities in iron metabolism are usual in patients with either alcoholic (AFL) or nonalcoholic fatty liver (NAFL). Serum ferritin, serum iron and transferrin saturation are frequently elevated in alcoholic patients, but this does not imply that liver siderosis has developed since ferritin synthesis is stimulated by alcohol and chronic liver disease may be responsible for abnormal serum iron tests in the absence of increased body iron stores. However, 50% of alcohohics present with hepatic iron overload - of whom 30% have significant hepatic siderosis - whether they have cirrhosis or not. This may be due to high iron intake (red wines), elevated nontransferrin-bound iron, chronic hemolysis, or increased iron absorption related or not to underlying HFE mutations. In patients with NAFL, hyperferritinemia is frequent and usually associated with normal transferrin saturation [1]. It corresponds to hepatic siderosis in 5% to 64% of cases according to series and means of assessment of iron stores (histology, determination of hepatic iron concentration or MRI) [2-3]. Insulin resistance - which is a common feature in patients with NAFL - could account for the development of iron overload in these subjects as suggested by the recent description of an insulin resistance-associated hepatic iron overload syndrome [4]. The role of HFE mutations has also been postulated on the finding of an increased prevalence of the C282Y mutation in patients with both NAFL and hepatic siderosis [2]. Whatever the cause of its deposition in AFL and NAFL, iron could aggravate the course of steatosis by triggering steatohepatitis (ASH and NASH) and, then, fibrosis. Indeed, by initiating oxidative stress and promoting lipid peroxidation - that can be responsible for cell damage and fibrogenesis through the production of various proinflammatory and profibrogenic products - iron could act as the second hit in the 2-hit process of steatohepatitis. This was suggested by the demonstration of an association between liver siderosis, the C282Y mutation, and the development of fibrosis in 51 Australian patients with NASH [2], but not supported by a study of 65 American patients with NAFL (3). Further experimental and human data - especially the effects of venesection therapy in cases of iron overloaded NAFL - are needed before understanding the cause and the role of iron deposition in steatohepatitis.


**Insulin Resistance in Non-Alcoholic Fatty Liver Disease. A clinical Perspective. Preliminary Results**

A. Lonardo, P. Loria*, P. Trande, P. Non, M. Pulvirenti, A. Borsatti*, A.M. Verrone* A.E. De Micheli, and N. Carulli*
Division of Internal Medicine and Gastroenterology, Modena City Hospital; anc
~Chair of Internal Medicine, Modena University Hospital - Modena, Italy

**BACKGROUND/AIMS** - Nonalcoholic fatty liver disease (NAFLD) is a common condition that defines a spectrum of alcohol-like liver disease in the absence of alcohol abuse, genetic, viral and autoimmune etiologies. Usual presentations of NAFLD in Italy include ultrasonographic evidence of fatty liver ("bright liver" based on liver-kidney contrast) with or without alteration of liver function tests, otherwise asymptomatic subjects. Recent literature supports the view that NAFLD might be related to insulin resistance. The latter condition is defined by a suboptimal biological response to insulin action that represents the shared pathophysiologic precursor for the components of the insulin resistance syndrome, namely obesity diabetes, hyperlipidemia and hypertension. To gain further insight into NAFLD - prospective study on this condition was planned by recruiting consecutive subject - referred to us by general practitioners participating in the study. **METHOD** -Criteria for referral were the evidence of a "bright liver" detected ultrasonographically - in a subject drinking <30 g of alcohol per day (<20 g for women). Referred subject underwent detailed history, physical examination, repeated US scanning using semiquantitative (moderate versus severe) scoring of liver-kidney contrast, biochemical profile, serum insulin determination, oral glucose tolerance test, a study of autoimmunity and HFE gene mutations, ECG, and ultrasound-doppler carotid examination whenever clinically indicated. In addition they were proposed liver biopsy in case of significant chronic elevation of serum transaminases. Insulin sensitivity index (181 ~) was computed through the formula: IO~ lnsulin ~ x Glucose ~. Chi-square and t-test were used as appropriate. **RESULTS** - The study is ongoing:

here we summarize the preliminary results. In the October 1999-April 2000 study period 49 consecutive cases were studied. Of these 10 were excluded from the analysis because of the following: use of alcohol slightly exceeding 30g/day (6 males); presence of autoantibodies (2 females); HCV-positivity (1 female) hypobetalinoproteinemia (1 female). The analysis of the 39 remaining subjects revealed as follows. Prevalence of male subjects (58%). Obesity (42%), hyperlipidemia (38%), diabetes/impaired glucose tolerance (35%), hypertension (7%) were present (often in combination) in most cases. Hypertransaminasemia was present in 55% of cases with a mean value in hypertransaminasemic patients of GOT 44.95 + 29.09; GPT 83.66+51.55. GPT/GOT ratio was >1 in all cases. The median value for 181 ~ in our series was 6.4. When compared to subjects with ISI ~ 6.4, patients with ISI ~ < 6.4 were more likely to be obese (54% vs. 25% p<.05) and to have elevated VGT values (49.78+31.03 vs. 27.62+26.59 p<.05). In addition, subjects with altered transaminase values were compared to those with normal transaminases. The former group included subjects with more elevated VGT values (54.95 i 35.11 vs. 23.68 i 12.14 p<.001); hyperuricemia (17.6% vs. 5.8% p<.05); and more abundant iron stores (serum iron 108.5+26.98 vs. 87.62 ~ 26.20 p <.05; serum ferritin 199.7 * 172.25 vs 99.93 * 62.7 p <.05).
CONCLUSIONS - Data analysed as of 10th May 2000 indicate that 1) NAFLD is a male-predominant condition. 2) Most subjects with NAFLD have at least one of the components of the insulin resistance syndrome as a concurrent disease. 3) Obesity and elevated VGT values are more prevalent in NAFLD patients with a lower insulin sensitivity index. 4) 55% of NAFLD cases have abnormal transaminase levels that cluster with elevated YGT, uric acid, iron and ferritin serum levels.