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ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

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From Alcohol Toxicity to Treatment

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This article presents the proceedings of a symposium held at the meeting of the International Society for Biomedical Research on Alcoholism in Mannheim, Germany, in October 2004. This symposium was dedicated to Charles S. Lieber in recognition of his contribution in alcohol research over the last 50 years. It was divided into two parts, namely effects of alcohol on the gastrointestinal tract and effects of alcohol on the liver. Major emphasis was given to recent discoveries elucidating mechanisms of alcohol-associated carcinogenesis. M. Salaspuro (Finland) discussed the role of acetaldehyde in the saliva and in the large intestine with respect to its role in the pathogenesis of alcohol-associated cancer, and H. K. Seitz (Germany) presented new data identifying individuals homozygous for the ADH1C*1 allele as high on risk for alcohol-associated upper aerodigestive tract cancer. M. Savolainen (Finland) discussed the role phosphatidylethanol as a bioactive lipid that can mediate beneficial and harmful effects of alcohol drinking. In the second part of the symposium, alcoholic liver disease was discussed. P. Haber (Australia) presented new data on hepatic transcriptome in alcoholic liver disease with the identification of new genes possibly involved in alcohol-initiated fibrogenesis of the liver, and H. Moshage (The Netherlands) described survival mechanisms of the cholestatic hepatocytes with implications for therapy in cholestatic liver disease. The role of the hepatic microsomal ethanol oxidizing system in the metabolism of alcohol in alcoholic liver disease was summarized by R. Teschke (Germany). H. Ishii (Japan) discussed the current status and treatment of alcoholic hepatitis in Japan. Finally, in a state-of-the-art lecture, Charles S. Lieber (USA) discussed the development of the understanding of the pathophysiology of alcoholic liver disease in the last 50 years. He emphasized the role of pathophysiology as an important prerequisite for better treatment strategies.

Key Words: Acetaldehyde, Gastrointestinal Bacteria, Hepatocellular Carcinoma, Phosphatidylethanol, Hepatic Transcriptome, Annexin A2, Alcoholic Hepatitis, Microsomal Ethanol Oxidizing System, Cholestasis, Cytochrome P4502E1, Hepatic Fibrogenesis.

FIFTY YEARS AGO, alcohol was considered to be nontoxic. In the mid-fifties of the last century, it was stated in Harrison's *Text Book of Medicine* that alcohol is probably as toxic as sugar and that alcoholic liver disease (ALD) is the consequence of malnutrition. Undoubtedly, it is the merit of Charles S. Lieber to have questioned this dogma and to have demonstrated alcohol toxicity on the liver and on various other organs. Charles S. Lieber, a giant in alcohol research, has pioneered the scientific field and has contributed to more than 1,000 scientific papers to the understanding of metabolic and toxic effects of alcohol on the liver and on the body in the last half of the century. This symposium bridges developments of knowledge and understanding of alcoholic liver disease from the past to the present and the future.

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REGULATION OF ACETALDEHYDE CONCENTRATION IN SALIVA AND IN THE CONTENT OF THE LARGE INTESTINE

M. Salaspuro

Some genetic factors, smoking, heavy drinking, and poor dental status are the major risk factors of upper digestive tract cancers. As well, they are also the strongest factors increasing salivary acetaldehyde production (Salaspuro, 2003).

A moderate dose of alcohol increases salivary acetaldehyde concentration to carcinogenic levels, and this is mainly due to the microbial oxidation of salivary ethanol to acetaldehyde (Homann et al., 1997). During smoking, salivary acetaldehyde level is further increased to seven-fold (Salaspuro and Salaspuro, 2004). Acetaldehyde of tobacco smoke is thus dissolved in part in the saliva and can be transported by swallowing further to the esophagus.

There exists a positive linear correlation between acetaldehyde production by oral microbes and cigarette use also *in vitro* (Homann et al., 2000). A smoker with a daily consumption of approximately 20 cigarettes has a 50–60% increased *in vitro* salivary acetaldehyde production from ethanol. Heavy drinking (>40 g/d) is also associated with increased *in vitro* acetaldehyde production in the saliva (Homann et al., 2000). When an increase is observed, it is dose-dependent and increases salivary acetaldehyde levels by about 50%. In a study with 132 volunteers, poor oral hygiene showed an approximately two-fold increase in salivary acetaldehyde production from ethanol, and this was confirmed after adjustment for smoking, alcohol consumption, age, and sex (Homann et al., 2001). The risk of ethanol-associated digestive tract cancers is remarkably increased in Asian subjects with partially inactive acetaldehyde dehydrogenase (ALDH)2 enzyme (Yokoyama et al., 1998).

After ingestion of a moderate dose of alcohol, ALDH2-deficient Asians have two to three times higher acetaldehyde levels in their saliva than those with the normal enzyme (Väkeväinen et al., 2000). The additional salivary acetaldehyde is derived from the salivary glands. Obviously, salivary glands of the individuals with the deficient ALDH2 enzyme are not able to detoxify acetaldehyde formed from ethanol by their own alcohol dehydrogenase (ADH)-enzyme.

ALDH2-deficient individuals had nine times higher acetaldehyde levels in their saliva than in their blood, which indicates that the additional salivary acetaldehyde cannot be derived from the blood (Väkeväinen et al., 2000). In subjects with the normal ALDH2 enzyme, the main source of salivary acetaldehyde are microbes representing the normal oral flora (Väkeväinen et al., 2001).

ALDH2-deficient Asian heavy drinkers form an exceptional human "knockout" model for long-term acetaldehyde exposure. Every time they drink alcohol, they are exposed for hours to abnormally high levels of salivary acetaldehyde, and this is associated with a remarkably increased risk of digestive tract cancers.

The ADH enzyme encoded by the ADH1C*1 allele metabolizes ethanol to acetaldehyde 2.5 times faster than that with the ADH1C*2 allele. In the homozygotic individuals, for this high activity, ADH the increased alcohol-related upper digestive tract cancer risk seems to be due to the elevated salivary acetaldehyde concentration after alcohol drinking (Visapää et al., 2004).

Two-thirds of carcinogenic acetaldehyde can be trapped in human saliva with slowly and continuously released L-cysteine (Salaspuro et al., 2002). It remains to be established in randomized trials whether this type of tablet formulas can be used for cancer prevention among abovementioned high risk groups.

Lactulose is a nonabsorbable disaccharide that is rapidly metabolized by cecal bacteria into short chain fatty acids, mainly lactate, thus inducing a consequent drop in the pH of the colonic contents. Lactulose has also been shown to decrease significantly the recurrence rate of colorectal adenomas (Roncucci et al., 1993). Lactulose feeding to rats significantly reduces ethanol elimination rate and intraluminal acetaldehyde concentration in the colon after ethanol administration (Zidi et al., 2003).

It can be concluded that smoking and alcohol together increase the salivary acetaldehyde production by about 100% as compared with nonsmokers and moderate drinkers.

Furthermore, during active smoking, salivary acetaldehyde concentration is increased further to seven-fold. Genetic factors—decreased capacity to eliminate salivary acetaldehyde or increased capacity to produce it—may further increase salivary acetaldehyde concentration after alcohol drinking by more than 100%. Therefore, all main risk factors for upper digestive tract cancer independently, jointly, and synergistically increase the salivary acetaldehyde concentration. This provides a new genetic and microbiologic approach for the pathogenesis and possible prevention of digestive tract cancers.

GENETIC PREDISPOSITION FOR ALCOHOL-ASSOCIATED UPPER AERODIGESTIVE TRACT CANCER AND HEPATOCELLULAR CARCINOMA IN HEAVY DRINKERS WITH THE ALCOHOL DEHYDROGENASE 1C*1 ALLELE

H. K. Seitz

Chronic alcohol consumption is associated with an increased risk for upper aerodigestive tract cancer and hepatocellular cancer (HCC). Acetaldehyde, a known carcinogen in rodents, is believed to be primarily involved in alcohol-associated carcinogenesis (Seitz et al., 2004).

Indeed, Asians with high acetaldehyde levels after alcohol ingestion, caused by a mutation of ALDH2, have an increased risk of UADTC. In whites, polymorphism of ADH1C occurs and the ADH1C*1 allele encodes for an enzyme with a higher capacity to generate acetaldehyde than ADH1C*2. Recent epidemiologic studies resulted in contradictory results regarding ADH1C polymorphism and alcohol-associated cancer (Seitz et al., 2004).

We therefore determined the ADH1C genotypes in heavy alcohol consumers with and without cancer. Altogether, 187 with UADTC (laryngeal cancer, 41; oropharyngeal cancer, 46; esophageal cancer, 100), 75 with alcoholic cirrhosis and HCC, 424 age-matched alcoholic patients without cancer (alcoholic cirrhosis, 164; alcoholic pancreatitis, 146, alcoholics without organic disease, 114) and 48 healthy control subjects were included. ADH1C genotypes were assessed by polymerase chain reaction followed by restriction fragment length polymorphism on white blood cell DNA. The allele frequency of the ADH1C*1 allele was significantly increased in heavy drinkers with upper UADTC and in patients with HCC in cirrhotic livers compared with age-matched alcoholic control subjects without cancer ($p < 0.01$). In addition, homozygosity of ADH1C*1 was also significantly higher in cancer patients (249 alcoholics with cancer: 35% vs. 424; alcoholics without cancer:

19%; $p < 0.001$). These data suggest that heavy drinkers homozygous for the ADH1C*1 allele carry a genetic risk for development of UADTC and HCC, respectively.

PHOSPHATIDYLETHANOL IS A BIOACTIVE LIPID MEDIATING THE BENEFICIAL EFFECTS OF ETHANOL

M. Savolainen

Even light to moderate alcohol consumption protects against coronary heart disease (Hannuksela et al., 2004). The protective effect could be partly mediated by an ethanol-induced increase in HDL cholesterol concentration, but the exact mechanisms are not known. In addition to the role in the reverse cholesterol transport, HDL particles may also regulate the expression or abundance of several growth factors, adhesion molecules, nitric oxide synthase, and cytokines. Furthermore, phospholipid composition of HDL lipoproteins has been shown to be of importance for the antiatherogenic functions of HDL. Phosphatidylethanol (PEth), an ethanol derivative of phosphatidic acid, is a compound formed only in the presence of ethanol. It is also present in the blood of subjects who have been drinking alcohol. We have recently shown that lipoprotein-associated PEth increases the endothelial secretion and the plasma concentration of vascular endothelial growth factor (VEGF) by signaling routes mediated by MAPK and PKC (Liisanantti et al., 2004a). We have also shown that PEth is transferred between lipoprotein particles by transfer proteins, both by cholesteryl ester transfer protein (CETP) and phospholipids transfer protein (PLTP) (Liisanantti et al., 2004b).

We hypothesized that lipoprotein-associated PETh enhances VEGF secretion also from smooth muscle cells, which may be more important because of their strong expression of VEGF and its receptors. An increase in the production of VEGF in cultured human smooth muscle cells by lipoprotein-associated PEth was time-dependent, and the maximal increase was obtained by 0.3% PEth-HDL. This content is lower than in our previous study and even lower than PEth concentration determined in HDL fractions isolated from alcohol drinkers (unpublished results).

Previous epidemiologic studies have shown that one drink twice a week may protect against atherosclerosis but has no effect on HDL cholesterol level. The beneficial effects of moderate drinking have therefore been hard to explain. The direct effects of ethanol on arterial wall are temporary if they are confined only to the period of ethanol abundance. For most of the hours of the day, ethanol is not present in the body of a social drinker.

A plausible explanation for the effect of moderate drinking may be provided by our new hypothesis suggesting that the long-term effect of occasional drinking is brought about by lipoproteins acting as carriers of PEth, an ethylated derivative of phosphatidic acid, a lipid second messenger. PEth is formed in the presence of ethanol and affects metabolism and function of cells by interfering with signal transduction. Association with lipoproteins could prolong the half-life of PEth from a few hours to several days even in an occasional drinker. In fact, the effect of PEth may last even longer, since erythrocytes may act as a reservoir of PEth and lipoproteins may transport PEth from erythrocytes to vascular wall cells.

Our research provides evidence that PEth molecules associated with HDL particles may alter the effect of the lipoprotein on vascular cells. The increasing effect of PEth-containing HDL on the secretion of VEGF from smooth muscle cells suggests that these cells are highly sensitive to the effects of altered HDL particles (Liisanantti and Savolainen, 2004c). Since these cells are in close contact with endothelial cells lining the vascular wall, this alteration may significantly affect the endothelial function.

In conclusion, ethanol not only modifies the quantity of lipoproteins or their components but also may cause incorporation of abnormal derivatives of lipids and proteins into lipoproteins and may therefore have marked and sustained effects on all vascular cells.

Further studies are needed to clarify more detailed mechanisms of how PEth regulates the interplay between the vascular wall cells during the development of atherosclerosis.

HEPATIC TRANSCRIPTOME IN ALCOHOLIC LIVER DISEASE

P. Haber

Alcoholic liver disease remains a major cause for morbidity and mortality in Australia. Despite the recognition of alcohol as a direct hepatotoxin, the precise molecular pathways that are important in causing liver injury are not well understood. One new approach is to study the effects of alcohol on hepatic gene expression. Microarray analysis is a powerful technique that enables simultaneous expression analysis of thousands of genes. It allows testing of established hypotheses and generation of new hypotheses concerning the pathogenesis of ALD. We have investigated the progression of pathogenesis of ALD by analysis of liver biopsy tissue using immunohistochemistry, reverse transcriptase polymerase chain reaction (RT-PCR), and DNA microarray technology.

RNA liver biopsies from baboon (B: alcohol-fed and control, $n = 2$ each), human noncirrhotics (NC, $n = 7$), alcoholic hepatitis (AH, $n = 7$) and explants from end-stage ALD (ES, $n = 7$) and nondiseased (ND, $n = 7$) were hybridized to human DNA microarrays. Analyses were performed with regression and using Bioconductor and *R*. t Statistics in *R* were used to rank differentially expressed (DE) genes. DE status of selected genes were confirmed by real time RT-PCR, using gene-specific primers and SYBR green. Frozen sections of human liver tissue were probed with primary antibodies to selected molecules and cell type marker antigens. Detection was with secondary antibodies AlexaFluor 488 and 594. RNA from hepatocyte carcinoma

cell lines (Huh7 and HepG2) treated with alcohol was used for real-time RT-PCR with annexin A1, A2, and p11 primers. Antibodies to the above molecules were used to detect cell surface expression of these molecules in response to alcohol treatment by using flow cytometry.

Using the novel approach of intrahepatic gene expression by cDNA array analysis, we have shown that the global gene expression profile of the Lieber-DeCarli baboon model of ALD is similar to that of human ALD. Expression profiling with microarrays identified several genes already implicated in alcohol and liver injury as well as novel genes/molecular pathways that are altered in this disease.

Gene expression data from the baboon ALD model is sparse in the literature; however, this study has identified a number of differentially expressed genes previously reported in other models of ALD including CYP2E1, Bcl-xL, LBP, MCP1, MMPs and TIMPs, ADH, GABA-R, PA, and collagens (Seth et al. 2003). Differential expression of CYP2E1 in the baboon is of particular interest because previous studies have demonstrated induction of CYP2E1 after chronic alcohol administration in several animal models. Advanced human alcoholic disease was identified by more fibrosis and immune related genes, whereas baboon with recent alcohol intake had more metabolism DE genes (Seth et al., 2003). Genes associated with fibrosis were the most prominent category of differentially expressed genes in human ALD, consistent with the fibrotic nature of ALD. However, the genes identified as differentially expressed were not specific to alcohol and have been reported in primary biliary cirrhosis (Shackel et al., 2001) and other forms of liver cirrhosis. We found genes such as alcohol dehydrogenases, acetaldehyde dehydrogenases, IL-8, *S*-adenosyl methionine synthetase (SAMeS), phosphatidylethanolamine N-transferase known to be involved in alcohol injury, collagens, MMPs, TIMPs, and integrins that are involved in fibrosis. This study also identified novel genes/pathways, such as claudins, osteopontin, CD209, selenoprotein, and several annexins. Comparison of transcriptomes from the baboon, end-stage ALD and alcoholic hepatitis revealed a small number of genes common to all three groups. Lumican, matrix Gla protein, and huntingtin-interacting protein 1 (HIP-1) were common in the three groups. Genes common to 1) baboon and AH belonged to toxin metabolism, oxidant stress, fibrosis, and coagulation [e.g., antithrombin III, paraoxanse 1, annexin A2, HMG-CoA reductase]; 2) baboon and ES were immune-related [e.g., CCL2]; 3) AH and ES belonged to ECM and fibrosis [FGF receptor, collagen type IV, integrin alpha V]. We have confirmed DE using real-time RT-PCR of selected genes [CXCL10, aldehyde dehydrogenase 1, Cyp2e1, frizzled related protein (FRITZ), glial cell–derived neurotrophic factor (GDNF), neuromodulin (GAP43), annexin A2, calpactin light chain (p11), annexin A1, annexin A7, IL-8, claudin 10, tumor necrosis factor receptor superfamily 14 (TNFRS*F*1,4), CD209, SAMeS, and osteopontin] in the different stages of alcoholic liver disease as compared with nondiseased liver. SAMeS required for conversion of methionine to SAMe is known to be reduced in alcoholic cirrhotics. We found SAMeS to be downregulated severalfold in alcoholic hepatitis, cirrhotics, and end-stage ALD. Several members of the annexin family were found to be DE in the baboon, AH, and ES. Annexin A2, a calcium and phospholipid-binding molecule, has been identified as a surface receptor for plasminogen and tissue plasminogen activator (tPA) on various cells. It has been reported to be upregulated in HUVECs in the presence of low levels of alcohol and is proposed to have a cardioprotective role (Falcone et al., 2001). Annexin A2 protein was upregulated on hepatocyte cell surface, bile duct cells, and portal-parencyhmal interface in the ALD cirrhotic liver as compared with the nondiseased tissue using immunofluorescence. Dual staining with annexin A2 revealed colocalization with cytokeratin 7 (proliferating bile duct cell marker), CD14 (macrophage/Kupffer cell marker), and CD3 (T-cell marker). Proliferation of bile ducts has been reported in human alcoholic hepatitis. These bile ductal changes correlated strongly with liver fibrosis, cirrhosis, portal inflammation, and overall histologic severity scores in patients with ALD. It is intriguing to note that annexin A2 expression was prominent in these proliferating bile ducts.

In Huh7 and HepG2 cells treated with 10 mM and 50 mM ethanol, Annexin A2 and p11 RNA (real-time RT-PCR) and protein (flow cytometry) expression was increased, indicative of a direct effect of ethanol on these molecules. AIIt is also involved in the activation as well as reduction of plasmin, thereby modulating ECM degradation.

We propose that upregulation of annexin A2 and p11 in response to alcohol may modulate alcohol-induced fibrosis in ALD and could be a potential therapeutic candidate.

ALCOHOLIC HEPATITIS: CURRENT STATUS AND TREATMENT IN JAPAN

H. Ishii

Recently, the incidence of ALD has been increasing in Japan; this has been associated with an increase in alcoholic beverage consumption. Multiple organ failure is frequently observed in patients with severe alcoholic hepatitis (SAH), most of whom have a poor prognosis similar to that of fulminant hepatitis; numbers of patients with SAH also appear to be increasing. Although the prognosis of fulminant hepatitis in Japan has recently been improved by intensive treatments such as plasma exchange (PE), that of SAH has not changed. The objective of this study was to determine whether any factor exists that could determine the prognosis of SAH. To that effect, we carried out a nationwide survey by asking hospitals certified by the Japanese Society of Gastroenterology and hospitals affiliated with ours hospital for the current status of patients with

SAH since 1998. We investigated correlations between various factors including blood laboratory data, presence of other organ failure, and prognosis. The mortality rate of patients with SAH was high (66.4%). The ratio of female patients with SAH (26%) was higher than that for women with other ALD (approximately 10%). Mean age was older (alive: 44 ± 11; dead: 50 ± 11 years), whereas blood cell (WBC) counts were higher (11,600 ± 5,200; 21,800 ± 13,400/ml), red blood cell counts were lower (320 ± 95; 265 ± 72 × 104/ml) and prothrombin time (PI) was prolonged (36.2 ± 10.4% and 29.1 ± 9.4%, respectively) in patients who had died compared with those who had survived.

However, as all differences diminished after carrying out PE, we analyzed data from patients without PE. The results were similar to those for all patients. Prevalences of infection, gastrointestinal (GI) bleeding, and disseminated intravascular coagulation (DIC) were higher in patients who had died. Although intensive treatments such as PE, hemodialysis, and anticoagulation therapy did not alter the prognosis of SAH, PE improved the prognosis of patients free of either GI bleeding or DIC. WBC counts were also higher in patients who had died after undergoing PE. Two cases had been cured by granulocytapheresis (GCAP). In conclusion, PE can compensate for and reduce the risk due to prolonged PT in patients with SAH. These results suggest that patients with markedly elevated WBC counts, prolonged PT, or anemia should, if possible, be treated at an earlier stage with an intensive treatment such as PE, hemodialysis, or anticoagulation therapy before infection, GI bleeding, or DIC occurs.

Furthermore, GCAP might be useful for patients with SAH with higher WBC counts.

HEPATIC MICROSOMAL ETHANOL-OXIDIZING SYSTEM (MEOS): EARLY ASSUMPTION AND SUBSEQUENT VERIFICATION REGARDING ITS NATURE AND ROLE

R. Teschke

The hepatic microsomal ethanol-oxidizing system (MEOS) was discovered and characterized in detail 35 years ago by Lieber and DeCarli (1970). There was, however, some debate whether MEOS represents an ethanol oxidizing system distinct from alcohol dehydrogenase (ADH) and catalase. Subsequent studies with solubilized hepatic microsomal fractions using DEAE-cellulose column chromatography showed that microsomal fractions devoid of ADH and catalase activities but containing cytochrome P-450, NADPH-cytochrome c reductase, and phospholipids were able of actively oxidizing ethanol to acetaldehyde (Teschke et al., 1972, 1974). These studies led to the conclusion that ethanol oxidation via MEOS proceeds by a mechanism similar to the hepatic microsomal metabolism of many other exogenous compounds including drugs, hepatotoxins, and carcinogens. It was also shown that cytochrome P–450 2E1 is the most important component of MEOS (Lieber, 1997). This form of cytochrome P-450 is specifically induced after chronic ethanol consumption in association with a proliferation of the smooth endoplasmatic reticulum of the liver cell.

Additional experimental and clinical studies have shown that the increased metabolism of ethanol *in vivo* was due to the enhanced MEOS activity in the liver. Moreover, interactions of ethanol that with drugs/carcinogens have been explained by competition or induction at the site of cytochrome P-450. Im summary, hepatic ethanol metabolism proceeds by MEOS through a mechanism independent from ADH and catalase and involving cytochrome P-450 2E1 as the main component. These findings explain many clinical important observations including increased ethanol metabolism and interactions between ethanol, drugs, and carcinogens.

SURVIVAL MECHANISMS OF THE CHOLESTATIC HEPATOCYTE

H. Moshage

In all chronic liver diseases, including ALD, hepatocytes are exposed to a potentially toxic combination of compounds. These compounds include proapoptotic and proinflammatory cytokines such as tumor necrosis factor (TNF) and transforming growth factor-β (TGF-β), oxidative stress (the inappropriate exposure to reactive oxygen species such as superoxide anions, hydrogen peroxide and hydroxyl radicals), and, in cholestatic conditions, the exposure to bile acids, some of which are proapoptotic (Moshage, 2004) In addition, in immune-mediated liver diseases, hepatocytes are exposed to proapoptotic FasLigand, expressed on various lymphoid cells (Gujral et al., 2004). All in all, one would expect in chronic (cholestatic) liver diseases extensive hepatocyte apoptosis. Indeed, many studies have reported apoptosis of hepatocytes in chronic liver diseases. However, these studies were often based on the use of rather aspecific methods to determine apoptosis, e.g., the TUNEL assay.

More specific methods are now available, e.g., the detection of activated forms of various effector caspases or the use of antibodies specific for caspase-cleaved protein fragments.

Studies performed by using these methods have now challenged the view that apoptosis is the predominant mode of cell death in chronic liver diseases. Indeed, in some of these studies a striking lack of apoptosis was observed, in particular in cholestatic liver diseases, suggesting that necrosis rather than apoptosis is the predominant mode of cell death in these disorders.

Since in chronic liver diseases the hepatocytes are exposed to a potentially proapoptotic environment, the question arises why apoptosis is not the predominant mode of cell death.

Recent studies have led to the emerging concept that in chronic liver diseases, the hepatocytes adapt to chronic exposure to toxic compounds by adopting a “survival” phe-

notype (Schoemaker and Moshage, 2004) The hepatocyte is able to activate a number of defense mechanisms that protect the hepatocyte against apoptosis (Moshage, 2004).

Activation of Transcription Factor NF-kB

Chronic liver diseases are invariably accompanied by some extent of inflammation and increased TNF levels. TNF induces activation of the transcription factor NF-kB and hence the transcription of NF-kB–regulated genes, including many antiapoptotic genes such as c-FLIP, A1, cIAP2, and so on.

Indeed, we and others have shown that activation of NF-kB protects hepatocytes against bile acid–induced apoptosis and that inhibition of NF-kB in an *in vivo* model of cholestasis leads to increased liver damage and apoptosis (Schoemaker et al., 2003). The NF-kB pathway is a transcriptionally regulated protective pathway and therefore takes some hours to be fully operational.

Activation of Antiapoptotic MAP-Kinases

These are more immediate survival pathways. Protective members of this family include the p38 MAP-kinase and ERK1/2 MAP-kinase. Activation of these pathways is essential in the protection against bile acid and oxidative stress–induced apoptosis.

Phosphatidyl-Inositol-3-Kinase/Akt Pathway

Activation of this pathway leads to a number of downstream protective events, including the phosphorylation and subsequent inactivation of caspase-9 and the apoptotic bcl-2 family member Bad.

Furthermore, Akt has been shown to augment TNF-induced NF-kB activation.

Reactive oxygen species (R)OS) have been shown to activate this pathway, possibly explaining why moderately increased intracellular ROS production may be protective.

Activation of Receptor Tyrosine Kinases

Bile acids are able to activate the EGF and insulin receptors. The exact consequences of bile acid–induced growth factor activation remain to be clarified, since bile acid–induced EGFR activation appears to be proapoptotic rather than antiapoptotic.

Activation of the Transcription Factor AP-1

AP-1 (fos/jun heterodimer) is a transcription factor that is activated by many compounds, including oxidative stress.

One of the target genes of AP-1 is heme-oxygenase-1 (HO-1). HO-1 is the enzyme responsible for the conversion of heme into iron, carbon monoxide, and biliverdin. Recently, it has been reported that both carbon monoxide and biliverdin (or bilirubin) protect against oxidative stress induced cell death.

Exclusion of Toxic Compounds

In cholestatic conditions, uptake of toxic bile acids is prevented by downregulation of the hepatocyte-specific bile acid importer ntcp (Na+-dependent taurocholate cotransporting polypeptide), resulting in decreased intracellular concentrations of bile acids. We have previously shown that uptake of bile acids is necessary to induce apoptosis and therefore this mechanism is very important.

Reduction of Caspase Levels

Recently, Black et al. (2004) have shown that cholestatic hepatocytes are less susceptible to TGF-β–induced apoptosis due to reduction of the intracellular amount of caspase-8 protein, a crucial initiator of apoptosis.

In Summary

In the chronically injured liver, the hepatocyte is able to adopt an apoptosis-resistant phenotype. However, this does not imply that these hepatocytes are resistant to cell death: In chronic liver diseases, there is extensive necrosis. This suggests that in these conditions, the resistance to apoptosis shifts the mode of cell death toward necrosis. This is of relevance for any attempt to therapeutically intervene in chronic liver diseases (Schoemaker et al., 2004). Apoptosis is highly regulated and amenable to specific intervention, but preventing apoptosis in chronic liver diseases is probably futile because of the lack of apoptotic cell death. In chronic liver diseases, preventing necrotic cell death by removing the cause of necrosis, e.g., oxidative stress (alcohol abuse) or bile acids (cholestasis), is probably much more effective. In fact, it could be argued that shifting the balance from necrosis to apoptosis may be preferred to reduce the inflammatory response resulting from necrotic cell death. An added benefit of this strategy might be avoiding the presence of hepatocytes resistant to apoptosis that may increase the risk for development of malignant cells and liver cancer.

ALCOHOLIC LIVER DISEASE OVER THE LAST 50 YEARS: FROM MALNUTRITION TO LIVER TOXICITY AND NEW THERAPY

C. S. Lieber

Fifty years ago, the dogma prevailed that alcohol was not toxic to the liver and that ALD was exclusively a consequence of nutritional deficiencies. This view was based on experiments carried out in rats fed alcohol in drinking water, which resulted in only negligible blood alcohol levels. When we overcame the aversion of the rats for alcohol by incorporating it into liquid diets, liver pathology developed even in the absence of malnutrition. This was also demonstrated in nonhuman primates and in clinical trials. This toxicity of alcohol was linked to its metabolism through ADH, which converts NAD to NADH, thereby contributing to the hepatic steatosis by inhibiting lipid ox-

idation and promoting lipogenesis. The same mechanism also explains alcohol-induced hyperuricemia and hypoglycemia (Lieber et al., 1963; Lieber and Davidson, 1962).

In these investigations, morphologic studies also revealed that chronic alcohol consumption is associated with proliferation of the membranes of the smooth endoplasmic reticulum, also called microsomes. This was confirmed by an increase in the enzyme activities of the hepatic microsomal membranes (Ishii et al., 1973). This led to the discovery of a new pathway of ethanol metabolism, namely the microsomal ethanol oxidizing system (MEOS) (Lieber and DeCarli, 1968). The activity of the main enzyme of the MEOS, namely cytochrome P-4502E1 (CYP2E1) is induced by chronic ethanol consumption, resulting in the metabolic tolerance to ethanol. It is noteworthy that the CYP2E1 induction is associated with an increase in the corresponding gene (Seth et al., 2003). The induction also involves other chemicals, including many drugs that are metabolized in the endoplasmic reticulum. This process of detoxification occasionally backfires with the production of toxic and even carcinogenic metabolites.

Electron microscopic studies also revealed morphological alterations, including swelling and abnormal cristae in the liver mitochondria of animals and humans (Lane and Lieber, 1966). These structural abnormalities were associated with functional impairments, especially decreased oxygen utilization (Lieber et al., 1989) with reduced oxidation of fatty acids and of a variety of other substrates, including acetaldehyde (Hasumura et al., 1976), and leakage of the enzymes succinic dehydrogenase (SDH) and glutamic dehydrogenase (GDH), biochemical parameters of mitochondrial damage. Thus it was found that alcoholic fatty liver, a reversible condition considered by many as benign, is in fact associated with striking alternations of the mitochondria. This was accompanied by a significant decrease in their level of phospholipids with a reduction in hepatic mitochondrial cytochrome oxidase activity.

However, there was a significant reactivation in the enzyme activity on the addition of phospholipids, with phosphatidylcholine being the most efficient. Thus, it appears that alterations in the phospholipid composition of the mitochondrial membranes are responsible, at least in part, for the depression of cytochrome oxidase activity produced by chronic ethanol consumption.

The activity of CYP2E1 is also associated with the production of free radicals with resulting tissue damage due to lipid peroxidation, as reviewed elsewhere (Lieber, 1997).

Oxidative stress results in depletion of mitochondrial reduced glutathione (GSH). To restore GSH, it is not useful to simply give GSH because it does not penetrate into the cells. Its ultimate precursor is methionine. However, methionine must to be activated to s-adenosylmethionine (SAMe), the main methylating agent of the liver. The enzyme involved is strikingly depressed in alcoholic liver disease and therefore a depletion of SAMe develops.

This prompted us to administer some SAMe to alcohol-fed baboons. It restored their liver functions and structure (Lieber et al., 1990a), and SAMe was subsequently found to be beneficial in patients with alcoholic cirrhosis (Mato et al., 1999). The activity of phosphatidyl ethanolamine methyl transferase (PEMT) was also found to be decreased, resulting in reduction of the phosphatidyl cholines (PC) required for maintenance of normal structure and function of the membranes. Administration of polyenylphosphatidylcholine (PPC), a PC mixture extracted from soybeans, restored the structure of the membranes and the function of the corresponding enzymes, including MT activity, as shown in baboons (Lieber et al., 1994a). This is also one of the mechanisms by which PPC prevents the development of alcohol-induced cirrhosis (Lieber et al., 1994b).

Furthermore, PPC also corrects the oxidative stress in baboons (Lieber et al., 1997). In terms of fibrogenesis, stellate cells were found to be transformed or activated by ethanol to myofibroblast-like cells (Mak et al., 1984), associated with active fibrogenesis. Stellate cells and myofibroblasts were isolated from the liver and cultured; acetaldehyde, the metabolite of ethanol, was shown to stimulate collagen production *in vitro* (Savolainen et al., 1984), with an associated increase in mRNA for collagen (Casini et al., 1991). The acetaldehyde-induced increase in collagen accumulation was prevented with stimulation of collagenase activity by PPC (Li et al., 1992) and by dilinoleoylphosphatidylcholine (DLPC) (Lieber et al., 1994b), the main and active component of PPC. Other possible modes of action were also found to be involved, including a significant reduction in the number of myofibroblast-like stellate cells (Lieber et al., 1990b) and a decrease in their activation (Poniachik et al., 1999).

In addition to the study of the hepatotoxicity of ethanol, we investigated associated effect on nutrients with implications for the liver. This included the discovery that liver microsomes harbor previously unrecognized pathways for retinol metabolism (Leo and Lieber, 1985), which were shown to play a role in the homeostatic control of hepatic vitamin A levels (Leo et al., 1989a). Using purified cytochrome P-450 isozymes, including the human CYP2C8 (Leo et al., 1989b), retinol (Leo and Lieber, 1985; Leo et al., 1989b) and retinoic acid (Leo et al., 1984; 1989b) metabolizing systems were reconstituted; administration of ethanol or some drugs was shown to result in the induction of the activity of these systems (Leo et al., 1986; Sato and Lieber, 1982), a possible mechanism for the striking hepatic vitamin A depletion that was discovered to result from chronic ethanol consumption in rats and nonhuman primates (Sato and Lieber, 1981) as well as in human beings (Leo and Lieber 1982). This hepatic vitamin A depletion was found to be associated with significant lysosomal lesions (Leo et al., 1983).

Depletion of vitamin A called for its replenishment, but it was found that vitamin A supplementation in alcohol users is complicated because of the marked exacerbation of

vitamin A hepatotoxicity by chronic ethanol consumption. Indeed, amounts of ethanol and vitamin A which, by themselves do not produce fibrosis, when combined, resulted in necrosis and fibrosis in the liver (Leo and Lieber, 1983), with development of severe mitochondrial injury (Leo et al., 1982). Furthermore, it was discovered in nonhuman primates that alcohol interferes with the clearance of the retinol precursor β-carotene, possibly by impairing its conversion to vitamin A, resulting in enhanced hepatic and blood levels in baboons (Leo et al., 1992) and also in human beings (Ahmed et al., 1994), with associated potentiation of their hepatotoxicity (Leo et al., 1992). In the aggregate, the above-listed studies have led to the recognition of a narrowed therapeutic window for vitamin A and β-carotene in moderate and heavy drinkers; in turn, this has prompted a redefinition of the optimal conditions for their therapeutic use to avoid adverse interactions with ethanol in terms of hepatotoxicity and carcinogenicity, as reviewed in Leo and Lieber (1999).

In conclusion, the better understanding of the pathogenesis of alcoholic liver disease resulting from extensive research carried out over the last half century has raised prospects for better treatment as well. Our present therapeutic approach is to first reduce excess alcohol consumption by the Brief Intervention technique which, in a recent controlled study, resulted in a significant and striking decrease of alcohol consumption from an average 18 to only 21/2 drinks per day sustained for up to 5 years (Lieber et al., 2003a). In a minority of subjects who still continued to drink (up to 5 drinks per day), supplementation with PPC had some favorable effects on parameters of liver damage such as jaundice and ascites (Lieber et al., 2003b). PPC also inhibited CYP2E1 activity (Aleynik et al., 1999), the induction of which is a key mechanism of alcohol induced liver pathology (vide supra). Similarly DLPC, its main component, also partially opposed the increase of CYP2E1 activity by ethanol (Aleynik and Lieber, 2001); a search is currently ongoing for agents even more active yet innocuous enough for chronic administration in man. In view of the favorable and complementary effects of SAMe and DLPC, their combination is now being considered.

SUMMARY

This symposium documented the long but fruitful path from the beginnings of alcohol research to our understanding today of the pathophysiology of ALD. Although 50 years of alcohol research have passed, the contribution of the exact role of various pathogenetic factors in ALD is far from clear. It is therefore not surprising that our attempts to treat ALD are still preliminary, and, obviously, we need more time to understand molecular mechanisms by which chronic alcohol consumption initiates and/or leads to the progression of liver disease. On the basis of the results of extensive research carried out in the past, new approaches are now being taken by the use of modern technologies. One is the use of DNA-array technology, as pointed out by P. Haber, which may identify changes in the gene expression of certain genes after chronic alcohol intake. This may be of relevance to explain the progression of ALD. Similarly, it is important to understand mechanisms by which hepatocytes may survive.

Thus, hepatocytes of chronically injured liver adopt an apoptosis resistant phenotype. The understanding of these survival mechanisms may then lead to new therapeutic strategies either by preventing apoptosis or by preventing necrotic cell death by involving the cause of the process. Similarly, a breakthrough in the understanding of the pathogenesis of alcohol-associated carcinogenesis has been demonstrated in the last years, including the identification of high-risk groups due to genetic predisposition and by the identification of the role of microbially produced acetaldehyde as a local and topical carcinogen in various body fluids, including saliva. These new findings led to a recent National Institutes of Health conference (26 years after the first in 1978) on this topic.

In conclusion, it seems that considerable research in the understanding of the pathogenesis of alcohol-associated organ damage has shifted our attempts to treatment strategies. Indeed, prevention and treatment is our goal for the future to decrease the number of patients with alcohol-associated diseases.

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