Session II

PATHOGENIC MECHANISMS OF NASH AND ASH (PART I)

GENETIC BASIS FOR NASH AND ASH

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The striking histological similarity between ASH and NASH strongly suggests that they are due to similar pathogenic mechanisms. We have proposed the hypothesis that both are diseases of "two hits". In this model the first hit is the development of steatosis and the second a trigger of necroinflammation, with the role of steatosis being to increase the liver's sensitivity to the second hit. Candidates for the necroinflammatory trigger are: endotoxin-induced cytokine release, oxidative stress and an increased supply of potentially toxic free fatty acids. Candidate genes with a potential role in genetic susceptibility to ASH and NASH are therefore those whose products are involved in: (i) hepatic fat accumulation; (ii) oxidative stress; (iii) fatty acid metabolism as well as cytokine genes. Evidence supporting a role for immunological mechanisms in the pathogenesis of ASH implicates immunoregulatory genes as further potential candidates.

Association studies with polymorphisms of candidate genes have thus far been largely restricted to studies of ASH and alcohol-related fibrosis/cirrhosis (ALC). Regarding oxidative stress, positive associations have been reported for the c2 allele of the cytochrome P450, CYP2E1, which generates ROS upon ethanol and fatty acid metabolism, while NASH, although not ASH/ALC has been associated with heterozygosity for the genetic haemochromatosis-associated HFE mutation, C282Y, in some studies. More recently, associations between ASH/ALC and promoter polymorphisms of the IL-10 and TNFα genes and the IL-4 receptor have provided support for the immune hypothesis and studies with the T cell molecule, CTLA-4, gene polymorphism, have now offered further support. Most of these genes appear worthy of study in patients with NASH and preliminary date will be presented.

OXIDATIVE STRESS IN ALCOHOLIC AND NON-ALCOHOLIC LIVER DISEASE

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Introduction

The state of oxidative stress occurs when the balance of production of reactive oxygen species (ROS) and its metabolites, exceeds the capacity to defend against or detoxify them. The consequent exposure to hydrogen peroxide (H_2O_2) and hydroxyl radical (.OH) can damage cellular macromolecules, including DNA, protein and lipids. It is becoming increasingly recognized that different levels of oxidative stress can have entirely different effects on cells, ranging from no apparent effect, to the stimulation of cell proliferation, growth arrest, apoptosis, and necrosis. Mitochondria constitute the greatest source of these

ROS as the mitochondrial electron transport system consumes about 90% of the oxygen consumed by the cell.

Among the factors recognized to participate in the development and progression of alcoholic liver disease (ALD), and important role has been ascribed to the oxidative stress and peroxidation of membrane lipids, caused by the oxidative metabolism of alcohol. Di Luzio was the first to suggest that ethanol could affect the oxidative balance in liver, by finding that an enhanced lipid peroxidation occurred both in liver homogenates following the in vitro addition of ethanol and in liver homogenates obtained from rats after oral ethanol administration. Furthermore, this effect could be prevented by the simultaneous addition of antioxidants. Thus, ethanol or its metabolites could induce an oxidative stress, either acting as prooxidants or reducing the antioxidant level. Recently, the in vivo detection of carbon-centred free radicals by electron spin resonance spectroscopy has provided unequivocal evidence for the formation of free radicals after ethanol consumption.

Formation of reactive oxygen species (ROS)

The liver is the primary site of ethanol metabolism. There are two key enzyme pathways to oxidize the ethanol that reaches the liver: the alcohol dehydrogenases (ADH), and the microsomal ethanol oxidizing system (MEOS). When blood ethanol levels are low, ADH is the enzyme responsible for alcohol metabolism. ADH may contribute to radical formation, through the redox shife; the excess of reduced nicotinamide-adenine dinucleotide (NADH) promotes mobilization of iron from ferritin, which in the reduced state can interact with H_2O_2 to produce .OH. However when ethanol levels are higher, there is an important contribution of the MEOS system, through the induction of the cytocrome P450 (CYP2E1), yielding reactive oxygen intermediates as by-products. The hepatic CYP2E1 is also increased in patients with non-alcoholic steatohepatitis (NASH). However, when CYP2E1 (-/-) mice were administered a methionine-choline deficient diet, neither lipid peroxidation, nor the development of NASH were prevented. Nonetheless CYP4E10 and CYP4E14 were upregulated, suggesting that CYP2E1 is not unique among P450 proteins in catalyzing peroxidation of endogenous lipids.

Another subcellular site of oxidative stress in alcoholism is the mitochondrion. In ALD it is believed that partial reduction of ubiquinone to produce ubisemiquinone free radical at complex III underlies 0_2 generation. In fact, an increased oxygen radical formation by mitochondria from ethanol-fed animals has been demonstrated. Besides, several factors in ALD may favour accentuation of oxidative stress in mitochondria. Chronic alcohol abuse and other conditions that promote hepatic steatosis increase the supply of fatty acids, induce an increase in substrate presented to the mitochondria, leading to an enhanced 0_2 generation. In addition, induction and release of tumour necrosis factor a (TNF a) by nonparenchymal cells such as Kupffer cells are likely to occur in ALD. This cytokine can promote generation of superoxide anion from mitochondrial complex III. Lastly, the mitochondrial pool of glutathione is depleted in alcoholism, because of an ethanol-induced defect in translocation of reduced glutathione (GSH) from cytosol to mitochondria. Besides, as mitochondria lack catalase they depend solely on GSH to cope with the toxic effects of hydrogen peroxide. On the other hand, mitochondria are not only the source of oxidative stress but are also a target for ROS, contributing to the ethanol-mitochondrial dysfunction.

EFFECTS OF ROS

Lipid Peroxidation

Free radical attack on unsaturated lipids initiates a chain reaction of lipid peroxidation. Ethanol feeding induces lipid peroxidation in experimental animals, an event that is associated both with acute liver injury and fibrosis. Lipid peroxidation is enhanced by the addition of polyunsaturated fat to the diet. Both ethanol-induced lipid peroxidation and liver injury can be abrogated in rats by administering ethanol along with an inhibitor of CYP2E1. During the last two decades, evidence has been produced in humans of an increased lipid peroxidation, associated with alcohol abuse. Suematsu et al., demonstrated increased lipid

peroxides in liver and serum of chronic alcoholics, by using the thiobarbituric acid method. Hepatic levels of conjugated dienes (an early product of lipoperoxidation) were found to be high in 16 patients with alcoholic liver disease than in 8 patients with non-alcoholic liver disease. Vendemiale et al., have shown an increase in plasma malondialdeyde (MDA) concentrations after an acute dose of ethanol. Ethane exhalation, an in vivo index of lipid peroxidation, was also increased in alcoholics, comparing with normal controls or patients with non-alcoholic liver disease. More recently it was shown that the hepatocytes in alcoholic liver disease contained high amounts of 4-hydroxy-2-nonenal (HNE)-protein adducts (a marker of lipid peroxidation-associated cellular damage). Lipid peroxidation causes cell demise and releases MDA and HNE. MDA and HNE may cause direct toxicity. MDA and HNE also increase the synthesis of collagen by stellate cells, while HNE is chemotactic for neutrophils. ROS may also induce Fas ligand expression and paracrine killing of Fas receptor positive hepatocytes, causing fratricidal killing.

Steatosis, whether acute or chronic, leads to lipid peroxidation in mice. Chronic lipid peroxidation might represent the missing link between chronic steatosis and the development of steatohepatitis lesions in human subjects. Our group demonstrated increased hepatic MDA (using the thiobarbituric acid method) in alcoholic steatohepatitis and NASH patients, comparing to controls. We did find that MDA correlated with the amount of fat in the liver. However, the more severe accumulation of fat in NASH was not associated with higher levels of MDA, when compared with alcoholic steatohepatitis, where steatosis was less marked, probably due to the above mentioned effects of chronic ethanol consumption in ROS production.

DNA lesions

Likewise, DNA is sensitive to oxidant stress. Mitochondrial DNA (mtDNA) is 10 to 16 times more prone to oxidative damage than nuclear DNA, due to the lack of protective histones in mtDNA, to incomplete repair processes in mitochondria, and to the proximity of mtDNA to the respiratory chain, which is the main source of ROS in the cell. In fact, multiple hepatic mtDNA deletions have been found in alcoholic patients, suggesting premature oxidative aging of mtDNA.

Antioxidants

The effects of ROS may be amplified if ethanol also reduces the hepatic anti-oxidant defences. Indeed, chronic alcohol consumption leads to depletion of several antioxidants in the liver including zinc, vitamin E, vitamin A and glutathione. Ethanol-induced glutathione depletion occurs selectively in hepatic mitochondria, preferentially by inhibiting the transfer of GSH from the cytosol to the mitochondria, and causing impairment of mitochondrial function. S-adenosyl-methionine (SAM), a glutathione precursor, can replenish mitochondrial glutathione stores, apparently by reverting the gluthatione transport defect. However, thus far, disappointing results have been obtained with the anti-oxidants, vitamin A and vitamin E. More encouraging results in human ALD have been obtained with SAM. The soybean lecithin extract, polyenylphosphatidylcoline (PPC), significantly attenuates ethanol induced oxidative stress, giving protection agains fibrosis and cirrhosis in baboons.

In conclusion, although there is evidence that oxidative stress is strongly implicated in the pathogenesis of ALD, the provision of anti-oxidants has not been a success story, although SAM looks promising. The relevance of ROS in the overall emerging liver disease is a matter of debate.