Current concepts in the pathogenesis of alcoholic liver injury

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ABSTRACT **Alcoholic liver disease (ALD) develops as a consequence of priming and sensitizing mechanisms rendered by cross-interactions of primary mechanistic factors and secondary risk factors. This concept, albeit not novel, is becoming widely accepted by the field, and more research is directed toward identifying and characterizing the interfaces of the cross-interactions to help understand individual predisposition to the disease. Another pivotal development is the beginning of cell type-specific research to elucidate specific contributions not only of hepatocytes, but also of hepatic macrophages, liver-associated lymphocytes, sinusoidal endothelial cells, and hepatic stellate cells to sensitizing and priming mechanisms. In particular, the critical role of hepatic macrophages has been highlighted and the priming mechanisms concerning this paracrine effect have been proposed. Glutathione depletion in hepatocyte mitochondria is considered the most important sensitizing mechanism. One of the contributing factors is decreased methionine metabolism. Remaining key questions include how altered methionine metabolism contribute to the pathogenesis of ALD; how cross-talk among nonparenchymal liver cells or between nonparenchymal cells and hepatocytes leads to ALD; how dysfunctional mitochondria determine the type of cell death in ALD; and what secondary factors are critical for the development of advanced ALD such as alcoholic hepatitis and cirrhosis.—Tsukamoto, H., Lu, S. C. Current concepts in the pathogenesis of alcoholic liver injury. *FASEB J.* 15, 1335–1349 (2001)**

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CONCEPTUAL APPROACH TO THE PATHOGENESIS OF ALCOHOLIC LIVER DISEASE (ALD)

Is ALD a disease of malnutrition or a disease caused by direct hepatotoxicity of ethanol? This question has fueled a debate over the latter half of the 20th century. Compelling evidence supports the role of nutritional deficiency in ALD (1–8). At the same time, Lieber and colleagues show that progressive ALD proceeds despite adequate nutrition (9, 10). The latter hypothesis was based primarily on the observation that baboons fed a nutritionally adequate liquid diet containing ethanol at 50% calories developed nearly the whole spectrum of ALD including cirrhosis, a finding that remains to be reproduced by other laboratories.

The intragastric ethanol infusion technique, which allows maximal ethanol consumption and absolute control over nutrient intake by rodents, has depicted the critical role of nutrition in determining sensitization and priming of the liver to ethanol-induced liver injury. Studies demonstrated profound effects on ethanol-induced liver injury by intake of nutrients such as polyunsaturated fat and iron in quantities that were never thought to be important. The concept of 'sensitization' and 'priming' is currently considered fundamental to our pursuit for elucidation of pathogenetic mechanisms of ALD. We define sensitization as a conditioning that makes the target cells, hepatocytes, more vulnerable to harmful effects triggered by ethanol and priming as the effect that promotes specific injurious mechanisms. The sensitizing and priming are rendered by the complex interactions of primary mechanistic factors and secondary risk factors (**Fig. 1**). Thus, in addition to independent in-depth studies focused on one mechanistic aspect, a more comprehensive and interactive approach is required to understand how sensitization and priming work together to determine individual predisposition to ALD and to precipitate the disease process. For example, intake of polyunsaturated fat in ethanol-fed rats, but not in pair-fed controls, results in a synergistic priming effect on induction of cytochrome P4502E1 (CYP2E1) with consequent oxidative injury to the liver (11). Conversely, saturated fat prevents this priming effect and abrogates depletion of a mitochondrial pool of glutathione (GSH) (12), one of the most crucial sensitization effects of ethanol on hepatocytes (13). Iron is another example. Whereas a slight increase in hepatic iron content by dietary iron supplementation is harmless in control rats, it exacerbates alcoholic liver injury via accentuation of oxidative stress (14). Further, increased iron storage in hepatic macrophages is a potential priming mechanism for

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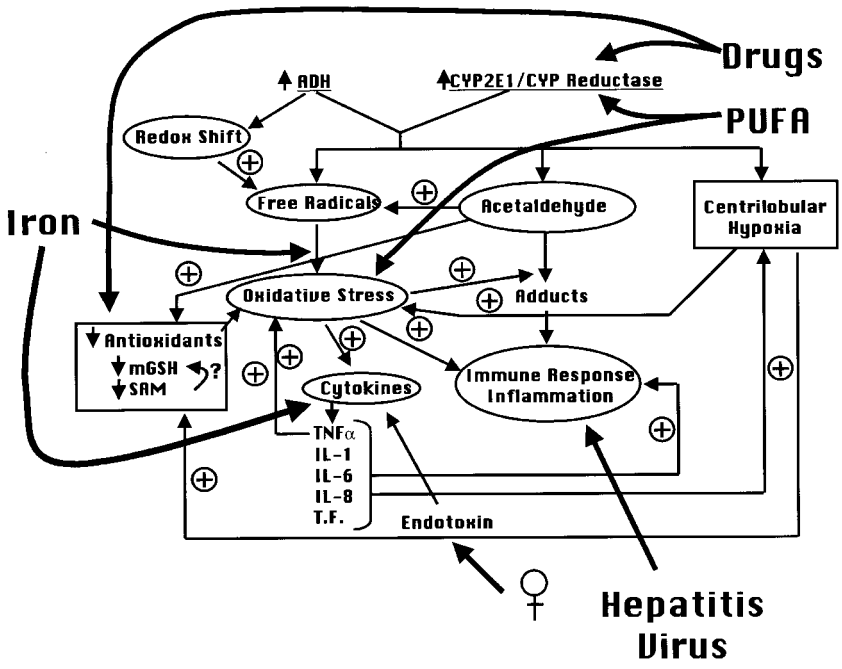

Figure 1. A schematic diagram depicting cross-interactions between primary mechanistic factors and secondary risk factors in the pathogenesis of ALD. Arrows with a plus sign represent the interfaces of interactions among the primary factors that result in either sensitization or priming. Boldface arrows indicate priming or sensitizing effects of secondary risk factors on the primary factors. For instance, induction of CYP2E1/CYP reductase by ethanol consumption leads to increased generation of reactive oxygen species (free radicals) and oxidative stress, which can also be promoted by redox shift associated with ethanol oxidation by ADH (alcohol dehydrogenase). Acetaldehyde, a metabolic product of ethanol from either ADH or CYP2E1-catalyzed oxidation, promotes oxidative stress via consumption of antioxidants or generation of free radicals via acetaldehyde oxidation. Acetaldehyde can incite immune responses via protein adduct formation; this adduct formation is accentuated by oxidative stress and the presence of lipid peroxidation aldehydic products. Induced activities of ADH and CYP2E1 cause enhanced oxygen consumption by the liver and consequently centrilobular hypoxia, which can be aggravated by endotoxin-mediated sinusoidal injury by TNF-α and TNF-α-inducible proinflammatory mediators. Centrilobular hypoxia in turn promotes oxidative stress in part via the release of iron and depletes S-adenosylmethionine (↓SAM). SAM depletion causes reduced synthesis of glutathione (GSH) and may impair transport of GSH to mitochondria, causing a selective decrease in the mitochondrial pool of GSH (↓mGSH). SAM depletion may also promote expression of proinflammatory and cytotoxic cytokines in hepatic macrophages via a NF-κB-dependent mechanism that is also responsive to oxidative stress. Drugs and polyunsaturated fat (PUFA) primes oxidative stress mediated by CYP2E1. Alcohol intake increases the iron concentration in both hepatocytes and macrophages, which promotes oxidative injury and cytokine expression, respectively, and additional iron loading via a dietary source accentuates these effects. The female gender appears to promote ethanol-induced entrance of endotoxin to the portal circulation and expression of proinflammatory cytokines. Concomitant infection with hepatitis viruses may prime immune and inflammatory responses induced by ethanol-associated liver injury.

enhanced expression of tumor necrosis factor α (TNF-α) in experimental ALD (15)

Besides nutritional factors, female gender, age, concomitant intake of other drugs that can induce CYP2E1, hepatitis virus infection, and genetic predisposition are all considered risk factors. Even among the primary mechanistic factors that include acetaldehyde, oxidative stress, immune response, hypoxia, and membrane alterations, there are cross-interactive relationships to render sensitization or priming effects. For instance, acetaldehyde, a potent toxic metabolite of ethanol, induces liver injury via its covalent binding to structural or functional proteins of the cells (16) while promoting oxidative stress via consumption of GSH. In turn, deleterious effects of acetaldehyde-protein adduct formation may be accentuated by oxidative stress since malondialdehyde, a lipid peroxidation end product, can increase the binding affinity of acetaldehyde by 13-fold (17). The resulting novel hybrid adducts are highly immunogenic and may incite immune response-mediated liver injury (18, 19). Although cellular immune response and inflammation are regarded as independent mechanisms of ALD, they can also lead to oxidative stress via the release of reactive oxygen species (ROS) by NADPH oxidase or action of TNF-α at the electron transport chain in target cells.

The multifactorial nature and complex interactions among primary mechanistic factors and between primary and secondary factors appear to be the basis for the heterogeneous response that alcoholics exhibit for ALD. Elucidation of the sensitization and priming mechanisms involving cross-interactions of these factors should allow us to gain insight into the most fundamental question, which is why only a small fraction of alcoholics develop advanced ALD. How, then, do we effectively approach the complicated and interactive mechanisms? First, investigators need experimental models or systems by which they can perform controlled deletion and addition analyses in order to identify what primary and secondary factors are required for the expression of a particular aspect or whole spectrum of experimental ALD. Second, investigation itself has to become interactive and multidisciplinary; experts in various disciplines need to work together to provide cutting-edge science for elucidating the precise nature and mechanisms that underlie interactions.

CELL TYPE-SPECIFIC MECHANISMS OF ALD PATHOGENESIS

One of the important recent developments in the field of ALD is cell type-specific research. The majority of

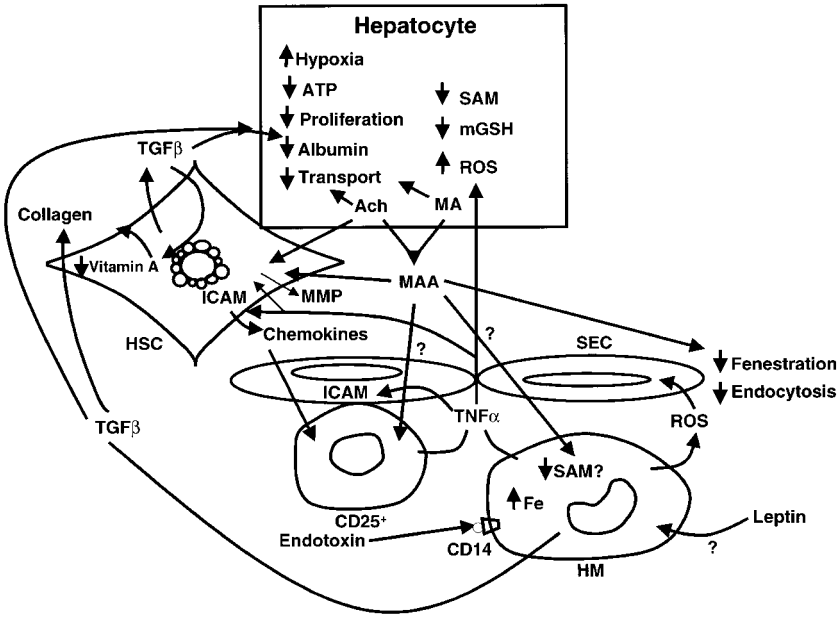

Figure 2. Cell type-specific mechanisms of ALD. Hepatocyte is the primary site of sensitization rendered by hypoxia, mitochondrial GSH depletion (↓ mGSH), and S-adenosylmethionine depletion (↓ SAM). It is also the site of ethanol-primed oxidant stress via increased generation of reactive oxygen species (↑ ROS) by CYP2E1 or mitochondria. Acetaldehyde (Ach) and lipid peroxidation aldehydes such as malondialdehyde (MA) may impair transport via protein adduct formation while giving rise to a new hybrid adduct, MAA. This adduct is highly immunogenic and may incite both humoral and cellular immune responses. Endotoxin-mediated activation of hepatic macrophages (HM) appears to be central to the ALD pathogenesis. Critical paracrine effects of activated HM include ROS or TNF-α-mediated damage to the sinusoidal endothelial cells (SEC), TNF-α-mediated mitochondrial oxidant stress in hepatocytes and cell death, and matrix remodeling and fibrogenesis initiated by TNF-α-mediated induction of matrix metalloproteinase (MMP) and proliferation by hepatic stellate cells (HSC). TNF-α, which is released by HM and CD25+ T lymphocytes (CD25+), can also incite inflammation via induction of intercellular adhesion molecule (ICAM) and chemokines by SEC and HSC. Primed TNF-α induction by HM in ALD may be attributable to induction of CD14, increased iron storage (↑ Fe), decreased SAM (↓ SAM), or regulation by leptin. TGFβ released by HM and HSC promotes fibrogenesis and suppresses proliferation and albumin synthesis by hepatocytes.

earlier studies analyzed the whole liver tissue, assuming that results obtained should reflect changes in hepatocytes. This assumption, in fact, may be mostly correct since hepatocytes account for 65% of total cell population and 85% of total volume of the liver. However, it unfortunately precludes cell type-specific research, cross-talk between parenchymal and nonparenchymal liver cell types, or even interactions among nonparenchymal liver cell types in the pathogenesis of ALD. The liver is composed of hepatocytes and five nonparenchymal liver cell types (endothelial cells, Kupffer cells/hepatic macrophages, hepatic stellate cells, bile duct epithelial cells, and pit cells/liver NK cells). Even though the nonparenchymal liver cells represent only one-third of total liver cells, they possess distinct and important cellular functions in support of liver homeostasis and actively participate in pathological processes. Thus, alcohol could potentially affect each of these cell types differently and the effects on each cell type may be significant in the evolution of ALD (**Fig. 2**).

Hepatocytes

How ethanol primes and sensitizes the hepatocytes is a topic of primary importance. After all, hepatocytes are the site of ethanol oxidation and ethanol-induced injury. For the past 20 years, much interest has been generated for the pathogenetic role of CYP2E1 in ALD. Alterations in methionine metabolism have also been well described in ALD, and several key changes in this metabolic pathway can sensitize and prime hepatocytes to injury. Changes in mitochondrial function have also received increasing attention as potential explanations for sensitization and priming mechanisms for hepatocellular injury.

Role of CYP2E1

CYP2E1 catalyzes the production of ROS (20) and lipid peroxidation of microsomal membranes in vitro (21, 22). It is well known that CYP2E1's expression and activity are induced after ethanol consumption (23, 24). Studies using cell lines stably transfected with a CYP2E1 expression vector have made a major contribution to research on this topic, confirming the role of CYP2E1 in ethanol-induced ROS production and cell death (25) and GSH depletion in both cytosol and mitochondria (26). However, the in vivo role of CYP2E1 in experimental ALD is controversial. Although there is a close correlation between induction of CYP2E1 and experimental ALD (11) and inhibitors for CYP2E1 ameliorates alcoholic liver injury (27), CYP2E1 knockout mice are just as susceptible to ethanol-induced liver injury and exhibit similar induction of other CYP families (CYP1A, CYP2A, CYP2B, and CYP3A) in response to ethanol feeding as wild-type mice (28). Furthermore, gadolinium chloride blocks experimental alcoholic liver injury despite induction of CYP2E1 (29), demonstrating dissociation of CYP2E1 induction from alcoholic liver injury. However, these latter two studies only examined the early stage of experimental ALD; whether CYP2E1 plays a role in progression of experimental ALD remains to be tested. It also has to be pointed out that CYP2E1 activity is

inherently much lower in mice than in rats or humans. Thus, the findings in mice may need to be interpreted with caution with respect to the role of CYP2E1 in ALD (M. Ingleman-Sundberg, personal communication). To this end, the effects of CYP2E1 overexpression on alcoholic liver injury in mice need to be evaluated.

Role of abnormal methionine metabolism

The liver plays a central role in methionine metabolism, as half of the daily methionine intake is catabolized here (**Fig. 3**). The first step in methionine metabolism is the formation of S-adenosylmethionine (SAM) catalyzed by methionine adenosyltransferase (MAT) (30, 31). In this reaction, the adenosyl moiety of ATP is transferred to methionine, forming a sulfonium ion that is a high-energy reagent and can easily transfer its methyl group to a large variety of acceptor substrates including nucleic acids, proteins, phospholipids, biological amines, and a long list of small molecules (31). SAM is the principal biological methyl donor, the precursor of aminopropyl groups used in polyamine biosynthesis and the liver, the precursor of glutathione (GSH) through its conversion to cysteine via the transsulfuration pathway (30, 31). Under normal conditions, most of the 6–8 gm of SAM generated per day is used in transmethylation reactions in which methyl groups are added to compounds and SAM is converted to S-adenosylhomocysteine (SAH) (31, 32). SAH is a potent competitive inhibitor of transmethylation reactions; both an increase in SAH level as well as a decrease in the SAM to SAH ratio are known to inhibit transmethylation reactions (31, 33). For this reason, the removal of SAH is essential. The reaction that converts SAH to homocysteine and adenosine is reversible and catalyzed by SAH hydrolase (30, 31). In vivo, the reaction proceeds in the direction of hydrolysis only if the products, adenosine and homocysteine, are rapidly removed (30, 31, 33).

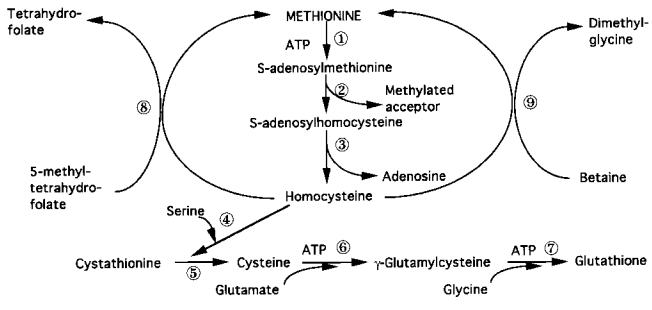

Figure 3. Hepatic methionine metabolism. Liver plays a central role in methionine metabolism, as half of the daily methionine intake is catabolized here. In the hepatocyte, methionine is sequentially converted to cysteine, the rate-limiting sulfur amino acid precursor for GSH synthesis. Methionine can also be resynthesized from homocysteine by methionine synthase and betaine homocysteine methyltransferase. 1) methionine adenosyltransferase (MAT); 2) transmethylation reactions; 3) S-adenosylhomocysteine hydrolase; 4) cystathionine β-synthase (CBS); 5) γ-cystathionase, 6) γ-glutamyl cysteine synthetase; 7) GSH synthetase; 8) methionine synthase (MS); and 9) betaine-homocysteine methyltransferase (BHMT).

There are three pathways that metabolize homocysteine. One is the trans-sulfuration pathway, which converts homocysteine to cysteine. This is a unique pathway present only in the liver and lens (34) that condenses homocysteine with serine to form cystathionine in a reaction catalyzed by cystathionine β-synthase (CBS) with vitamin B_6 as a cofactor (32, 34). Cleavage of cystathionine, catalyzed by another vitamin B_6-dependent enzyme, γ-cystathionase, then releases free cysteine, the rate-limiting precursor for GSH synthesis (34). The other two pathways that metabolize homocysteine resynthesize methionine from homocysteine. One is catalyzed by methionine synthase (MS), which requires normal levels of folate and vitamin B_{12}; the other is catalyzed by betaine-homocysteine methyltransferase (BHMT), which requires betaine, a metabolite of choline (30, 32). If homocysteine metabolism is impaired in these pathways, homocysteine may be released into sinusoidal blood (resulting in hyperhomocystinemia) and the ratio of hepatic SAM to SAH may decrease (leading to inhibition of transmethylation reactions). Given the critical role of methylation in determining various cellular processes, these changes have far-reaching effects. Hyperhomocystinemia is associated with increased risk for arteriosclerosis (35) and was recently shown to induce tissue inhibitor of metalloproteinases-1 (TIMP-1) and collagen gene expression in a hepatic stellate cell (HSC) line (36). As discussed below, ALD affects many of these enzymatic steps in methionine metabolism. It is therefore vital to have a better understanding of the abnormalities in the hepatic methionine metabolism for both the pathogenesis of the disease and the design of therapy against alcoholic liver injury.

Methionine adenosyltransferase MAT is a critical cellular enzyme because it catalyzes the only reaction that generates SAM. The MAT gene is one of 482 genes required for survival of an organism (32). In mammals, the genes MAT1A and MAT2A encode for two homologous MAT catalytic subunits, α 1 and α 2 (37, 38). MAT1A is expressed only in the liver and encodes the α 1 subunit found in two native MAT isozymes, which are either a dimer (MAT III) or tetramer (MAT I) of this single subunit (38). MAT2A encodes for a catalytic subunit (α 2) found in a native MAT isozyme (MAT II), which is widely distributed (38). MAT2A and its gene product also predominate in the fetal liver and are progressively replaced by MAT1A during development (39). In adult liver (40), MAT1A is primarily expressed. However, an intriguing switch in the gene expression from MAT1A to MAT2A is demonstrated in liver cancer (41, 42), liver regeneration after partial hepatectomy (42), and hepatotoxicity induced by thioacetamide (43). Using a cell line model that differs only in the type of MAT expressed, the type of MAT expressed by the cell significantly influences the rate of cell growth (44). The mechanism is likely via a change in the steady-state SAM level and methylation status. This is

because MAT isozymes differ in kinetic parameters and regulatory properties, so a switch in MAT expression can affect the steady-state SAM level and methylation (45).

Non-liver-specific MAT (MAT II) has the lowest K_m for methionine but is tightly regulated by SAM with an IC_{50} of 60 μM, which is close to the normal intracellular SAM concentration (30, 47, 48). In contrast, SAM has a minimal inhibitory effect on MAT I (IC_{50}=400 μM) and stimulates MAT III (up to eightfold at 500 μM concentration of SAM) (47). Thus, the SAM level in cells that express only the non-liver-specific MAT isoform is relatively unaffected by fluctuations in methionine availability because of the negative feedback inhibition. In contrast, the rate of SAM synthesis and the SAM level increase with increasing methionine availability in cells that express mostly liver-specific MAT (30). Consistent with this, we found that cells that express MAT1A have much higher levels of SAM and DNA methylation but lower rates of cell growth than cells that express MAT2A (44). A caveat to this is the recently described regulatory subunit (β) associated with the MAT enzyme in lymphocytes (48, 49). The β subunit was shown to lower the K_m of MAT II for methionine and render the enzyme more susceptible to feedback inhibition by SAM (49). Whether it exists in other cells is unknown. In liver, the current data suggest the relative expression of MAT isozymes is likely to influence the rate of liver growth and possibly hepatocarcinogenesis.

It has long been realized that patients with alcoholic cirrhosis often have hypermethioninemia and delayed plasma clearance of methionine after intravenous injection (50, 51). Subsequent studies showed that hypermethioninemia in cirrhotic patients is attributed to a 50–60% decrease in the activity of the liver-specific MAT (52, 53). This likely contributes to a decreased hepatic GSH level in patients with ALD, as SAM administration prevented the fall in GSH (54). The fall in hepatic GSH sensitizes the liver to oxidative injury and sets up a vicious cycle since it can further contribute to inactivation of liver-specific MAT. A 30% reduction in hepatic GSH levels results in a 60% reduction in hepatic MAT activity and a 40% reduction in SAM level (55). The decrease in MAT activity is thought to occur primarily by post-translational mechanisms (37). A fall in MAT activity can be a result of a change in the oligomeric equilibrium of the liver-specific MAT (decrease in the ratio of tetramer to dimer) or covalent modification of the enzyme. The liver-specific MAT contains several critical cysteine residues, modifications of which can inactivate the enzyme by direct interference with the substrate binding site(s) or by causing dissociation of the oligomers (56–59)

In end-stage liver disease, the decrease in MAT activity affects the tetramer selectively (52). Since the intracellular GSH/GSSG ratio is known to be an important modulator of the oligomeric equilibrium of the hepatic MAT isozymes (56), part of the selective loss of the tetramer is due to a reduction in the GSH/GSSG ratio (31). The cysteine at position 121, conserved in rat and human liver-specific MAT and absent in non-liver-specific MAT, is a target of covalent modification (58–60). Although this cysteine is not essential for activity, as substitution of serine for this residue has no effect on MAT activity, when cysteine is modified either by oxidation or by the formation of a nitrosothiol, the enzyme is inactivated (57–60). The inactivation could be reversed by GSH and other thiol-reducing agents. The amount of GSH required to reverse the inactivation is 3 mM for the dimer and 25 mM for the tetramer (59). Since normal hepatic GSH concentration is 5–10 mM, the difference in reversibility of inactivation by GSH may also contribute to the selective loss of the tetramer in liver disease. Nitrosylation of Cys 121 of liver-specific MAT and its inactivation have been demonstrated both in vitro and in vivo in animals treated with lipopolysaccharide (LPS) (58, 60). Both oxidative stress and endotoxemia occur in ALD and contribute to the liver injury. One of the mechanisms may well be inactivation of the liver-specific MAT.

Although liver-specific MAT is inactivated in end-stage ALD in humans, changes in MAT expression have been largely unknown. Finkelstein et al. showed induction of hepatic MAT activity in rats given 50% ethanol by gavage and a low protein diet for up to 10 days (61). Thus, changes in MAT may depend on the stage of ethanol-induced liver disease. Since a change in hepatic MAT expression can affect the steady-state SAM level, methylation status, and cell growth, we examined MAT expression and SAM homeostasis using the Tsukamoto-French intragastric ethanol feeding model (62). In this model, fatty liver develops by the 4th week, centrilobular steatonecrosis, mononuclear cell infiltration, and increased TNF-α gene expression occur by the 9th week, and early fibrosis by the 16th week (62). We found a twofold increase in the mRNA level of both MAT1A and MAT2A 9 wk after ethanol feeding (63). However, the protein level of MAT1A was essentially unchanged whereas that of the MAT2A was greatly induced. Hepatic levels of methionine, SAM, and DNA methylation fell by ~40%. The question is whether these changes are of pathogenetic importance.

Reduced SAM levels and methylation can affect gene expression, membrane fluidity, and GSH levels in liver (32, 64). Effects on membrane fluidity (32, 65, 66) and GSH levels (32, 64) have been well studied. How changes in DNA methylation affect ethanol-induced liver injury is unclear. ALD is associated with increased risk of liver cancer, but the molecular mechanism is unclear. Global DNA hypomethylation is observed in the development of many types of cancer, although there is often regional hypermethylation (67). A common hypothesis is that hypomethylation of growth-promoting proto-oncogenes and/or hypermethylation of tumor suppressor genes will alter transcription factor binding and expression of these genes to promote a selective growth advantage for the initiated cell (68). Alternatively, DNA hypomethylation may promote malignant transformation by inducing regional alterations

in DNA conformation and chromatin structure, rendering affected regions more accessible to DNA-damaging agents (68). We found hypomethylation and increased expression of c-*myc* in the ethanol-fed livers (63). We also detected increased genome-wide DNA strand break accumulation (63). Thus, even at the prefibrotic stage of ethanol-induced liver injury, there are already changes that may predispose the liver to malignant degeneration.

In summary, available data show in ALD a relative switch in MAT expression due primarily to inactivation of liver-specific MAT and increased expression of non-liver-specific MAT. As a result, there is depletion of hepatic SAM and GSH levels and decreased transmethylation. Important consequences include impairment in antioxidant defense, altered phospholipid composition and membrane fluidity, gene expression, and DNA stability. Further studies should define when these changes occur in the course of ALD and whether they can be corrected or prevented by SAM administration (see Treatment section for a discussion). Genes whose expression are affected by a change in methylation status should be further defined to improve our understanding of the pathological consequence of altered DNA methylation in this chronic liver disease.

Resynthesis of methionine In addition to changes in MAT, decreased MS activity and increased BHMT activity occur in animals fed ethanol (61, 69–71). These changes can also affect the availability of SAM and homocysteine metabolism. In rats given 50% ethanol by gavage and a low protein diet for up to 10 days, Finkelstein et al. showed decreased MS activity and increased hepatic BHMT activity (61). Other investigators also showed the same changes in MS and BHMT activities in rats fed a Lieber-DeCarli ethanol liquid diet for 2–4 wk (69, 70). In these models, the methionine pool is conserved at the expense of betaine. Betaine supplementation greatly increased the hepatic SAM level and protected against fatty infiltration of the liver (69). However, whether this is true for other animal models or in rats with more advanced ethanol-induced liver disease is unclear. In fact, hepatic methionine level depended on the stage of liver injury in rats fed ethanol intragastrically. Our previous study using a lower amount of fat (25%) combined with intragastric ethanol feeding found increased hepatic methionine level after 5 wk but a 50% reduction in methionine level after 16 wk (72). Our recent study used a diet regimen that contains additional fat, which is known to result in accentuated ethanol-induced oxidative liver injury (73). The most likely explanation for the fall in hepatic methionine level is decreased resynthesis of methionine from homocysteine. MS has already been shown to be inactivated (61, 69–71). It is likely that the induction in BHMT was not sufficient to maintain the methionine pool. Another possibility to be examined is decreased methionine uptake as ethanol-induced liver injury progresses. Although the changes in MS and BHMT activity have been described, the mechanisms are not clear. Thus, although folate and B_{12} deficiency contribute to the fall in MS activity, the enzyme is also postulated to be inactivated by possibly forming adducts with acetaldehyde or nitrosylated (69, 71, 74). Recently we found the mRNA levels of three key enzymes for homocysteine metabolism—MS, BHMT, and CBS—to be markedly lower in 10 unselected patients with alcoholic cirrhosis (75). Thus, multiple mechanisms likely contribute to decreased resynthesis of methionine and homocysteine catabolism.

How would impaired methionine resynthesis contribute to the live injury in ALD? One mechanism is decreased SAM availability with the possible consequences discussed in the preceding section. Another mechanism is impaired homocysteine metabolism, resulting in increased homocysteine release by hepatocytes. This may contribute to alcoholic liver fibrogenesis (see the Hepatic Stellate Cells section).

Role of the mitochondria

An important cellular organelle that contributes to both priming and sensitizing effects of ethanol is the mitochondria. Mitochondria isolated from ethanol-fed rats produce more ROS (76). Ceramide, an intracellular signaling molecule for TNF-α, acts on mitochondrial electron transport chain to generate ROS (77) and represents a key mechanism of TNF-α-induced oxidative stress in mitochondria. Depletion of mitochondrial GSH is the most important sensitizing mechanism rendered by ethanol feeding (78, 79) to TNF-α-induced cell death (13). This ethanol-induced defect is caused by impaired transport of GSH into mitochondria and corrected by administration of SAM but not N-acetylcysteine (66). It is not known what molecular mechanisms underlie the ethanol-induced impairment in mitochondrial GSH transport except for an observation that fluidization of mitochondria with a fatty acid derivative restored their ability to transport GSH (66). This finding and the demonstrated efficacy of SAM suggest the possibility that ethanol-feeding induced alterations in mitochondrial membrane phospholipid and fatty acid compositions with resulting impairment in GSH uptake. It is well known that phospholipid methylation is required for maintenance of normal membrane fluidity and function (64). Thus, reduced SAM level as a result of abnormalities in the methionine metabolism can affect mitochondrial GSH transport and sensitize the cell to oxidative stress-induced injury by this mechanism. We do not know why the depletion in GSH occurs selectively in mitochondria and more profoundly in perivenular hepatocytes. Nor do we know whether it requires oxidative stress mediated by CYP2E1, acetaldehyde, or TNF-α. It remains to be determined how significant this defect is in the mitochondrion's regulatory role in determining the type of cell death (13).

Chronic ethanol consumption causes decreases in cellular ATP content and mitochondrial membrane potential, particularly in perivenular hepatocytes (77), and these functional impairments are corrected by

repletion of mitochondrial GSH by administration of SAM (80). Ethanol feeding also results in increased oxidative modification and single-strand breaks of mitochondrial DNA (81). It is not known whether these changes can be corrected by repletion of mitochondrial GSH. Ethanol was shown to induce apoptosis in human primary hepatocytes and HepG2 cells in culture (82), although this effect has been difficult to reproduce by others. Mitochondria from rats chronically fed ethanol are more sensitive to induction of the mitochondrial permeability transition by ceramide, GD3 ganglioside, and Bax (83). Whether and how ethanol sensitizes hepatocytes for apoptosis need to be pursued. Reasons for the discrepancy in ethanol-induced apoptosis of hepatocytes in vitro may be related to different cellular conditions in culture resulting in differential sensitivity to apoptotic and/or anti-apoptotic signaling. What determines the type of cell death (apoptosis vs. necrosis) in ALD is another important question. The extent of ATP depletion may be critical. Ethanol-fed animals are vulnerable to hepatocellular ATP depletion and necrosis caused by ischemia and hypoxia (84). The role of uncoupling protein-2 (UCP-2) in ALD also needs to be addressed. UCP2 expression is induced in hepatocytes of obese mice; their livers have reduced mitochondrial membrane potential and ATP stores and are sensitive to necrosis caused by ischemia (85). LPS induces UCP-2 expression in hepatocytes via a TNF-α-dependent mechanism (86). Thus, UCP-2 is expected to be up-regulated in ALD, which may make hepatocytes more vulnerable to cell death by compromising the mitochondrial membrane potential and ATP generation. Cell biology related to the role of mitochondria in hepatocellular cell death is an exciting area of science. Further research on how ethanol perturbs the underlying mechanisms should serve as an insightful model to generate basic information in this important area of research.

Effects of ethanol on hepatocyte growth

The effect of ethanol on hepatocyte growth is an interesting and complex topic. It is well known that animals with ALD exhibit impaired liver regeneration (87). Ethanol feeding has been shown to inhibit calcium mobilization and DNA synthesis in hepatocytes induced by HGF, the most potent mitogen for hepatocytes (88) and TGFα-stimulated receptor autophosphorylation (89). Ethanol intake also inhibits a TNF-α-dependent increase in nuclear factor κB (NF-κB) binding in partially hepatectomized rat livers but does not affect IL-6-induced Stat-3 phosphorylation and DNA binding (90). Administration of HGF improved recovery from alcohol-induced fatty liver (91). This illustrates the importance of the liver's regenerative state in opposing progression of degenerative processes. It is important to understand how ethanol affects signaling of growth factors and comitogens in hepatocytes, which future investigations should help to achieve.

Although the regenerative capacity after partial hepatectomy of ethanol-fed animals is impaired, chronic alcoholic liver disease is associated with an increased risk of cancer. This seemingly opposing effect of ethanol illustrates the importance of differentiating normal regenerative growth from abnormal malignant transformation. In micropigs fed ethanol, both increased apoptosis and regenerative proliferation occur in the same liver specimen (71). We have also reported increased expression of proto-oncogene c-*myc* in ethanol-fed rats with prefibrotic liver injury (63). A common finding is the development of decreased hepatic SAM level and global DNA hypomethylation after ethanol feeding (63, 70, 71). We and others speculate that this change in methylation status may be causally related to the propensity for this chronic liver disease to undergo malignant transformation. Future studies to define changes in gene expression as a result of the change in DNA methylation status may help us understand the molecular mechanism(s) responsible for increased risk of malignant degeneration. Studies to examine whether preventing the fall in SAM level will prevent induction in the expression of proto-oncogene should also be insightful.

Hepatic macrophages

Some of the best support for the essential role of hepatic macrophages in the pathogenesis of ALD is offered in a study by Thurman's group on Kupffer cells (92), which demonstrates amelioration of early alcoholic liver injury in the intragastric ethanol infusion model by depletion of Kupffer cells with gadolinium chloride. Even though this method of Kupffer cell elimination is not without confounding effects on the liver, such as enhanced NF-κB activation and increased TNF-α expression by bile duct epithelium and vascular endothelium (93, 94), the study highlights the direct regulatory role of the Kupffer cells in hepatocyte injury caused by ethanol. The role of Kupffer cells is also supported by studies in which a similar protective effect is seen in ethanol-fed animals given polymyxin B and neomycin (95) or lactobacillus (96), which theoretically reduce portal endotoxin levels and Kupffer cell activation. In fact, administration of antibodies against TNF-α, the cytokine predominantly expressed by Kupffer cells, attenuates alcoholic liver injury (97), and the importance of TNF-α is confirmed by the absence of alcoholic liver injury in TNF receptor 1 knockout mice (98). These findings collectively support the pathogenetic role played by Kupffer cell-derived TNF-α in experimental ALD. However, we need to be more precise with respect to the source of TNF-α. Is it resident macrophages (Kupffer cells), newly recruited monocyte-derived macrophages, or both that serve as a primary effector cell type for expression of this proinflammatory cytokine? Since the latter is a possibility in any liver disease including ALD, we should use the term hepatic macrophages. The next question has to be why TNF-α expression by hepatic macrophages is induced

and sustained in ALD. Macrophages are arrayed with the control mechanisms for their immune functions, including negative feedback regulation for expression of TNF-α. As postulated for other chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease, there may be defective regulation of cytokine expression in hepatic macrophages in ALD. Such possibilities include defective release of IL-10 (99), which may be associated with polymorphism of IL-10 promoter (100), or increased TNF-α expression due to polymorphism of its promoter (101). Alternatively, hepatic macrophages may acquire phenotypic alterations due to intracellular changes. If so, what are they? Suppression of NF-κB and TNF-α expression by antioxidants in monocytes from alcoholic hepatitis patients suggests dysregulation of TNF-α gene transcription driven by NF-κB (102). Increased iron storage by hepatic macrophages may prime NF-κB activation in experimental ALD (15). Altered methionine metabolism may also play a role in dysregulation of TNF-α gene expression in hepatic macrophages as described below.

Until recently it was believed that the expression of MAT1A and liver-specific MAT isozymes was restricted to hepatocytes. Shimizu-Saito and colleagues showed that both hepatic macrophages and endothelial cells express the liver-specific and non-liver-specific MATs whereas HSC express only non-liver-specific MAT (103). The relative contribution of the two MAT isoforms to the overall MAT activity is unknown in hepatic macrophages. Since endotoxin and oxidative stress both selectively inactivate liver-specific MAT, a relative switch in MAT expression is conceivable in hepatic macrophages of alcoholic livers that could affect the SAM level, which in turn can influence the expression of TNF-α. This idea is supported by studies from Chawla et al. showing that rats with decreased hepatic SAM levels are predisposed to liver injury caused by LPS, which was prevented with exogenous SAM treatment (104, 105). Rats with deficient hepatic SAM levels had much higher levels of basal serum TNF-α and secreted more TNF-α after LPS challenge (105); treatment of murine macrophage cells (RAW cells) with SAM in the culture medium down-regulated TNF-α mRNA and protein synthesis upon stimulation by LPS (106). These findings emphasize the importance of understanding changes in MAT and SAM in hepatic macrophages during the course of ethanol-induced injury.

A potential role for leptin in regulating proinflammatory immune response has also been demonstrated by recent studies from Diehl's group (107). The circulating level of leptin is increased in alcoholic patients (108). Thus, this *ob/ob* gene product that regulates appetite, metabolic rate, and fat depot size may modulate the phenotype of hepatic macrophages in ALD. Diehl and colleagues draw an intriguing analogy between pathophysiology of fatty livers caused by obesity and ethanol feeding. Notably, the livers in both conditions are susceptible to injury induced by LPS (109). Underlying mechanisms for this resemblance may include induction of UCP-2 and proinflammatory cytokine expression as well as mitochondrial oxidative stress (110, 111). Further cell type-specific analysis of hepatic macrophages should provide more insight into this interesting proposal. Elucidation of priming mechanisms for TNF-α expression by hepatic macrophages is a critical prerequisite for potential development of antiinflammatory therapeutic modalities for ALD. It is also important to recognize that TNF-α serves as a comitogen for liver regeneration (112). Thus, future research must address how to differentially suppress proinflammatory signaling of TNF-α while preserving its pro-regenerative effect by understanding the kinetic of TNF-α induction and intracellular signaling induced by this cytokine in different target cell types in the liver.

Liver-associated T lymphocytes

It is important to note that ethanol also sensitizes the liver for concanavalin A (Con A) -mediated injury (113). This T lymphocyte-mediated injury accompanies accentuated increases in plasma levels of TNF-α and IL-6 and the percentage of activated CD25+ T cells in both blood and liver. Ethanol feeding increases TNF-α production ex vivo by CD4+ T cells isolated from the liver, and ConA-induced liver necrosis is associated with infiltration of CD4+ T cells. These potentiating effects of ethanol on Con A-mediated TNF-α production and liver injury are reproducible in naive normal rats after adoptive transfer of liver-associated T cells obtained from ethanol-fed rats and Con A administration (114). These two studies demonstrate that ethanol primes liver-associated T lymphocytes for Con A-mediated TNF-α production and suggest that this priming mechanism may be sufficient to induce liver injury in the normal liver. These provocative findings deserve further research because alcoholic hepatitis is associated with an increased ratio of Th1/Th2 cytokine production and characterized by infiltration of not only neutrophils, but also T lymphocytes. Remaining questions include *1)* how ethanol feeding primes T lymphocytes; *2)* what polyclonal mitogens can be implicated in alcoholic hepatitis; and *3)* how ethanol affects cross-talk between hepatic macrophages and T lymphocytes.

Sinusoidal endothelial cells

In contrast to the research on hepatic macrophages, only a limited number of studies have investigated the role of sinusoidal endothelial cells in ALD. Ethanol and LPS induce similar morphological and functional changes in sinusoidal endothelial cells typically characterized by reduced fenestration and hyaluronan uptake (115, 116), both of which are preventable by elimination of Kupffer cells, suggesting a role in mediating the effects of ethanol and LPS. Chronic ethanol intake impairs receptor-mediated endocytosis by sinusoidal endothelial cells at the level of internalization (117). It remains to be determined whether these cells experience two chronological stages of ethanol-induced ab-

normalities in receptor endocytosis (receptor inactivation, followed by reduced receptor expression), as shown for asialoglycoprotein receptors on hepatocytes (118). Studies of sinusoidal endothelial cells are important for understanding the mechanisms underlying ethanol-induced hepatic inflammation. Sinusoidal endothelium is the site of inflammatory cell adhesion and transmigration. Intracellular adhesion molecule 1 (ICAM-1) expression by endothelial cells is up-regulated in experimental ALD and correlates with plasma endotoxin, hepatic TNF-α mRNA, and liver inflammation and injury (119). Expression of a receptor component for the adhesion molecule such as CD18 (β 2-integrin) is up-regulated on neutrophils of ethanol-fed rats (120). However, ethanol-induced neutrophilic inflammation in the liver, the hallmark of alcoholic hepatitis, has not been successfully reproduced in experimental animals, suggesting the models are missing a critical factor(s) required for this pathological change. Endotoxin challenge to ethanol-sensitized livers results in coagulative necrosis and neutrophilic inflammation, but most critical changes appear to occur initially at the sinusoidal endothelium such as cellular swelling, blood cell aggregation, and microcirculation disturbance (121). Elucidation of biochemical and molecular basis for sensitizing and priming sinusoidal endothelium for initiation of hepatic inflammation appears to be a pivotal prerequisite for understanding what controls progression of ALD, namely, alcoholic hepatitis and liver fibrosis. The potential importance of cross-talk between sinusoidal endothelial cells and hepatic stellate cells in the evolution of ALD needs to be emphasized.

Hepatic stellate cells

Research on HSC has seen exponential growth in recent years. This is partly because the mechanisms of HSC activation in liver fibrogenesis have been increasingly recognized as a clinically relevant and important area. Growing interest also stems from the fact that the biology of HSC is now known to be involved in many different aspects of liver homeostasis including vitamin A storage, regulation of sinusoidal blood flow, communication with hepatocytes, maintenance of hepatocyte phenotype, matrix remodeling and deposition, and regulation of local inflammation. Although development of the HSC isolation technique was largely responsible for advancement in this field, our knowledge of the mechanisms of HSC activation in ALD is limited because of difficulties in both inducing diffuse liver fibrosis in ethanol-fed animals and isolating sufficient numbers of HSC from ethanol-fed rats. HSC from the intragastric ethanol infusion model of ALD show the expected changes of cellular activation such as increased collagen and DNA synthesis (122), induced expression of α-smooth muscle actin, and depletion of retinyl palmitate (123). HSC isolated from rats fed a diet high in polyunsaturated fat with or without ethanol show increased responsiveness to hepatic macrophage-derived factors for stimulation of either collagen or DNA synthesis, providing the potential cellular basis for increased alcoholic liver fibrogenesis under the high polyunsaturated fat diet regimen (122). Besides the paracrine mode of HSC stimulation involving soluble factors, oxidative stress may be particularly relevant to the mechanisms of HSC activation in alcoholic liver fibrogenesis. Human HSC collagen synthesis is induced by 4-hydroxynonenal, one of the common lipid peroxidation by-products, via its interaction with JNK and AP-1 activation (124). In contrast, manipulation of GSH content in cultured rat HSC does not affect collagen synthesis despite its theoretical influence on endogenous lipid peroxidation and aldehyde adduct formation (125). Stable transfection of a rat HSC line with CYP2E1 cDNA results in increased production of ROS, collagen type I mRNA synthesis, and stability, effects that can be abrogated by treatment with antioxidants but are exacerbated by reduction of cellular GSH with L-buthionine sulfoximine (126). Thus, although the role of oxidative stress in hepatocellular injury in ALD is convincing, the direct involvement of oxidative stress in HSC activation is still controversial. Central questions now being addressed in the field of HSC biology can be directly applicable to alcoholic liver fibrogenesis. They include *1)* do acetaldehyde and lipid aldehydes have direct and major roles in activation of HSC in vivo and *2)* do extracellular or intracellular ROS serve as pivotal signals for expression of HSC activation phenotype? A potential mechanistic link between vitamin A depletion and HSC activation needs to be further investigated.

Recent studies suggest hyperhomocystinemia, a consequence of altered methionine metabolism, may also contribute to activation of HSC. Hyperhomocystinemia, a well-recognized independent risk factor for atherosclerosis (35), can be a result of genetic factors such as CBS deficiency, nutritional deficiencies in folate, vitamin B_6 or B_{12}, and impaired liver function (127, 128). Although homocysteine is metabolized in the liver efficiently under normal conditions, this amino acid may also be protein-bound and its excess may be released by hepatocytes into the extracellular medium (32, 129). Plasma homocysteine is believed to be derived largely from the sinusoidal release of homocysteine via a mechanism that is poorly understood. Increased plasma levels of this amino acid occur in patients and animals with impaired liver function (71, 130–133) and after a methionine load (129). This attests to the central role of the liver in the metabolism of methionine and the subsequent catabolism of homocysteine (134). An elevated plasma homocysteine level in patients with ALD is likely to be a consequence of deficiencies in vitamin B_6, B_{12}, or folate (132, 135) as well as decreased activities of enzymes that metabolize homocysteine as described above. Homocysteine exerts multiple effects on a variety of cells. It caused endothelial injury, leading to impaired platelet-modulating activity (136, 137). In vascular smooth muscle cells (VSMCs), homocysteine promoted DNA synthesis and

enhanced collagen production (138–141) possibly via activation of ERK2. These results suggest that homocysteine may serve as a direct fibrogenic mediator for vascular atherosclerosis. Since the role of smooth muscle cells in atherosclerosis is considered analogous to that of HSC in liver fibrosis (142), whether homocysteine also exerts stimulatory effects on HSC is an intriguing question. Indeed, Torres and co-workers demonstrated induction of α 1(I) procollagen and TIMP-1 mRNA expression by a HSC line and TIMP-1 mRNA and AP-1 activation by cultured hepatocytes (36). These findings support the potential role of homocysteine in liver fibrogenesis. A plausible hypothesis is that increased homocysteine release in ethanol-induced liver injury by hepatocytes due to abnormal methionine metabolism can exert paracrine effects on stellate cells to induce fibrogenesis. This remains to be examined.

GENETIC BASIS OF ALD

A genetic basis for the pathogenesis of ALD has long been proposed. The best supporting evidence for this proposal comes from twin studies where twin concordance was shown for ALD (143, 144). In fact, alcoholism is believed to result from interactions between genetic predisposition and environmental factors. To this end, the genes encoding enzymes involved in alcohol metabolism have been studied. Both alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) exhibit functional polymorphism. ADH2*2, ADH3*1, and ALDH2*2 alleles are suggested to confer protection from developing alcoholism presumably via faster production or slower elimination of acetaldehyde, a metabolite that induces unpleasant biological effects (145, 146). Thus, these polymorphisms should theoretically protect individuals from developing ALD. An association of functional polymorphisms of CYP2E1 and alcoholism or ALD has so far been negative (147, 148). Since cytokines are considered to be central mediators of ALD, as discussed earlier, polymorphisms in cytokines genes have been studied for their association with ALD. Polymorphism of TNFα promoter was reported to be associated with alcoholic steatohepatitis (101), but the functional significance of the polymorphism and confirmation of the association in larger and diverse populations need to be assessed. Recently, the single base pair substitution at −627 (C→A) in IL-10 promoter, which results in reduced IL-10 expression, was shown to be associated with an increased risk of developing advanced ALD (100); this important observation awaits confirmation. Genetic studies of ALD are just beginning to identify potential associations. Further exploration is needed especially for alcoholic hepatitis; recent developments in genetic epidemiology and microarray techniques may promote this area of research in the future.

POTENTIAL NEW TREATMENTS OF ALD

S-adenosylmethionine

SAM has been used increasingly to treat liver disease, although the protective mechanisms remain unclear. Controversy has surrounded whether SAM can be taken up by hepatocytes (32). However, in animal models of alcoholic liver disease and carbon tetrachloride hepatotoxicity, exogenous administration of SAM prevented the depletion of SAM and GSH levels and significantly ameliorated liver injury, including fibrosis (32, 64, 149, 150). It has been proposed that SAM's protective effect is via increased GSH levels. SAM has also been used to prevent the development of hepatocarcinogenesis. Decreased hepatic SAM levels and hypomethylation of certain proto-oncogenes have correlated with the development of experimental hepatocarcinogenesis, which were completely prevented by exogenous administration of SAM (151). Here, the mechanism of protective action was proposed to be via a change in the DNA methylation status. Impaired mitochondrial uptake of GSH has been postulated to be an important pathogenetic factor in alcoholic liver injury, and Colell et al. showed that SAM administration prevented impaired GSH uptake into mitochondria in chronically ethanol fed rats by preventing changes in mitochondrial membrane fluidity (66). Another important protective mechanism is SAM-mediated inhibition of TNF release from macrophages (105, 106). It is clear from these studies that SAM exerts numerous effects, from increasing membrane fluidity to altering gene expression. Recently a randomized double-blind trial using SAM in patients with alcoholic liver disease reported improved survival in SAM-treated patients with less advanced liver disease (152). However, the mechanism of protection was not investigated. With the wide availability of SAM, it is important to examine the molecular mechanisms of SAM's protective effect at different stages of liver disease.

Phosphatidylcholine

Depletion of SAM may also lead to hypomethylation of phosphatidylethanolamine and a consequent reduction in the hepatic level of phosphatidylcholine, the methylation product. This condition is aggravated by known inhibition in ALD of the activity of phosphatidylethanolamine methyltransferase (153), an enzyme that catalyzes this methylation process. Administration of phosphatidylcholine has been advocated for prevention and treatment of ALD. Polyenylphosphatidylcholine is shown to protect the baboon against alcohol-induced fibrosis and cirrhosis (154). Underlying mechanisms for the observed anti-fibrotic effects may include induction of collagenase activity (155) or suppressed platelet-derived growth factor (PDGF) -induced proliferation of hepatic stellate cells (156), protection against oxidant stress (157), and suppression of CYP2E1 inducibility (158); a clinical trial is under way.

Additional experimental and clinical studies are needed to fully understand the mechanisms and specificity of the therapeutic effects of polyenylphosphatidylcholine on ALD.

Antioxidants

Antioxidants represent a potential group of therapeutic agents for ALD. They likely provide beneficial effects on hepatocytes via desensitization against oxidant stress while inhibiting priming mechanisms for expression of proinflammatory and cytotoxic mediators via suppression of NF-κB (102, 159, 159). The question remains as to how one can achieve antioxidant-mediated, antiinflammatory effects without suppressing the anti-apoptotic effects of NF-κB. Potential approaches may include cell type-specific targeting of antioxidant therapy and development of modalities for more specific and selective regulation of NF-κB-mediated signaling.

Insulin sensitization

Insulin resistance is a common finding in ALD (160). As discussed above, the livers of alcohol-fed animals exhibit features similar to obese mice with insulin resistance, including induced TNF expression, hepatocellular ATP depletion, susceptibility to necrosis induced by ischemia, and hypoxia (85). These findings suggest that insulin resistance may be of pathogenetic importance. A recent work demonstrates striking beneficial effects of metformin, an insulin-sensitizing agent, on fatty liver disease in insulin-resistant, obese mice including reversal of hepatomegaly, hepatic steatosis, plasma aminotransferase levels, hepatic ATP depletion, and TNF induction (161). This suggests the potential therapeutic effects of insulin-sensitizing agents such as PPARγ ligands on ALD. FJ

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