levels of TNF-α on the surface of rat tracheal epithelial cells in explants. Increasing levels of TNF-α (TNF5 = 5 ng/mL, TNF20 = 20 ng/mL) lead to increasing dust adhesion. Adhesion was determined by morphometry of scanning electron microscopic images of the explant surface, as described by Xie et al.18

environment tend to increase the potential for dust-induced remodeling of the airway wall.

CONCLUSION

Our studies show that mineral dusts and PM particles can induce airway wall remodeling and thus presumably COPD. Although our model uses large airways (tracheal explants), it very likely applies to the small airways, the crucial site of airway obstruction. These processes represent intrinsic reactions to dust and are particularly, although not exclusively, mediated by surface transition metals through an NF-κB-activation pathway. Coexposures to dust-evoked mediators such as TNF-α or to other pollutants, such as cigarette smoke or ozone, all appear to be able to potentiate the intrinsic reactions.

REFERENCES

13 Dai J, Churg A. Relationship of fiber surface iron and active oxygen species to expression of procollagen, PDGF-A, and TGFβ1 in tracheal explants exposed to amosite asbestos. Am J Respir Cell Mol Biol 2001; 24:427–435

Chronic Ethanol Ingestion Increases Susceptibility to Acute Lung Injury* 

Role of Oxidative Stress and Tissue Remodeling

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Clinical studies have demonstrated that chronic alcohol abuse is an independent outcome variable in acute lung injury. The Emory Center for the Study of Acute Lung Injury is determining the mechanisms by which ethanol increases susceptibility to acute lung injury. We developed a rat model of chronic ethanol ingestion and demonstrated that ethanol predisposes rats to edematous lung injury elicited by endotoxemia or sepsis. Chronic ethanol ingestion in rats led to decreased levels of glutathione, an important antioxidant in the lung, and this defect was associated with alterations in epithelial cell permeability, decreased alveolar liquid clearance, decreased cell
viability, and decreased surfactant production. Chronic ethanol ingestion also led to the activation of lung tissue remodeling as demonstrated by the increased expression of profibrotic growth factors, matrix components, and metalloproteases. In cultured fibroblasts, the induction of the matrix glycoprotein fibronectin by ethanol was mediated via nicotinic acetylcholine receptor-dependent signal transduction. We speculate that these alterations render the host susceptible to acute lung injury by diminishing the protective mechanisms of the lung and promoting exaggerated inflammatory and tissue repair responses elicited against injurious agents.

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Key words: acute lung injury; ethanol; fibronectin; glutathione; tissue remodeling

Abbreviations: α-BGT = α-bungarotoxin; bp = base pair; cAMP = cyclic adenosine monophosphate; CRE = cyclic adenosine monophosphate responsive element; CREB = cyclic adenosine monophosphate responsive element binding protein; nAChR = nicotinic acetylcholine receptor

ARDS is an important and common disease process that afflicts approximately 75,000 to 150,000 individuals per year in the United States.1 The most common at-risk diagnoses associated with the development of ARDS are sepsis, trauma, and the aspiration of gastric contents. The mechanisms that lead to the development of this syndrome in some patients and not others are unknown, but a recent discovery points to alcohol abuse as an important predisposing factor. Herein, we describe our findings linking chronic alcohol abuse with alterations in lung structure and function that render the host susceptible to acute lung injury.

ETHANOL AND ACUTE LUNG INJURY

The association between alcohol abuse and ARDS was first identified by the work of Moss and colleagues,2 who demonstrated that chronic alcohol abuse in humans independently increases the incidence of ARDS in at-risk patients, and is associated with increased mortality related to multiorgan failure. In their study,2 they evaluated the hospital courses of 351 consecutive patients with one of seven diagnoses associated with the development of ARDS. Thirty-four percent of the patients (121 of 351 patients) were defined as chronic alcoholics. Using a strict definition of ARDS, 43% of the alcoholics acquired ARDS, as opposed to 22% of the nonalcoholics (p < 0.001; relative risk, 1.98; 95% confidence interval, 1.32 to 2.85). Furthermore, in the patients who acquired ARDS (n = 102), the in-hospital mortality rate was higher in chronic alcoholics (65%) than in nonalcoholics (36%) [p = 0.003].

This observation was compelling because it provided an important clue as to why some patients are at increased risk of acquiring ARDS. We reasoned that if we could identify the precise mechanisms by which alcohol abuse predisposes to acute lung injury, we could learn more about the pathophysiology of the syndrome and design treatment strategies capable of limiting the development and/or severity of ARDS in this important subset of patients. To this end, we first developed a rat model of ethanol-mediated susceptibility to acute lung injury. As seen in humans, chronic ethanol ingestion (>3 weeks) increased endotoxin-mediated acute edematous injury in rat lungs isolated and perfused ex vivo.3,4 In addition, ethanol increased sepsis-mediated lung dysfunction in vivo (unpublished data).

These novel observations linking alcohol with increased susceptibility to clinical and experimental lung injury are important for two reasons. First, they strengthen the idea that alcohol has direct effects on the lung. Second, they provide a unique opportunity to explore the factors and mechanisms involved in the development of acute lung injury. We next focused our efforts on identifying the discrete mechanisms by which ethanol ingestion renders the lung susceptible to acute edematous injury. This work implicated oxidative stress and tissue remodeling as key determinants of this process and is summarized below.

ROLE OF GLUTATHIONE IN ETHANOL-MEDIATED SUSCEPTIBILITY TO ACUTE LUNG INJURY

Previous studies of alcohol-mediated liver disease provided strong evidence that depletion of the antioxidant glutathione is a critical step in the pathogenesis of that disorder. Most importantly, they demonstrated that glutathione replacement with treatments that restore and/or maintain mitochondrial glutathione levels prevents ethanol-mediated hepatotoxicity.5,6 However, there had never been any parallel investigations in the lung because a link between alcohol abuse and ARDS had not been identified previously.

Using our rat model, we determined that ethanol ingestion dramatically decreased the levels of glutathione in the alveolar lining fluid and within the alveolar epithelial type II cells. We also determined that chronic ethanol ingestion, via glutathione depletion, altered surfactant synthesis and secretion,3,7 cell viability,3,8 and tissue remodeling during endotoxemia.4 These abnormalities rendered the lung intrinsically susceptible to endotoxin-mediated acute edematous injury.3 Because acute lung injury in rats was decreased by glutathione supplementation prior to endotoxin administration, we believe that many of the defects demonstrated are related to glutathione depletion3 (Fig 1).

Having established a role for glutathione depletion in ethanol-mediated susceptibility to acute lung injury, we studied barrier function within the alveolar epithelium of the alcoholic lung.8 This work led to the observation that alveolar type II cells isolated from ethanol-fed rats and...
grew in culture formed a more permeable monolayer (as reflected by inulin leak) than type II cells harvested from control-fed rats. In contrast, type II cells isolated from ethanol-fed rats given the glutathione precursor procys-teine in their ethanol diet formed a tighter monolayer in vitro that resembled monolayers derived from control-fed rats. In ethanol-fed rats, these abnormalities were associated with decreased alveolar liquid clearance (ie, the ability of the lung to remove pulmonary edema fluid) and increased protein leak across the alveolar epithelium in vivo. In contrast, ethanol-fed rats that had their diets supplemented with procysteine had the same protein leak and alveolar liquid clearance as control-fed rats. Overall, these studies indicate that chronic ethanol ingestion significantly alters alveolar epithelial barrier function, and that these defects can be prevented by glutathione replacement.

Because the animal studies described above pointed to glutathione as a protective molecule against the development of acute lung injury in the presence of chronic ethanol ingestion, we questioned whether chronic alcohol abuse produced similar alterations in lung glutathione homeostasis in humans. We studied 13 otherwise healthy alcoholics defined by daily alcohol use (Short Michigan Alcohol Screening Test score $>3$) who were $\leq 45$ years old and had no prior medical history of cardiac, hepatic, renal, or pulmonary disease, HIV, diabetes mellitus, or hypertension. The glutathione concentrations in the epithelial lining fluid of these patients (obtained by BAL) were significantly lower when compared to nonalcoholic control subjects, showing a decrease of $>80\%$. Additionally, the percentage of glutathione in the oxidized form was higher in the chronic alcoholic group when compared with control subjects, indicative of oxidative stress and increased utilization of glutathione in the epithelial lining fluid of the lung. Further, the lung lavage fluids from alcoholic subjects had approximately twice as much protein as lung lavage fluids from control subjects, a finding that is consistent with a subclinical defect in alveolar epithelial barrier function. This study provided the first evidence that chronic alcohol abuse profoundly decreases the levels of the critically important antioxidant glutathione in the lungs of humans.

ROLE OF TISSUE REMODELING IN ETHANOL-MEDIATED SUSCEPTIBILITY TO ACUTE LUNG INJURY

Acute and chronic forms of lung injury are associated with dramatic alterations in connective tissue remodeling. The control of connective tissue remodeling in lung is key for wound healing since alterations in this process lead to excess extracellular matrix expression and/or degradation with subsequent effects on lung structure. In addition, newly deposited matrix components interact with signal-transducing matrix receptors expressed by lung cells. In this fashion, alterations in the composition of the lung extracellular matrix lead to effects on cellular functions ranging from adhesion and migration to proliferation, differentiation, and cytokine expression. Accordingly, regulation of tissue remodeling is considered an important determinant of disease outcome in different forms of lung injury, but the factors that control tissue remodeling in the lung are poorly understood.

We hypothesized that another mechanism by which ethanol could affect the lung is by the activation of connective tissue remodeling. In activating tissue remodeling, ethanol can alter the content and overall composition of the pulmonary extracellular matrix. Furthermore, we speculated that this new matrix, through its interactions with lung cells, contributes to the susceptibility to acute lung injury. Data in support of ethanol-induced activation of tissue remodeling are available for the liver. For example, alcohol intake and alcohol-related cirrhosis in humans are associated with increased deposition of the extracellular matrix glycoprotein fibronectin in the perivascular areas of the liver. In rat liver, ethanol induces fibronectin expression in stellate cells. Studies designed to define the mechanisms responsible for the induction of matrix that ethanol induces $\alpha_1(I)$ procollagen expression in co-cultures of liver stellate cells and freshly isolated hepatocytes. In those studies, the stimulatory effect of ethanol occurred at the level of gene transcription. Also, the metabolism of ethanol by alcohol dehydrogenase was found to be important since 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, prevented the ethanol-induced response. The latter has been corroborated by others who have found that the metabolite of ethanol, acetaldehyde, is capable of inducing collagen and fibronectin expression in human hepatic stellate and fat-storing cells. These latter findings are interesting, particularly since others have identified the presence of alcohol dehydrogenase in the lung.

In view of this, we examined whether chronic ethanol ingestion results in activation of lung tissue remodeling as has been demonstrated in the liver. Consistent with the hypothesis, we found that rats fed with the Lieber-DeCarli isocaloric liquid diet (36% of total calories provided as ethanol) for 6 weeks showed increased expression in the lung of the pro-fibrotic molecule transforming growth factor-$\beta1$ (unpublished data), and increased activity for
the matrix-degrading endopeptidase matrix metalloproteinase-9. In addition, ethanol induced an increase in the expression (determined by reverse transcriptase-polymerase chain reaction) and deposition (determined by immunohistochemistry) of the matrix molecule fibronectin (unpublished data). Fibronectin is a multifunctional glycoprotein highly expressed in acute lung injury. In vitro, fibronectin is capable of stimulating the chemotaxis of immune cells, the proliferation of fibroblasts, and the production of proinflammatory cytokines by mononuclear cells. This and other functions suggest that fibronectin might prime cells to injurious stimuli thereby implicating fibronectin in wound healing and repair in acute lung injury.24

To define the mechanisms responsible for the ethanol-mediated deposition of fibronectin in lung, we engaged in the following studies with cultured fibroblasts. We demonstrated that ethanol induced the expression of fibronectin messenger RNA and protein in lung fibroblasts. These data suggested that the effects of ethanol occur at the level of gene transcription. To test this, we used NIH3T3 fibroblasts permanently transfected with a construct containing the human fibronectin promoter fused to a luciferase reporter gene as previously reported.25 This plasmid construct contains approximately 1200 base pairs (bp) of the 5′ flanking region of the human fibronectin gene that includes previously identified regulatory elements including three cyclic adenosine monophosphate (cAMP) response elements. To verify the association between CREs and the fibronectin promoter fused to a luciferase reporter gene, we measured fibronectin expression in cells stimulated after transfection with a competing oligonucleotide. The CRE oligonucleotide greatly diminished the serum-induced fibronectin expression, whereas the control oligonucleotide had no effect.

We next explored the possibility that ethanol induces fibronectin gene transcription via receptor-mediated signals. Ethanol has been shown to act on nicotinic acetylcholine receptors (nAChRs) in neuronal cells.22,23 Because nAChRs have been demonstrated in NIH3T3 fibroblasts and monkey lung fibroblasts,24,25 we examined the role of these receptors in our system. We demonstrated that NIH3T3 cells and human lung fibroblasts express messenger RNA coding for α7 nAChRs. Moreover, the α7 messenger RNA was increased after ethanol stimulation, thereby providing a potential amplification step for the ethanol-induced fibronectin response. Further evidence for the presence of α7 nAChRs was derived from α-bungarotoxin (α-BGT)-binding assays. α-BGT is a specific competitive ligand for α7 nAChRs.26 Consistent with the expression of α7 nAChRs, we found binding sites for α-BGT on the surface of fibroblasts. The binding of α-BGT was increased after the exposure of the cells to ethanol for 24 h, and α-BGT binding was decreased by excess unlabeled α-BGT. To confirm the role of α7 nAChRs in the ethanol-induced fibronectin response, we pretreated fibroblasts with α-BGT prior to exposing them to ethanol. α-BGT completely prevented the expression of the fibronectin gene in response to ethanol.27

These observations indicate that ethanol induces fibronectin gene expression in fibroblasts by acting on nAChRs. Consistent with this, we found that nicotine mimicked the effects of ethanol and that this stimulatory effect was mediated via specific intracellular signals. Of note, ethanol induction of fibronectin was associated with increased accumulation of intracellular cAMP. Induction of cAMP was followed by phosphorylation and increased DNA binding of the cAMP responsive element (CRE) binding protein (CREB) to nuclear DNA. To confirm the role of CREs in the fibronectin response, we tested fibroblasts transfected with deletion constructs of the fibronectin gene promoter and found that the induction of fibronectin after stimulation of nAChRs was abolished in the absence of all three CREs. To verify the association between CREs and the response tested, we measured fibronectin expression in cells stimulated after transfection with a competing CRE oligonucleotide. The CRE oligonucleotide greatly diminished the serum-induced fibronectin response, whereas the control oligonucleotide had no effect.

The mentioned work suggests that ethanol has direct effects on the lung and that these effects can be mediated, at least partially, via nAChRs expressed by fibroblasts. The interaction of ethanol with these cells (and perhaps others) through nAChRs induces a signal-transducing cascade characterized by increased intracellular cAMP, phosphorylation of CREB, and binding of CREB to CREs present on the production and deposition of fibronectin in the extracellular spaces of the lung (Fig 2). We speculate that this effect can be further enhanced by oxidative stress, another consequence of chronic ethanol ingestion.

**IMPLICATIONS FOR ACUTE LUNG INJURY AND CONCLUSIONS**

Together, our studies indicate that chronic alcohol abuse predisposes humans to acute lung injury by decreasing lung glutathione levels. This abnormality is associated with alterations in epithelial cell permeability, decreased alveolar liquid clearance, decreased cell viability, and decreased surfactant production. In addition, alcohol activates tissue remodeling in the lung leading to increased expression of profibrotic growth factors and extracellular matrix components, and the activation of matrix metalloproteinases. How ethanol induces these effects is unclear, but α7 nAChRs might mediate many of these processes at the cellular level.

These studies present a potential new paradigm for the understanding of the development of ARDS in a large subset of patients at risk for the syndrome (Fig 3). Our clinical epidemiologic studies provide important new evidence that chronic alcohol abuse significantly increases the incidence of ARDS in at-risk patients. How important is this newly identified “pre-risk” factor? In our more recent study of > 200 patients with septic shock, 50% of the patients who acquired ARDS were chronic alcoholics, even though they comprised a minority of the total population at risk for the syndrome (unpublished data). If we extrapolate these observations to the problem at large, we can speculate that alcohol abuse contributes to the development of ARDS in hundreds of thousands of patients each year worldwide. If we can define in even greater detail the mechanisms by which chronic ethanol ingestion renders the lung more susceptible to injury, other therapeutic approaches to the problem at large will be more feasible.
susceptible to acute edematous injury, we will not only make important new discoveries about pulmonary pathophysiology in general, but we can also develop and test new therapeutic strategies that could prevent or at least minimize the severity of acute lung injury in both alcoholic and nonalcoholic patients. While glutathione replacement is a logical strategy, particularly based on our experimental findings, it may not be feasible in the acutely ill patient in which alveolar injury is already established. However, combinations of glutathione precursors and agents capable of controlling tissue remodeling may prove to be important components of a multifaceted approach to the treatment of these highly vulnerable patients. Ongoing studies are examining discreet signaling pathways that may prove even more specific targets for novel intervention strategies in the acutely ill alcoholic patient.

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REFERENCES


Oxidant-Antioxidant Balance in Acute Lung Injury*

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ARDS is a disease process that is characterized by diffuse inflammation in the lung parenchyma. The involvement of inflammatory mediators in ARDS has been the subject of intense investigation, and oxidant-mediated tissue injury is likely to be important in the pathogenesis of ARDS. In response to various inflammatory stimuli, lung endothelial cells, alveolar cells, and airway epithelial cells, as well as activated alveolar macrophages, produce both nitric oxide and superoxide, which may react to form peroxynitrite, which can nitrate and oxidize key amino acids in various lung proteins, such as surfactant protein A, and inhibit their functions. The nitration and oxidation of a variety of crucial proteins present in the alveolar space have been shown to be associated with diminished function in vitro and also have been identified ex vivo in proteins sampled from patients with acute lung injury (ALI/ARDS). Various enzymes and low-molecular-weight scavengers that are present in the lung tissue and alveolar lining fluid decreased the concentration of these toxic species. The purpose of this brief chapter