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14. ABSTRACT

Acute lung injury (ALI) is a major cause of morbidity and mortality in the U.S., and sepsis is a common cause. We have found that chronic ethanol abuse renders the host susceptible to ALI. This project explores the mechanisms by which ethanol affects the lung focusing on lung fibroblasts. Specifically, it uses in vitro and in vivo models to evaluate the mechanisms of lung tissue remodeling in the setting of chronic ethanol exposure and sepsis.

15. SUBJECT TERMS
Acute lung injury, sepsis, tissue remodeling, fibronectin

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I. INTRODUCTION

Acute lung injury is a major cause of morbidity and mortality in the U.S. Its most dramatic manifestation is the Acute Respiratory Distress Syndrome or ARDS, an illness that affects over 150,000 Americans each year and that leads to death in up to 40-50% of cases (1). Acute lung injury is characterized by the activation of tissue remodeling which is responsible for the excessive deposition and turnover of extracellular matrices (2). Ultimately, it is the ability of the host to control tissue remodeling that determines the final outcome in acute lung injury (2,3). Although external factors capable of eliciting acute lung injury have been identified (e.g., infection, trauma), little is known about the factors that control the tissue remodeling response. This project addresses this very aspect. It was prompted by an intriguing report published in 1996 linking chronic ethanol ingestion to outcomes in ARDS (4). This report identified ethanol as an independent outcome variable in ARDS, a finding that is considered one of the most significant observations made in the area of acute lung injury. Today, it is believed that the development of acute lung injury is related to chronic ethanol ingestion in over 50% of cases (5). Because of its importance, we began to explore the mechanisms by which ethanol affects tissue remodeling and predisposes the lung to acute lung injury. Preliminary observations made in this area led us to hypothesize that ethanol ingestion renders the lung susceptible to acute lung injury by acting on $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) expressed by fibroblasts, and stimulating their expression of tissue remodeling genes; in particular that of fibronectin (FN). The consequent aberrant deposition of FN in the lung parenchyma induces the expression of potent transcription factors (e.g., AP-1, NFkB) in macrophages and other cells that come in contact with the newly deposited fibronectin-containing matrix. This promotes a "proinflammatory state" that primes resident and incoming immune cells recruited by diverse pulmonary insults (e.g. infection) thereby amplifying inflammatory responses in the lung that promote the development of acute lung injury. The following objectives were designed to address the hypothesis:

Objective I. Determine the role of $\alpha 7$ nAChRs (and perhaps other nAChRs) in ethanol induction of FN.

Objective II. Delineate the intracellular pathways responsible for the induction of FN in fibroblasts in response to ethanol.

Objective III. Elucidate the effects of ethanol-induced FN expression on cytokine expression.

Objective IV. Study the effects of ethanol-induced FN expression in the rat model of sepsis-induced acute lung injury.

II. BODY

This work has led to important observations that are described below under each of the objectives proposed in the initial application:

Objective I. **Determine the role of α7 nAChRs (and perhaps other nAChRs) in ethanol induction of fibronectin.**

As reported in the previous progress report, we have found that ethanol stimulates transformed and primary lung fibroblasts to express fibronectin mRNA and protein both *in vitro* and *in vivo*. These effects are mediated via α7 nAChRs and require protein kinase C activation and DNA binding by the transcription factor CREB. We also explored the role of ethanol metabolism in our system. We found that 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, inhibited ethanol-induced fibronectin expression in fibroblasts. This suggests that the main player in this process is acetaldehyde. To test this, we exposed cells to acetaldehyde and found that this molecule indeed stimulated fibronectin expression. The latter observation suggests that lung fibroblasts contain alcohol dehydrogenase and that metabolism through this enzyme is required to allow for the effects of ethanol on fibronectin expression. This work was published *(Roman et al., Am J Physiol, 2005).*

The evidence implicating α7 nAChR in this process included blockade of ethanol-induced fibronectin expression in fibroblasts by α-bungarotoxin, a snake venom that is considered somewhat specific antagonist for α7 nAChR. However, in view that this agent is not 100% specific, we obtained C57BL mice with knockout mutations in α7 nAChRs. This allowed us to harvest primary murine lung fibroblasts from these animals for further study. Unexpectedly, w found that ethanol was able to stimulate fibronectin expression in fibroblasts lacking α7 nAChRs. This suggests that other nAChRs are involved in this process. These observations contrast with those made with nicotine where we show that nicotine also stimulates fibronectin expression in wildtype fibroblasts, but not in α7 nAChR knockouts. This work was published *(Roman et al., Am J Physiol, 2005).* These data open a relatively new area of investigation that links chronic ethanol abuse with lung tissue remodeling. It also highlights the need for further research directed at elucidating the role of distinct nAChRs in lung cells.

Objective II. Delineate the intracellular pathways responsible for the induction of fibronectin in fibroblasts in response to ethanol.

In the previous year, we determined that ethanol stimulates fibronectin expression in fibroblasts by stimulating the transcription of the fibronectin gene. We delineated a number of signaling pathways and the transcription elements on the fibronectin gene promoter that were involved in this process. We also investigated if endotoxin was capable of stimulating fibronectin expression. The initial observations regarding this area were discussed in our previous progress report. They are considered important because our hypothesis suggests that ALI in alcoholics is triggered by a second hit; infection representing a common example of this. In fact, sepsis is the most common cause of ALI in the U.S., and it is often the consequence of infection with gram negative bacteria (e.g., pseudomonas) that release endotoxins. We failed to detect a change in fibronectin expression in response to endotoxin. However, we found that endotoxin was capable of stimulating human monocytic cells to express a fibronectin receptor, the integrin α5β1. This is an important finding because it suggests that endotoxemia during sepsis might promote increased recognition of fibronectin matrices by monocytic cells through induction of fibronectin receptors. In turn, this would facilitate cellular migration into tissues

with subsequent damage upon activation. We found that the lipid A portion of endotoxin was most responsible for this effect and that it depended on CD14 expression on the surface of the cells. Through induction of protein kinase C activation and DNA binding by the transcription factor NfκB, endotoxin induced the transcription of the gene encoding for the α5 subunit of the α5β1 integrin. These changes were associated with increased adhesion to fibronectin. This work was published *(Roman et al., Am J Physiol, 2005).*

Objective III. Elucidate the effects of ethanol-induced fibronectin expression on cytokine expression.

Our hypothesis suggests that excessive deposition of fibronectin in lung may alter the behavior of immune cells recruited to the lung. To test this possibility, we harvested alveolar type II cells from rats exposed to ethanol for 6 weeks and cultured them for up to 3 days thereby allowing the cells to deposit an insoluble matrix. At the end of the culture period, the cells were eradicated and the remaining matrix-coated plates were used for further experiments. Human monocytic U937 cells were cultured on the matrices followed by testing for expression of the pro-inflammatory cytokine interleukin-1β (IL-1β). As reported before, we found that cells cultured on matrices derived from cells harvested from ethanol-treated animals produced more IL-1β than those cultured on matrices derived from control animals. Furthermore, we found that an antibody to α5β1 integrin inhibited the response, whereas a control antibody did not. In collaboration with Dr. LouAnn Brown (Department of Pediatrics, Emory University), we found that the matrix produced by alveolar type II cells harvested from rats on the alcohol diet was very different than that produced by control cells. Specifically, the matrix contained a relative increase in fibronectin content and its assembly (as determined by immunofluorescense staining) appeared disturbed. This and related work is being prepared for submission for publication.

In other work, we began to investigate the effects of oxidant stress on matrix expression in lung fibroblasts. This work is based on observations generated in collaboration with the collaborator, Dr. David Guidot, who has shown that chronic ethanol ingestion causes oxidant stress in the lung. This is due, in part, to derangements in the function of alveolar type II cells which are largely responsible for the transport of glutathione into the lung to maintain homeostasis. Thus, we speculated that, in addition to the direct effects of ethanol on lung fibroblasts through nAChRs, ethanol could also influence the cells indirectly by affecting the extracellular redox state. To test this possibility, we developed an *in vitro* system where we could test the effects of extracellular redox state on cells by changing the relative concentrations of cysteine and cystine (Cys/CySS redox). As demonstrated in Figure 21, our system is reliable since measured Cys/CySS redox state correlates well with the estimated redox. NIH3T3 fibroblasts exposed to different ratios of Cys/CySS (0 = most oxidized, -150 = most reduced) showed increased fibronectin gene expression in oxidized media (Figure 1B). Primary lung fibroblasts show the same effect.

In parallel work, we showed that increased gene transcription by oxidized Cys/CySS redox is associated with increased fibronectin mRNA and protein expression (Figure 1C and D). We also showed that oxidized Cys/CySS redox causes activation of transcription factors like NFkB known for their ability to stimulate the expression of IL-1β and other pro-inflammatory cytokines (not shown). We are currently testing this *in vivo* using the rat model of chronic

ethanol exposure and sepsis. Together, these studies suggest that ethanol might promote lung tissue remodeling indirectly by affecting the extracellular redox state of the lung. This is a novel area of investigation, particularly in view of work from our collaborators showing that anti-oxidants (e.g., N-acetylcysteine and procysteine) inhibit ethanol-induced susceptibility to acute lung injury *in vivo*.

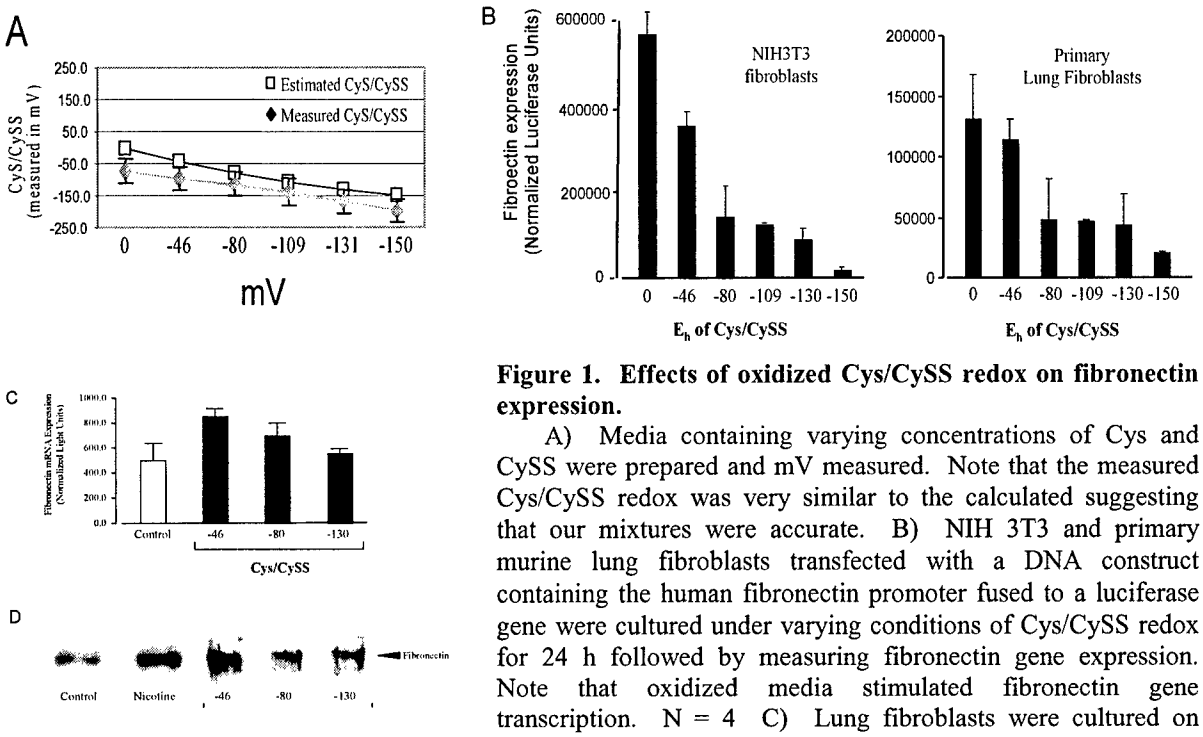

Figure 1. Effects of oxidized Cys/CySS redox on fibronectin expression.

A) Media containing varying concentrations of Cys and CySS were prepared and mV measured. Note that the measured Cys/CySS redox was very similar to the calculated suggesting that our mixtures were accurate. B) NIH 3T3 and primary murine lung fibroblasts transfected with a DNA construct containing the human fibronectin promoter fused to a luciferase gene were cultured under varying conditions of Cys/CySS redox for 24 h followed by measuring fibronectin gene expression. Note that oxidized media stimulated fibronectin gene transcription. N = 4 C) Lung fibroblasts were cultured on varying Cys/CySS redox followed by RT-PCR for fibronectin mRNA expression. D) Lung fibroblasts were cultured on varying Cys/CySS redox followed by western blotting with anti-fibronectin antibody. Note that oxidized media (-46 mV) induced fibronectin protein expression as did nicotine (positive control).

Finally, we have collaborated with Dr. Guidot in defining the potential beneficial effects of granulocyte-macrophage colony stimulating factor (GMCSF) in restoring alveolar epithelial cell function in the setting of chronic ethanol abuse. His group has found that the administration of GMCSF to cultured cells or to rats chronically exposed to ethanol restores cell homeostasis. We have now shown that this effect relates to the ability of GMCSF to activate intracellular pathways that include the transcription factor PU.1 *(Joshi et al., J Immunol, accepted).*

<u>Objective IV</u>. Study the effects of ethanol-induced fibronectin expression in the rat model of sepsis-induced acute lung injury.

As indicated in the previous report, we have begun to explore other tissue remodeling molecules in the alcoholic lung as it relates to sepsis. This work led us to study Matrix Metalloproteinase-9 (MMP-9), a matrix degrading protease that has been implicated in ALI. Because fibronectin can stimulate MMP-9 expression, we studied MMP-9 in the lungs of rats exposed to ethanol for 6 weeks. This work in animals, together with work *in vitro*, suggests that

chronic alcohol abuse and endotoxemia might interact to stimulate tissue remodeling through a number of pathways that include the increased deposition of connective tissue (e.g., fibronectin), while stimulating its degradation through the stimulation of MMP-9 expression. We predict that such scenario would lead to alterations in matrix composition in the lung rendering it susceptible to injury. This work is being prepared for publication and was presented during the recent American Thoracic Society Meeting held in San Diego, CA (May 2005). As stated before, we are also evaluating the potential role of GMCSF administration on acute lung injury related to sepsis in rats chronically exposed to ethanol.

We also began collaboration with Dr. Ellen Burnham who is evaluating the role of chronic alcohol abuse in susceptibility to acute lung injury in humans. She has obtained clinical samples (broncho-alveolar lavage fluid) from chronic alcoholics, chronic alcoholics that smoke, and smokers that don't drink who are otherwise 'healthy'. We have examined these samples using various assays to detect: 1) induction of specific transcription factors in the macrophages obtained through alveolar lavage and 2) induction of MMP-9 and fibronectin by the lavage fluid using unique bioassays. This work revealed that the alveolar lavage fluid of chronic alcoholics stimulates MMP expression in human monocytic cells; this effect is different than what we observed with clinical samples obtained from smokers. Some of this work was presented during the recent American Thoracic Society Meeting held in San Diego, CA (May 2005), and it is considered important because it provides a translational approach that will allow us to relate our findings *in vitro* and in the animal model to the human condition.

III. KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that ethanol stimulates fibronectin expression in lung fibroblasts through effects on nAChRs (not $\alpha 7$ nAChRs) via signaling mechanisms that include activation of protein kinase C, phosphorylation of CREB, and alcohol metabolism. This work is published.

- We have found that endotoxin, a product of sepsis with gram negative bacilli, increases the recognition of fibronectin matrices in monocytic cells by stimulating the expression of functional fibronectin $\alpha 5\beta 1$ receptors. This work is published.

- We confirmed that alveolar type II cells harvested from animals exposed to ethanol chronically produce an aberrant matrix characterized by, among other things, increased relative content of fibronectin and abnormal assembly. This work is being prepared for publication.

- We have found that ethanol might induce abnormal tissue remodeling in lung by affecting extracellular redox state of this organ. Specifically, we found that oxidized extracellular Cys/CySS redox induces fibronectin expression and activates a number of transcription factors in primary lung fibroblasts. This, together with data showing that chronic exposure to alcohol in both humans and animals leads to oxidant stress, suggests that extracellular redox may be affected by ethanol and this, in turn, can influence matrix expression and other processes relevant to lung injury and repair.

- We have examined the potential beneficial effects of GMCSF in restoring homeostasis in lung epithelial cells both *in vitro* and *in vivo*. Specifically, we have found that ethanol affects the expression and signaling of GMCSF receptors and that this defect might be overcome by exogenous GMCSF which acts through specific intracellular pathways that affect the transcription factor PU.1. This work is accepted for publication.

- We have worked to elucidate the effects of chronic alcohol abuse on MMP expression in lung because of the potential roles that MMPs are considered to play in lung injury. This work is being prepared for publication.

- We have begun to translate our work to the situation in humans by examining clinical samples obtained from alcoholics who are otherwise healthy. This work suggest that these individuals already show alterations in the expression of tissue remodeling genes, and this may help explain the increased susceptibility to acute lung injury that these subjects show.

IV. REPORTABLE OUTCOMES

- **Manuscript in press or submitted for publication in peer-reviewed journals**

Roman J, Ritzenthaler JD, Bechara R, Brown LA, Guidot DM. Ethanol stimulates fibronectin expression through protein kinase-dependent signals that activate CREB. *Am J Physiol*, 288:L975-L987, 2005.

Roman J, Ritzenthaler JD, Boles B, Lois M, Roser-Page S. Lipopolysaccharide induces the expression of fibronectin $\alpha5\beta1$ integrin receptors on human monocytic cells in a Protein Kinase C-dependent fashion. *Am J Physiol*, 287:L239-L249, 2004.

Joshi PC, Applewhite L, Ritzenthaler JD, **Roman J**, Fernandez AL, Eaton DC, Brown LAS, Guidot DM. Chronic ethanol ingestion in rats decreases granulocyte/macrophage colony-stimulating factor receptor expression and downstream signaling in the alveolar macrophage. *J Immunol*, 2005, accepted for publication.

- **Abstracts/Presentations**

Burnham EL, Ritzenthaler JD, Moss M, **Roman J**. Fibronectin gene transcription is increased in individuals with a history of chronic alcohol abuse. AJRCCM, A840, 2005. Presented during 2005 ATS Meeting, San Diego, CA.

Mehta A Ritzenthaler, JD Roser-Page S, Guidot DM, Roman J. Ethanol and endotoxin affect MMP expression in lung: Implications for understanding acute lung injury. AJRCCM, 2005. Presented during 2005 ATS Meeting, San Diego, CA.

V. CONCLUSIONS

Our data suggest that ethanol predisposes subjects to ALI during sepsis by stimulating lung tissue remodeling characterized by increased expression of fibronectin and MMP-9. This effect appears to work through direct pathways that include nAChR activation and alcohol metabolism, and indirect pathways that result from the development of an oxidized extracellular Cys/CySS redox state. The newly deposited matrices can stimulate monocytic cells to produce increased amounts of pro-inflammatory cytokines such as IL-1β. These incoming cells recognize fibronectin matrices through endotoxin-induced upregulation of surface fibronectin α5β1 integrin receptors. Our work in humans suggests that they present the same derangements seen in animals chronically exposed to alcohol. Therefore, it is likely that we will be able to translate our efforts to the clinical arena. Currently, there are no effective treatments for acute lung injury. Our work with GMCSF suggests that administration of this agent to patients might be helpful, but further work is needed to determine the utility of this strategy.

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