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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. The consequent aberrant deposition of fibronectin 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 fibronectin.

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

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

Objective IV.   Study the effects of ethanol-induced fibronectin 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.**

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, 288:L975-L987, 2005)* and represents the first demonstration of these pathways as they relate to ethanol and fibronectin in lung.

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, we 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, 288:L975-L98, 2005)*. These data opened a relatively new area of investigation that link chronic ethanol abuse with lung tissue remodeling. They also highlight 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.

As described above, 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. More recently, we engaged in work directed at elucidating the role of oxidant stress in ethanol-induced fibronectin expression. Others have shown that chronic ethanol ingestion induces oxidant stress in lung by diminishing the levels of glutathione, an important antioxidant (5). However, the mechanisms that link oxidant stress to activation of tissue remodeling in lung remain unclear. We speculated that extracellular oxidant stress could stimulate redox signaling in lung fibroblasts thereby inducing fibronectin expression. To study this, we cultured lung fibroblasts in media containing varying concentrations of cysteine (Cys) and cystine (CySS). This allowed for manipulation of the extracellular redox state. We focused on this because Cys/CySS is the largest extracellular thiol disulfide couple that affects extracellular and intracellular glutathione concentrations. We found that oxidized Cys/CySS

redox (-46 mV) induced fibronectin expression in NIH3T3 and lung fibroblasts, whereas normal redox conditions (-80 mV) and reduced conditions (-130 mV) had less of an effect on this process. **Figure 1** shows the effects of extracellular Cys/CySS redox state on fibronectin gene transcription in NIH3/T3 (left panel) and primary lung fibroblasts (right panel). We have demonstrated similar findings for fibronectin mRNA and protein (not shown). These studies suggest that ethanol can stimulate increased expression of fibronectin directly by stimulating nAChR-mediated signals and indirectly by causing oxidant stress (*Ramadan et al., in preparation*).

Figure 1. Effect of oxidized Cys/CySS redox potential on fibronectin expression. Media containing varying concentrations of Cys and CySS were prepared and mV measured. Then, NIH3T3 and primary murine lung fibroblasts transfected with a DNA construct containing the human fibronectin promoter gene fused to a luciferase gene were cultured under varying conditions of Cys/CySS redox potential (E_h) for 24 hours followed by measuring fibronectin gene expression (n = 4).

The above work is interesting in view that it links chronic oxidant stress to increased fibronectin expression and lung remodeling in the setting of ethanol abuse. Of note, oxidant stress also induces the expression of adenosine by lung epithelial cells. This is important because adenosine may stimulate fibronectin expression in lung by acting on purinergic receptors expressed on lung fibroblasts. We have confirmed this possibility in published work (*Roman et al., Am J Physiol, 290:L317-L325, 2006*).

In other work, we investigated whether endotoxin was capable of stimulating fibronectin expression. The latter is 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 $\alpha5\beta1$. 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 $\alpha5$ subunit of the $\alpha5\beta1$ integrin. These changes were associated with increased adhesion to fibronectin. This work was published *(Roman et al., Am J Physiol, 287:l239-l249, 2004).*

We previously reported that ethanol and endotoxin stimulate the activation of matrix metalloproteinases (MMPs), proteases capable of degrading connective tissue and implicated in regulation of inflammation (6). However, that work did not examine the mechanisms responsible for MMP upregulation in lung in the setting of chronic ethanol exposure. In more recent work, we showed that ethanol alone does not stimulate increased expression/activation of

MMP-9 in lung or in monocytic cells *ex vivo*. Instead, this requires a second hit such as endotoxin. This observation led to other studies evaluating in detail the mechanisms that control endotoxin-induced MMP-9 expression in lung and how this might be affected by ethanol (*Metha et al., in preparation*).

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 (*Roman et al., in preparation*).

We have collaborated with Dr. Guidot in testing the role of other soluble factors in the control of ethanol-induced derangements in lung. This work strongly suggested an important role for epithelial-derived transforming growth factor β1 (TGFβ1) in this process (*Bechara et al., Alcohol Clin Exp Res, 27:1006-1014, 2003; Bechara et al., Am J Respir Crit Care Med, 170:188-194, 2004*). This is tightly linked to lung tissue remodeling since TGFβ1 is a pro-fibrotic molecule with pro-remodeling effects including stimulation of fibronectin expression. With Dr. Guidot, we also began to define 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, 175:6837-6845, 2005)*.

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

The collaborator used the rat model of sepsis-induced acute lung injury to test the effects of chronic ethanol ingestion. He showed that ethanol ingestion, via glutathione depletion, increased sepsis-mediated lung dysfunction, and these effects could contribute to the increased risk of ARDS seen in alcoholic patients (7). These studies indeed suggested that chronic ethanol ingestion rendered the host susceptible to acute lung injury. In collaboration with Dr. Ellen

Burnham (Assistant Professor of Medicine at Emory), we have begun to examine the implications of our *in vitro* and animal data in humans. Specifically, we wanted to determine if chronic alcohol abuse was associated with activation of tissue remodeling in human lung. To this end, we tested bronchoalveolar lavage fluid (BALF) harvested from smokers and alcoholics who were otherwise healthy. These clinical samples were then submitted to bioassays designed to test the ability of the sample to stimulate the expression of fibronectin. As noted in **Figure 2A and B**, the BALF obtained from alcoholics induced fibronectin expression in fibroblasts. Note that the BALF fluid harvested from alcoholics induced fibronectin expression independent of their smoking status (right panel). The stimulatory effect of the BALF from alcoholics was inhibited by N-acetylcysteine again suggesting a role for oxidant stress in the ability of the fluid to promote tissue remodeling (not shown).
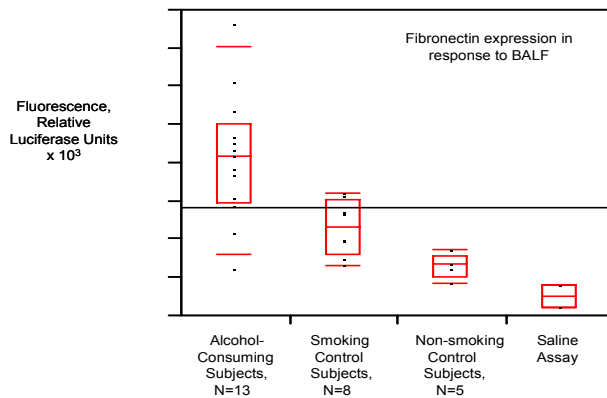

Figure 2A. Stimulation of fibronectin gene transcription in lung fibroblasts by BALF from 3 subject cohorts. Fibroblasts were obtained from transgenic mice containing the full length human fibronectin promoter connected to the luciferase reporter vector. Boxplots signify the median value for each cohort (middle line), and 25th and 75th quartiles (bottom and top of box). Whiskers are 10th and 90th percentiles. By Kruskal-Wallis rank-sums test, alcohol consuming subjects vs. smoking controls, p<0.01; and alcohol consuming patients vs. non-smoking controls, p<0.004.
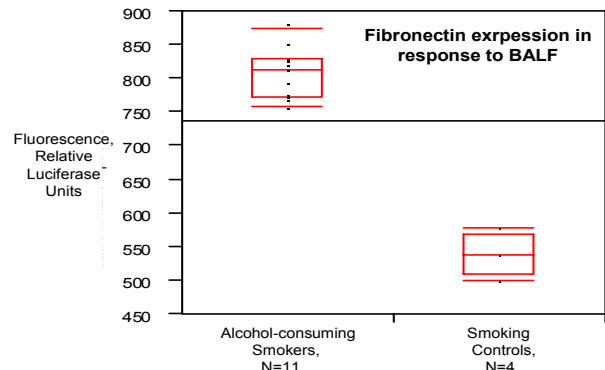
Figure 2B. Stimulation of fibronectin transcription in stably transfected fibroblasts by BALF from smoking and alcohol consuming subjects and smoking controls. Boxplots signify the median value for each cohort (middle line), and 25th and 75th quartiles (bottom and top of box). Whiskers are 10th and 90th percentiles. P<0.005 between these two cohorts by Kruskal-Wallis rank sums test.

In parallel studies, we harvested the alveolar macrophages of alcoholics and smokers through bronchoalveolar lavage. The macrophages were then submitted to RT-PCR to detect mRNA for a number of tissue remodeling genes (**Figure 3**). These studies showed an increase in fibronectin, and in its isoforms EDA and EDB in alcoholics independent of their smoking status. This information suggests different roles for fibronectin isoforms in ethanol-induced lung susceptibility to injury. The studies also showed increased mRNA expression for the $\alpha 5$ integrin, the fibronectin receptor. This suggests an increase in the ability of macrophages in alcoholics to recognize fibronectin. In contrast, MMP-2 and MMP-9 mRNAs were most prominent in smokers not exposed to alcohol.
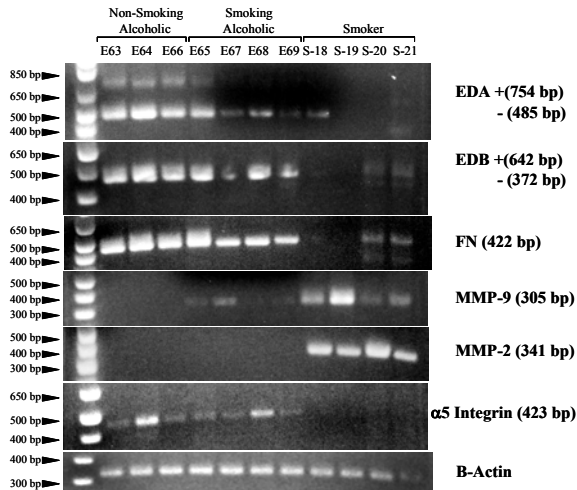
Figure 3. Pro-remodeling activity in alveolar macrophages harvested from alcoholics.

Alveolar macrophages obtained via bronchoalveolar lavage from alcoholics and smokers were processed for RT-PCR to examine fibronectin, fibronectin EDA and EDB isoforms, MMP-2 and MMP-9, $\alpha 5$ integrin. β-actin was used as control. Note that increased mRNA accumulation was noted for fibronectin, its isoforms, and $\alpha 5$ integrin in alcoholics.

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 potential may be affected by ethanol and this, in turn, can influence matrix expression and other processes relevant to lung injury and repair. This work is being prepared for publication.

● We have examined the potential effects of adenosine, TGF$\beta 1$, and GMCSF in affecting homeostasis in lung epithelial cells both *in vitro* and *in vivo*. Specifically, we have found that ethanol promotes adenosine expression which induces fibronectin production. Ethanol also stimulates TGF$\beta 1$ expression which has pro-remodeling activity, among other detrimental effects. Ethanol also diminishes the expression and signaling of GMCSF receptors which is also detrimental. This defect might be overcome by exogenous GMCSF which acts through

specific intracellular pathways that affect the transcription factor PU.1. This work is published.

- 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 suggests 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. This work is being prepared for publication.

IV.   REPORTABLE OUTCOMES

This work has led to the following reportable outcomes.

- **Manuscripts published in peer-reviewed journals or in preparation**

1. Bechara RI, Brown LAS, Eaton DC, Roman J, Guidot DM. Chronic Ethanol Ingestion Increases Expression of the Angiotensin II Type 2 (AT2) Receptor and Enhances Tumor Necrosis Factor-alpha- and Angiotensin II-Induced Cytotoxicity Via AT2 Signaling in Rat Alveolar Epithelial Cells. Alcohol Clin Exp Res. 27:1006-1014, 2003.

2. 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.

3. Bechara RI, Brown LAS, Roman J, Joshi PC, Guidot DM.   Transforming growth factor $\beta1$ expression and activation is increased in the alcoholic rat lung. Am J Respir Crit Care Med, 170:188-194, 2004.

4. 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.

5. 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, 175:6837-45, 2005.

6. Roman J, Rivera HN, Roser-Page, S, Sitaraman SV, Ritzenthaler JD. Adenosine induces fibronectin expression in lung epithelial cells: Implications for airway remodeling. Am J Physiol, 290:L317-L325, 2006.

7. Mehta A, Tomic R., Ritzenthaler J, Guidot DM, Roman J. Effects of chronic ethanol exposure on MMP expression *in vitro* and *in vivo*. In preparation.

8. Roman J, Ritzenthaler J, Guidot DM, Brown LA. Chronic ethanol exposure affects alveolar epithelial cell phenotype and promotes their deposition of a proinflammatory matrix. In preparation.

● **Reviews**

1. **Guidot DM** and **Roman J**. Chronic ethanol ingestion increases susceptibility to acute lung injury: role of oxidative stress and tissue remodeling. Chest. 122:309S-314S. 2002.

● **Abstracts/Presentations**

1. Ritzenthaler, JD, Boles, B, Lois, M, **Roman, J**. Lipopolysaccharide induces the expression of the fibronectin integrin receptor α5β1 on monocytic cells. Oral presentation at the 2002 Am Thor Soc International Meeting, Atlanta, GA. *Am J Respir Crit Care Med*, 165:A417, 2002.

2. **Roman J**, Ritzenthaler JD, **Guidot DM**, Brown LAS. Ethanol induces alveolar type II cells to deposit a matrix that promotes monocyte activation. Presented during the 2004 Research Society on Alcoholism Annual Meeting, June, Seattle, WA.

3. **Roman J**, Ritzenthaler JD, Sitaraman S, Rivera H, Roser-Page S. Adenosine stimulates fibronectin gene transcription in pulmonary epithelial cells and fibroblasts: implications for lung remodeling. AJRCCM, A214, 2005. Presented during 2005 ATS Meeting, San Diego, CA.

4. 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.

5. 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.

6. Fernainy K, Joshi PC, Mitchell PO, Roman J, Guidot DM. Granulocyte-macrophage colony stimulating factor receptors are expressed in rat lung epithelium and are decreased by chronic ethanol ingestion. SAFMR/SSCI Annual Conference, Atlanta, GA (SAFMR/SSCI Trainee Research Award Winner).

7. Ramadan B, Ritzenthaler, JD, Rivera HD, Roser-Page S, Jones DP, Roman J. Oxidation of the thiol disulfide cysteine/cystine redox couple stimulates lung fibroblast proliferation and matrix expression through redox signaling. SAFMR/SSCI Annual Conference, Atlanta, GA (SAFMR/SSCI Trainee Research Award Winner).

- **Grant Submissions**

1. Date: February 1, 2006
   Agency: NIH
   PI and Co-PI: Jesse Roman and David M. Guidot
   Title: Ethanol and chronic oxidant stress in regulation of tissue remodeling in lung.
   Outcome: Pending

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 other matrix molecules (e.g., MMPs). This effect appears to work through <u>direct</u> pathways that include nAChR activation and alcohol metabolism, and <u>indirect</u> 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. Together with TGFβ1 production, these effects promote the remodeling of the lung connective tissue. Incoming immune cells recruited after injury 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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