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Research Article

Aspiration of Gastrointestinal Material and Induction of Fibronectin Expression in Lung Transplant Recipients: Implications for Early Airway Remodeling

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Abstract

***Background*:** Gastroesophageal reflux disease has been associated with the development of chronic lung allograft dysfunction following lung transplantation. While the mechanisms are unclear, it is postulated that microaspiration of gastrointestinal material (GIM) leads to

inflammation and airway remodeling that culminates in obliterative bronchiolitis. As the expression of the matrix glycoprotein fibronectin has been shown to be an early marker of this fibroproliferative process, its induction could suggest a causal mechanism for allograft dysfunction in lung transplant (LTX) recipients experiencing microaspiration.

***Methods*:** De-identified bronchoalveolar lavage fluid (BALF) samples sequentially collected during surveillance bronchoscopies throughout the first post-transplant year were analysed. Microaspiration was defined by the detection of bile salts in the BALF via an ELISA assay. An *in vitro* bioassay then detected fibronectin expression as a marker of activation of airway remodeling via incorporation of murine NIH/3T3 fibroblasts transfected with the human fibronectin promoter connected to the luciferase reporter vector. Fibroblasts were cultured with BALF for 24 hours and the ability of the BALF to stimulate fibronectin induction was measured via luciferase activity units.

***Results*:** Sequential bronchoscopies on fifteen LTX recipients yielded 101 BALF samples for analysis. Six recipients had at least one BALF 'positive' for bile salts indicating GIM aspiration while the remaining nine recipients had no 'positive' analyses. Increased fibronectin induction stimulated by the BALF correlated with the presence of one or more episodes of GIM aspiration occurring during the first post-transplant year (r_s =0.22, p=0.03). Furthermore, fibronectin luciferase activity in the BALF from recipients experiencing aspiration was significantly greater than that from those recipients who did not aspirate (1.05 $\pm$ 0.19 vs. 0.92 $\pm$ 0.28 luciferase activity units/µg protein) [p< 0.02].

***Conclusions*:** Increased fibronectin induction is implicated in LTX recipients who experience microaspiration. This may herald early airway remodeling that can lead to later allograft dysfunction.

Keywords

Lung transplant; fibronectin; aspiration; microaspiration; remodeling; luciferase; gastroesophageal reflux; bile salt

1. Introduction

Lung transplantation (LTX) is a critical treatment for patients suffering from chronic progressive lung disease, with nearly 30,000 procedures having been performed since 1988 [1]. Presently, recipients of lung allografts can expect median survivals of 85.0% at one year, 68.2% at three years, and 55.5% at five years post-procedure [2]. The major impediment to long term survival following LTX is the development of chronic lung allograft dysfunction (CLAD) which predominately manifests as obliterative bronchiolitis (OB), an immune rejection response characterized by progressive obliteration of small airways in the lung allograft leading to irreversible airflow obstruction [3, 4]. Approximately 50% of lung recipients experience OB within five years of their transplant procedure resulting in median survival being between three and five years once this diagnosis has been established [5]. The other less common phenotype of CLAD, the Restrictive Allograft Syndrome (RAS), is an entity characterized by parenchymal interstitial fibrosis most commonly involving the upper lobes of the lung allograft.

While the mechanisms leading to OB are not completely elucidated, it is thought to be the result of repetitive insult to bronchial epithelium through the combined interaction of both immunologic and nonimmune factors. One of the nonimmune factors that has been strongly implicated in the genesis and propagation of OB is the clinical development in the transplant recipient of gastroesophageal reflux disease (GERD). The resulting microaspiration of gastrointestinal material (GIM) is postulated to be the promoter of chronic inflammation in the lung allograft [6, 7]. It has been well established that lung recipients experiencing GERD have a higher prevalence of Bronchiolitis Obliterans Syndrome (BOS) – the clinical manifestation of those with histologic OB [8-10].

A current hypothesis suggests that repetitive insults arising from microaspiration of GIM promote local chronic inflammation which is characterized by a fibrotic reparative response of activated fibroblasts. This inflammatory response stimulates extracellular matrix expression resulting in remodeling and ultimately obliteration of the airway lumen [11]. A hallmark of this inflammatory reparative response is the expression of fibronectin, a matrix glycoprotein upregulated after airway injury and implicated in many pulmonary disorders [12]. Likewise, fibronectin induction might also be expected to be part of the early injury/reparative events occurring in the lower airway of LTX recipients who experience microaspiraton. The purpose of this investigation was to ascertain whether evidence of fibronectin induction was indeed manifested in the lower airway of LTX recipients experiencing microaspiration of GIM.

2. Materials and Methods

2.1 Patient Population and Sample Collection

For the purposes of this investigation, de-identified samples of bronchoalveolar lavage fluid (BALF) were retrieved from an Institutional Review Board approved lung transplant specimen repository. These specimens, along with corresponding clinical data, had been prospectively collected on participating recipients at pre-determined intervals following their lung transplant procedure. The collection intervals were designated at 30, 90, 180, 270 and 365 days following transplantation and took place during routine scheduled bronchoscopies while additional collections made during clinically indicated bronchoscopies. During each of these bronchoscopies, BALF was collected by first instilling sequential 60 ml aliquots of normal saline (total instillation of 120 – 180 cc) into either the right middle lobe or the lingula of the lung allograft followed by suction retrieval of a portion of that fluid. The recovered fluid was then filtered, centrifuged and stored at -80°C until retrieved for analysis. Specimens were retrieved on all LTX recipients who completed all of their first post-transplant year surveillance bronchoscopies for the interval June 2015 through October 2017 (i.e. - all recipients utilized in this investigation survived at least one year following their transplant).

All recipients were maintained with standard immunosuppressive medications including a calcenurin inhibitor (i.e. tacrolimus), an anti-metabolite (i.e. –mycophenolate or azathioprine), and a corticosteroid. Additionally, azithromycin was routinely introduced within the first month following transplantation. Also following transplantation, recipients were assessed for the presence of GERD via 24 hour esophageal pH probe monitoring with the results analyzed by an experienced gastroenterologist who was blinded to other aspects of this investigation.

This study was submitted to the University of Louisville Institutional Review Board (IRB) – Human Subjects Protection Program Office and to the hospital research review committees for approval prior to evaluating samples from the repository. The lung transplant biorepository is an IRB approved (IRB Number 15.0339, approved 15/18/15) function of routine follow up for which patients provided an informed consent prior to their transplantation.

2.2 Bile Salts and Fibronectin Induction Assay

Supernatant from the LTX BALF samples was assessed for the presence of bile salts using a commercially available enzyme-linked immunosorbent assay (ELISA) (Bioquant, San Diego, CA). The bile salts were measured using a Beckman Coulter AD 340C Absorbance detector (Fullerton, CA) at 540nm and concentrations were expressed as nmol/ml BALF. Concentrations $\geq$ 0.312 nmol/ml were considered 'positive' and thus indicative of aspiration of GIM [13]. An *in vitro* luminescent bioassay was used to determine fibronectin luciferase activity in LTX BALF. Briefly, murine NIH/3T3 fibroblasts permanently transfected with the human fibronectin gene promoter connected to a luciferase reporter vector (pFN LUC) were used [14]. Here, the transfected fibroblasts were cultured in the presence or absence of LTX BALF for 24 hours. Afterwards, the cells were washed, harvested and fibronectin luciferase activity was measured using luciferase assay reagent (Promega, Madison, WI) and a Luminoskan Ascent luminometer (Labsystems, Helsinki, Finland). All samples were run in duplicate, normalized to total protein and results were reported as luciferase activity units/ug protein.

2.3 Statistical Analysis

Data are expressed as means and standard deviations. A statistical difference between the means of groups was determined by the student's t-test. Correlations between dichotomous variables were made utilizing the Spearman's rho coefficient. A 'p' value of <0.05 was considered significant. All analyses were performed using IBM SPSS 22.0 statistical software package (Armonk, NY).

3. Results

Outlined in Table 1 are the demographics for the fifteen LTX recipients who met criteria for study inclusion. Each recipient had between five and nine BALF samples obtained during surveillance bronchoscopies within the year following their transplant. This yielded 101 BALF samples for analysis. Six recipients (represented by 42 of the obtained samples) had at least one BALF 'positive' for bile salts via the bile salt assay thus indicating aspiration of GIM. Even though every BALF sample collected in these six recipients may not have demonstrated a result 'positive' for detection of bile salts, for the purposes of this investigation, these recipients were characterized as those experiencing aspiration. The remaining nine recipients (represented by 59 of the obtained BALF samples) had no 'positive' analyses when assessing for bile salts in the BALF, and were consequently characterized as never having aspirated.

The relative induction of fibronectin expression (determined by luciferase activity) in recipients characterized as having experienced aspiration was 1.05 $\pm$ 0.19 luciferase units/µg protein, and was significantly greater than the 0.92 $\pm$ 0.28 luciferase units/µg protein identified in the samples

from recipients without evidence of aspiration ($p< 0.02$) (Figure 1). Furthermore, this increased fibronectin luciferase activity positively correlated with the presence of one or more episodes of aspiration ($r_s = 0.22$, $p=0.03$).

Table 1 Lung recipient demographics.

Item	Mean/Count	Percent/Range
Gender		
Male	10	66.7%
Female	5	33.3%
Age	56.5 years	25-74 years
Indication for Transplant		
IPF	7	46.7%
COPD	4	26.65%
CF	4	26.65%

Legend: IPF = idiopathic pulmonary fibrosis; COPD = chronic obstructive pulmonary disease; CF = cystic fibrosis

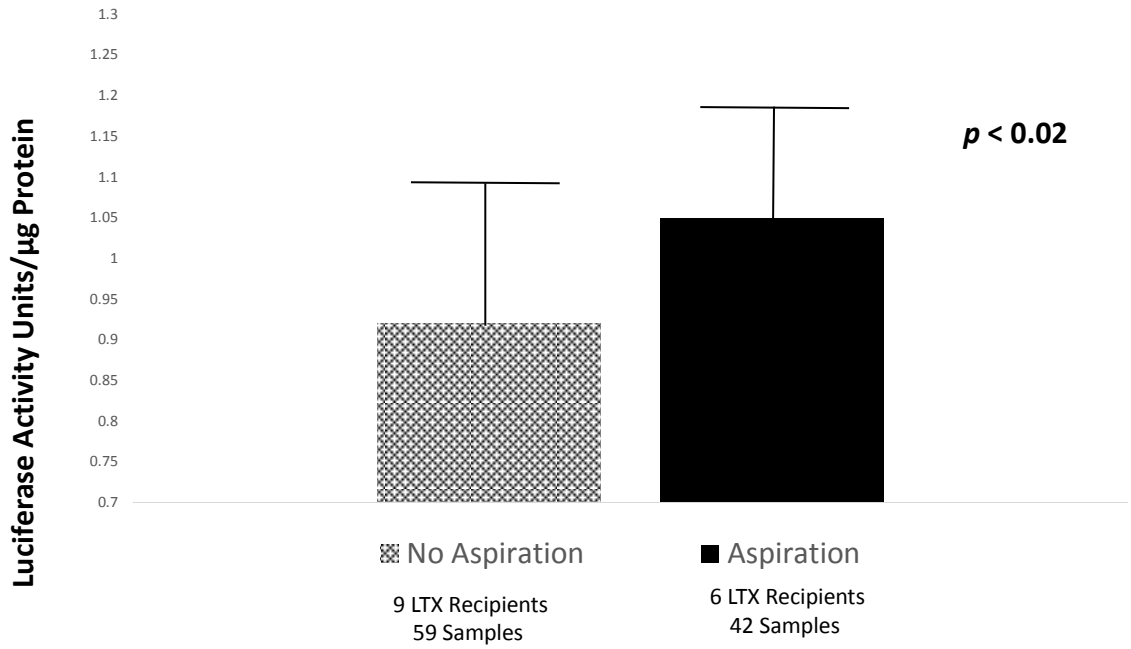

Figure 1 Fibronectin expression via luciferase activity in lung recipients.

Thirteen of the fifteen recipients underwent esophageal pH probe monitoring following their transplant procedure. This esophageal testing occurred at median post-operative day 112 (range 56-305). Eight of these thirteen recipients were interpreted to have pH probe monitoring consistent with acid-reflux. Of these eight recipients only three also had evidence of

accompanying microaspiration via detection of bile salts in their BAL fluid. Of the five recipients who did not have evidence of acid-reflux on esophageal pH probe monitoring, two had bile salts detected in their BALF suggesting some degree of microaspiration.

4. Discussion

The association of GERD with the later development of the BOS phenotype of CLAD is well known and current clinical practice is to be vigilant in its detection in LTX recipients [15]. Both the anatomic manipulations inherent in the transplant surgery and the side effects of necessary immunosuppressive medications required for the health of the allograft have been implicated in the development of both gastroparesis and GERD [13]. Indeed, GERD has been shown to be twice as prevalent in LTX recipients [16-19]. While there is general agreement that once identified GERD should be treated, there is however currently no unanimity regarding the best method of screening this population. Current practices for screening range from relying on only symptomology to performing invasive esophageal studies on all lung recipients. Likewise, there is no unanimity on treatment with some transplant programs employing proton pump inhibitors or other antacid preparations while other programs perform surgical fundoplication procedures to halt the reflux [20]. Much of this variability in practice stems from an uncertainty as to what agent(s) and/or mechanism(s) are responsible for initiating the injury in the lower airway that leads to the obliteration of bronchioles.

While the use of antacid medications is very effective in reducing the symptoms associated with GERD experienced by LTX recipients, there is mounting evidence that the simple control of gastric acid reflux alone may not be sufficient to obviate the inflammatory response in the allograft. One hypothesis posits that the reflux and subsequent aspiration of *particulates* rather than gastric acid may be the primary injurious insult [21]. A second hypothesis proposes that it is the aspiration of the bile salts themselves that directly leads to impairment in the innate immune system of the lung tissue, making it the most threatening element to the health of the allograft [22]. Thus, the implication is that it is not merely the act of GERD, but the consequent microaspiration of GIM that promotes repetitive insults to the airway that then initiates a cascade of inflammation and maladaptive remodeling culminating in the dysfunction of the lung allograft. Recent evidence has accumulated revealing that the examination of BALF may provide the most sensitive/accurate methods for detecting aspiration of non-acid contents into the lung allograft. The assay of this lavage fluid for gastric enzymes and refluxed bile salts has been shown to be both reproducible and definitive for the identification of aspiration resulting from GERD [13, 23, 24]. As such, analysis of this fluid for these aspirated elements and markers of inflammation may provide a ‘window’ through which a better understanding of their effects in the lower airways can be elucidated as well as their potential connection with the development of CLAD.

Although the expression of tissue remodeling genes could have been tested, fibronectin induction and expression was chosen for this investigation as it is triggered early after lung injury. It is expressed in many cell types and is involved in several cellular functions which play an integral part in the early fibroproliferative process involved in airway remodeling including modulation of cellular adhesion, migration and proliferation [25]. Fibronectin fragments are also known to be chemotactic to monocytic cells [26], a process that might help perpetuate inflammation. It is this altered composition of the extra-cellular matrix, which includes fibronectin that has been shown

to be involved in pathological and functional changes reported in many lung diseases including asthma [27], chronic obstructive pulmonary disease [28], idiopathic pulmonary fibrosis [29], and pulmonary arterial hypertension [30]. Because of this proposed role in both acute and chronic lung injury, fibronectin has importance as an early marker of lung disease [31]. Given these findings in other lung disorders, fibronectin induction could be expected to signal early events leading to allograft dysfunction in LTX recipients who experience microaspiration of GIM. In fact, earlier investigation (which did not evaluate aspiration) has reported greater induction of fibronectin expression in the BALF from LTX recipients who would later progress to BOS compared to those who did not develop BOS. These investigators further reported that the induction of fibronectin by BALF correlated with the amount of transforming growth factor-beta (TGF-β), a pro-fibrotic growth factor known to stimulate fibronectin [32]. Thus, aspiration of GIM could induce the production of fibronectin via the upregulation of TGF-β or other agents in lower airway epithelial cells and/or alveolar macrophages. Furthermore, there has been some preliminary evidence that similar pro-fibrotic mechanisms may be involved in the development of other CLAD phenotype – RAS, not only in the pathogenesis of BOS [33-35].

Because fibronectin has been implicated in lung injury and repair, and is considered an early marker of activation of tissue remodeling, this investigation was performed to test if evidence of its induction correlated with markers of aspiration of GIM in LTX recipients. Herein GIM was confirmed via the detection of bile salts in the BALF of six LTX recipients who were sequentially sampled throughout their first post-transplant year. By using the *in vitro* bioassay, increased fibronectin induction was inferred to occur in the lung allografts of these six recipients confirmed to have one or more episodes of GIM aspiration occurring during that first post-transplant year; an amount that was significantly greater than that in contemporaneous samples from the nine recipients who never had identification of bile salts in their sequential BALF samples. Despite these findings, it should be noted that detection of this activity did not faithfully discriminate between recipients with aspiration of GIM and those without. However, its positive correlation with bile salts content in BALF suggests a mechanistic link between aspiration of GIM and activation of tissue remodeling in the lower airway. It is important to emphasize that this investigation did not directly measure fibronectin in the BALF samples, but instead tested the ability of BALF to stimulate fibronectin gene expression in a fibroblast-based bioassay.

The finding that recipients with as few as a single 'positive' bile salt assay still had increased amounts of fibronectin luciferase activity could suggest one of two scenarios. One possibility is that recipients with one or few 'positive' assays in fact experienced many other episodes of aspiration of GIM throughout the first post-transplant year that were simply not identified due to the bronchoscopy sampling schedule (i.e. – a given bronchoscopy and recovery of BALF may have been sufficiently removed from an aspiration episode so as not to have significant bile salts for detection). A second possibility is that these recipients may have indeed experienced only one or a few aspiration events, but even in the absence of multiple episodes this was enough insult to the airway to initiate a continual inflammatory/reparative cascade hallmarked by fibronectin expression.

It is still unclear whether it is the injurious effect of the bile salts themselves which initiates this inflammatory/reparative cascade or whether they simply have a spurious connection to other particulates that may be aspirated at the same instant. Similarly, as previously mentioned, the detection of bile salts might simply be a marker for increased concentrations of other fibronectin-

stimulating agents such as growth factors (e.g. TGF-β) or oxidants. Of course, whether these LTX recipients ultimately develop clinical manifestations of CLAD out of proportion to the other nine recipients remains to be seen and will be the subject of future investigations.

There are several potential limitations to this study that deserve notation. While this investigation has focused on microaspiration of GIM being a potential stimulus to pro-fibrotic inflammation and airway remodeling, it should be noted that the coexistence of other clinical entities also associated with the development of CLAD could have potentially affected airway remodeling and thus confounded the results displayed herein. Among these, are infectious insults to the lung allograft including bacterial and viral infections, both of which have been implicated in the later development of BOS [36-39]. Non-infectious complications such as primary graft dysfunction and high grade acute rejection episodes too have been associated with later allograft dysfunction [40-44]. Unfortunately, as this investigation involved airway samples from only fifteen LTX recipients, it was not possible to correct for all of these variables.

With respect to the samples themselves, several potential confounders that could manifest a sampling error need to be emphasized. First, all of the samples were obtained from very specific anatomic locations in the allografts (i.e. – either the right middle lobe or the lingula of the left lung). Obviously, depending on the amount of aspiration and the postural configuration of the recipient, there could be differences in anatomic distribution of aspirated material that may have been over or under appreciated because of the sampling technique employed. Second, the interval at which BALF was collected (i.e. - via routine scheduled surveillance bronchoscopies) may have been inadequate to detect all of the aspiration episodes. This is particularly germane to the nine recipients who had no detectable bile salts in the BALF of their lung allografts. Given that the maximum number of samples obtained from any one recipient in the first post-transplant year was only nine, there was clearly opportunity to miss detection of episodes of GIM aspiration. Lastly, although over one hundred BALF samples were analyzed, as a single center study, this investigation involved only fifteen transplant recipients. Clearly, samples from a greater number of recipients for confirmatory analysis would be beneficial.

5. Conclusions

In the first year following LTX, recipients who experience microaspiration of GIM demonstrate an association between increased bile salt content and increased activity promoting fibronectin expression in the bronchioalveolar milieu. This suggests the presence of matrix-inducing activity which may reflect maladaptive airway remodeling. The clinical manifestations and consequences of this paradigm remain to be ascertained.

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Author Contributions

Conception and design: Jill Gualdoni, Jeff Ritzenthaler, David Nunley, Jesse Roman. Analysis and interpretation: Jill Gualdoni, Jeff Ritzenthaler, Ibrahim Elkhawas, Gerene Bauldoff, David Nunley,

Jesse Roman. Drafting the manuscript for important intellectual content: Jill Gualdoni, Jeff Ritzenthaler, David Nunley, Jesse Roman. All authors read and approved the final manuscript.

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Competing Interests

The authors have declared that no competing interests exist.

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