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Activin Biology After Lung Transplantation

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Background. Activins A and B, members of the TGF-β superfamily, are produced as part of the physiological response to tissue damage and the resulting proinflammatory response. Given that lung allograft reperfusion results in an inflammatory response, it is likely that the activins and their binding protein follistatin will form part of the regulatory response. There is a need to document the response of these proteins to allograft reperfusion to determine if there is a role for the use of follistatin to control the biological actions of the activins because some of these are potentially damaging. **Methods.** Serum from 48 consecutive patients undergoing lung transplantation (LTx) was collected at 2, 6, 12, and 26 weeks post-LTx. The serum levels of activin A and B and follistatin were measured by enzyme-linked immunosorbent assay and specific radioimmunoassays and compared with clinical events. **Results.** Serum activin A and B levels were at the upper limit of the normal ranges at 2 weeks post-LTx decreasing thereafter to 12 weeks post-LTx ($P < 0.05$). In contrast, serum follistatin levels were unchanged between 2 and 12 weeks, with a late significant increase at 24 week post-LTx ($P < 0.01$). Patients with primary graft dysfunction had lower serum follistatin levels (7.7 vs 9.5 ng/mL; $P = 0.04$) and a higher activin A/follistatin ratio (13.1 vs 10.4; $P = 0.02$) at 2 weeks post-LTx. **Conclusions.** Activin and follistatin levels vary with time form LTX and reflect a proinflammatory environment. Future studies will elucidate associations with chronic lung allograft dysfunction and the therapeutic potential of exogenous follistatin administration.

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Lung transplantation (LTx) has a useful and often lifesaving role in the management of end-stage lung disease. By its very nature however, LTx involves a large surgical procedure on a very ill recipient and long-term outcomes are challenged by the additive burdens of immunosuppressive therapies, infection and rejection. It is apparent that both perioperative and late postoperative complications of LTx can create an inflammatory environment that results in a deterioration of graft function with resultant morbidity and mortality. Although there is no universally agreed definition, the term chronic lung allograft dysfunction (CLAD) has been widely used and this concept includes spirometric and clinical features that describe and quantify this deleterious outcome of LTx.[1,2]

The inflammatory milieu seen after LTx is complex, with the activation of innate-immune and alloimmune pathways.[1] The development of a better understanding of these pathways will allow improved therapeutic targeting to decrease end effects with the potential to diminish CLAD. Activins A and B are members of the transforming growth factor β super-family and their role as key regulators of inflammation and fibrosis is gaining recognition.[3] Activin A promotes inflammation by stimulating production of inflammatory mediators including IL1β, IL6, tumor necrosis factor, and nitric oxide.[3-5] The activins are produced in many organs and tissues, and their biological actions are regulated primarily by follistatin which binds activin A with a very high affinity and targets the complex to a lysosomal degradation pathway.[3-5] Follistatin also binds activin B although with lower affinity than activin A. High activin to follistatin ratio therefore favors a proinflammatory fibrotic state.[3] Activin A and its antagonist follistatin have been recognized as regulatory factors in a number of acute and chronic diseases including cirrhosis,[6] rheumatoid arthritis,[7] chronic kidney disease,[8] cachexia,[9] and several lung diseases.[10-14]

Serum activin A levels have been noted to correlate inversely with lung function and nutritional status in cystic fibrosis (CF) patients.[11] In a CF mouse model, follistatin administration reduced activin levels, mucus hypersecretion, and airway neutrophilia.[11] Activin A has been shown to be associated with increased CD8 T cells and subsequent neutrophilic airway infiltration in asthma and chronic obstructive pulmonary disease (COPD) patients.[12,13,15,16] These conditions share clinical and pathological features with the bronchiolitis obliterans syndrome form of CLAD.[1] Furthermore, activin A has also been associated with neutrophilic infiltration and fibrosis in patients with acute respiratory failure in the intensive care unit. Activin overexpression mouse models show pathological features similar to the restrictive allograft syndrome form of CLAD.[1,10,12]

Our studies have already established that serum activin A, activin B, and follistatin increase acutely during LTx surgery, in part due to the use of heparin and secondly during the reperfusion of the transplanted lung.[17]

The current study aims to explore the longitudinal dynamics of activin A, activin B, and follistatin after clinical LTx, correlating these with clinical outcomes (including, acute rejection, intercurrent infections, and CLAD. Because increased activin A levels are elevated in a large variety of inflammatory and fibrotic conditions, the use of follistatin as an anti-inflammatory and antifibrotic agent has significant therapeutic potential in these disease states.[3,11,12,18]

MATERIALS AND METHODS

Patient Population

Consecutive and locally followed up patients undergoing LTx at the Alfred Hospital between February 2014 and January 2015 were invited to participate in a study to investigate the dynamics of serum activins A and B and follistatin levels during the longer-term follow-up of such patients. The censor date for the study was July 2015, such that all patients had at least 6 months of follow-up post-LTx. All patients provided written consent, and the study was approved by the Alfred Hospital Ethics Committee.

Study Design

Serum levels of activin A, activin B, and follistatin were measured when patients attended for surveillance bronchoscopy. Surveillance bronchoscopy and transbronchial biopsies were performed at 2, 6, 12, 26, 39, 52 weeks post-LTx, and at other times as indicated by clinical circumstances. Additional serum sampling was performed at times of clinical instability if patients had to be admitted to hospital due to complications related to their LTx.

The relationship between cross-sectional and longitudinal levels of activins/follistatin with clinical outcomes was studied. Clinical variables analyzed included primary graft dysfunction (PGD) grade 3,[19] the duration spent in intensive care unit (hours of mechanical ventilation and length of inotropic support), acute cellular rejection,[20] cytomegalovirus (CMV) reactivation (positive CMV PCR in blood or bronchoalveolar lavage [BAL]), allograft infection (positive BAL bacterial or fungal isolate), measured lung function (forced expiratory volume in 1 second and vital capacity), and survival at 6 months post-LTx.[1,2]

Recipient and Donor Selection, Assessment, and Recipient-Donor Matching

Recipient selection was based on National and International Guidelines.[21] Donor assessment routinely considered extended criteria donor lungs for LTx.[22] Donor-recipient matching was undertaken according to our standard protocol which has been described previously.[23,24] Prospective donor-recipient T and B cell lymphocytotoxic crossmatching was performed in all patients,[23,24] and over the last 3 years included additional Luminex (Luminex Corp, Austin, Texas) testing to identify anti-HLA donor-specific antibodies.

Lung Procurement, Preservation, and Transplantation

Donor lung retrieval was performed according to standard practice.[24,25] Preservation was initiated with Perfadex (Vitrolife, Goteborg, Sweden), which includes 25 000 units of sodium heparin. Intraoperatively patients also received boluses of 2500 to 5000 units of heparin to maintain activated clotting times at twice baseline with an average dose of 5000 units per patient.

Postoperative Management

A postoperative fluid management guideline was used, encompassing both respiratory and cardiovascular management algorithms, targeting a central venous pressure less than 7 mm Hg, where mean arterial pressure and cardiac index permitted.[26] The utilization of inhaled nitric oxide and extracorporeal membrane oxygenation (ECMO) as specific therapies for PGD followed standard practice.[27,28] All LTx recipients received standard triple immunosuppression with tacrolimus, azathioprine, or mycophenolate mofetil and corticosteroids.[20] All patients received prophylactic antibiotics based on known or suspected donor and recipient microbiology results. All patients at high risk of CMV reactivation (either donor- or

TABLE 1.

Demographics

Characteristics	
Age (years)	55.4 ± 12.9 (23–70)
Male sex	30 (62.5)
Indication for LTx	
Chronic obstructive lung disease	24 (50.0)
Pulmonary fibrosis	11 (22.9)
CF	7 (14.6)
Pulmonary arterial hypertension	4 (8.3)
Bronchiectasis	2 (4.2)
Type of transplant	
Bilateral sequential lung transplant	47 (98)
Single lung transplant	1 (2.1)
Graft Ischemic time (mean, minutes)	268 + 78 (112–505)
Use of cardiopulmonary bypass	7 (14.5)
Duration intubated, h	36.5 ± 37 (5–166)
PaO$_2$/FiO$_2$ ratio at 24 h	325.5 ± 98.5 (138–571)
Hospital days posttransplant	22.8 ± 10.4 (11–63)
Cytomegalovirus status	
Donor positive/recipient negative	12
Donor positive/recipient positive	15
Donor negative/recipient positive	12
Donor negative/recipient negative	9

Continuous data are shown as mean ± standard deviation (range) and categorical.

recipient-positive CMV serostatus) received prophylaxis with 2 weeks of intravenous ganciclovir and then oral valganciclovir for a minimum of 5 months.

Definitions

Acute rejection was diagnosed by histopathological changes identified on transbronchial biopsy specimens, and treated with daily intravenous corticosteroid for 3 days, if grade 2 or greater rejection was detected.[20] CLAD was defined, diagnosed, and treated according to standard protocols and practice.[1,2,20]

Immunoassays

Blood samples were drawn before initiation of bronchoscopy and, after clotting, serum was separated and stored frozen until analyzed. Total serum activin A, activin B, and follistatin levels were measured by enzyme-linked immunosorbent assay and by specific radioimmunoassay, respectively.[10] Normal ranges were provided from previous studies based on samples from 138 normal controls.[10,29] Reference values were calculated from the 95% confidence intervals of these normal controls.

Statistics

Data are expressed as means unless otherwise stated. Comparisons were made between groups using the Pearson χ^2 test for categorical variables, the unpaired Student t test for parametric continuous data, the Mann-Whitney test for nonparametric continuous data and Kaplan-Meier method of survival analysis. Differences in survival were compared by log rank (SPSS, Statistics, Version 17.0). A P value less than 0.05 was considered significant.

RESULTS

Study Cohort

The study cohort consisted of 48 patients who underwent LTx between February 2014 and December 2014 and were available for long-term follow-up. The demographics of the study cohort are shown in Table 1. One patient was bridged to transplant on ECMO, whereas 2 patients with pulmonary arterial hypertension were electively placed on ECMO perioperatively.

Longitudinal Dynamics of the Secretion of Activin A and B After LTx

The levels of serum activin A and activin B decreased with time from transplant. However, over the same time, serum follistatin levels remained mostly unchanged with a significant increase at the 6-month time point (Figure 1). The serum activin A levels at 2 weeks posttransplant (152.6 ± 45.1 pg/mL) were significantly higher than those measured at 6 weeks (138.1 ± 40.9 pg/mL; $P = 0.02$) and at 6 months posttransplant (125.6 ± 37.8 pg/mL; $P < 0.001$). Likewise, levels of serum activin B at 2 weeks posttransplant (91.0 ± 41.6 pg/mL) were significantly higher compared with levels at 6 weeks (70.0 ± 28.9 pg/mL; $P > 0.001$) and 6 months posttransplant (60.8 ± 19.2 pg/mL; $P > 0.0001$). In contrast, mean follistatin levels were unchanged during the initial early posttransplant period, but were higher at 6 months posttransplant (8.7 ng/mL vs 10.8 ng/mL; $P < 0.001$).

Compared with the normal reference ranges (activin A, 110 ± 41 pg/mL; activin B, 70 ± 20 ng/mL; follistatin, 12.6 ± 0.4 ng/mL),[10,28] activin A levels were higher and follistatin levels were lower, especially during the early posttransplant period. The ratio of activin A/follistatin and of activin B/follistatin, markers of the bioactivity of the activins, decreased with time from transplant
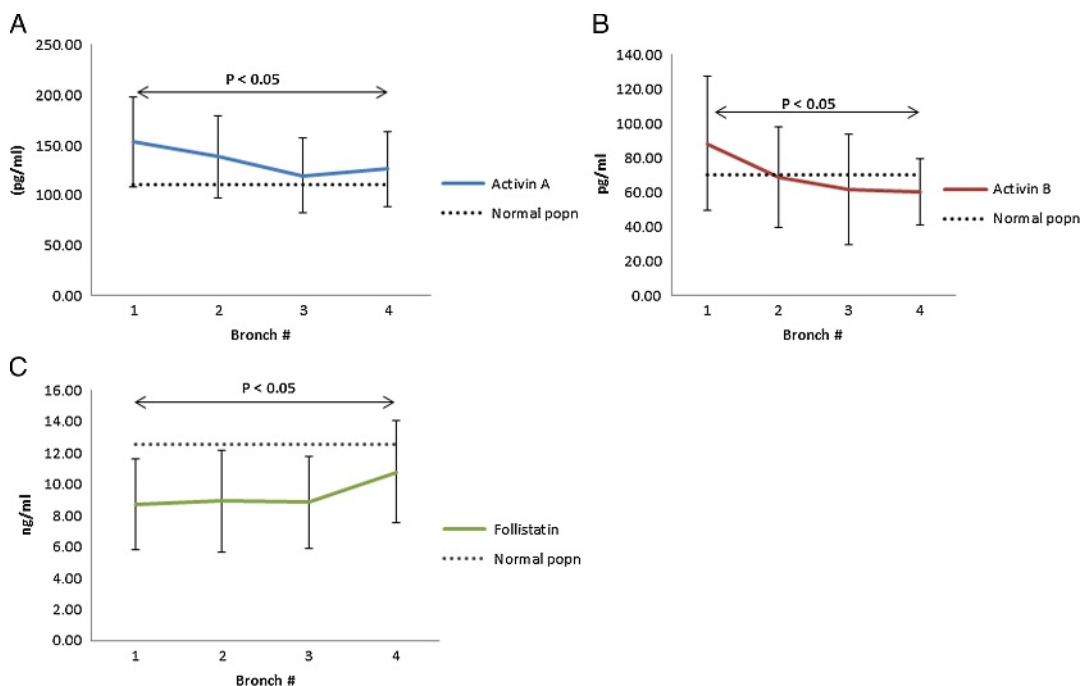

FIGURE 1. Longitudinal changes in mean serum levels of (A) activin A, (B) activin B and (C) follistatin measured at 2 weeks (bronchoscopy 1), 6 weeks (bronchoscopy 2), 3 months (bronchoscopy 3) and 6 months (bronchoscopy 4) postlung transplant. Comparative normal values (———).[28]
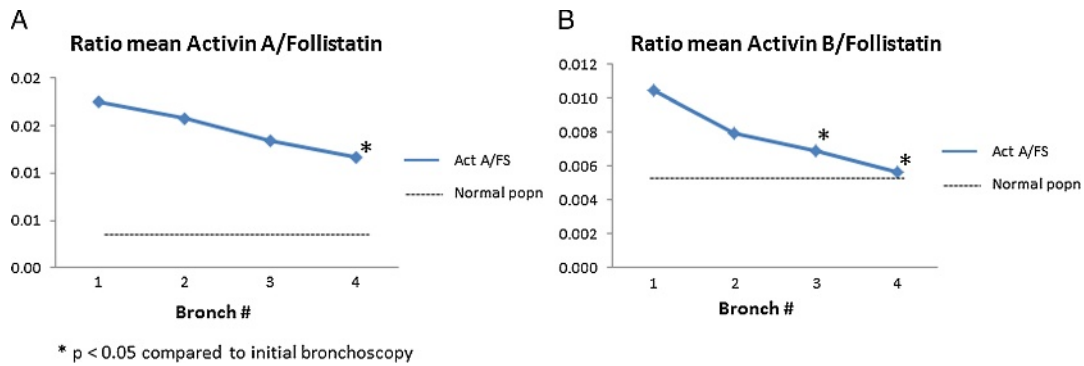
FIGURE 2. Longitudinal changes in (A) activin A/follistatin and (B) activin B/follistatin ratio after LTx.

(Figure 2), indicating a decrease in proinflammatory markers. The levels of serum activin A, activin B, or follistatin did not differ per the underlying lung disease leading into transplant (data not shown).

Primary Graft Dysfunction Associated With Proinflammatory Profile

Twenty patients had evidence of PGD as defined by a PaO_2/FiO_2 ratio < 300 at 24 hours posttransplant (PGD grade 3, n = 7; PGD grade 2, n = 13), none of whom required ECMO support. Compared to the rest of the cohort, poor initial gas exchange was associated with lower levels of follistatin (7.7 vs 9.5 ng/mL; $P = 0.04$) and an elevated activin A/follistatin ratio 13.1 vs 10.4; $P = 0.02$). Time of intubation, ECMO and or cardiopulmonary bypass use, renal function, length of stay in the ICU or total length of stay did not impact on serum activin levels when assessed at 2 weeks' posttransplant (data not shown).

Clinical Outcomes

Six-month survival for this cohort of 48 LTx recipients was 100%. Clinical outcomes in the 1st 6 months post-LTx are shown in Table 2. CMV reactivation was rarely seen during this period of CMV prophylaxis. Treated acute rejection (≥A2) was seen in 8 patients (17%). Respiratory virus screening by PCR was only performed if patients presented with viral symptoms and was positive in 13/48 LTx recipients (27%). BAL cultures were positive (typically *Pseudomonas* and *Aspergillus* spp identified) in 27/48 patients (56%). Activin B levels tended to be higher in patients with positive BAL culture with the difference being statistically significant at the 6-week post-LTx bronchoscopy (59.2 pg/mL vs 78.4 pg/mL; $P = 0.02$) (Figure 3). Serum activin A levels were not significantly different in patients undergoing acute cellular rejection compared with those with no rejection (142.7 pg/mL vs 131.6 pg/mL; $P = 0.4$), or in those presenting with upper respiratory tract viral infections (133.8 pg/mL vs 133.2 pg/mL; $P = 0.95$).

DISCUSSION

In this study, we have extended our previous observations of the response of activins A and B after LTx.[17] The data demonstrates that while serum levels of activins A and B remain elevated above the normal ranges for up to 2 weeks post-LTx, in contrast, follistatin levels remain below the normal range for up to 6 months. This is surprising since higher activin A levels stimulate follistatin. The increase in serum

activins A and B levels in response to transplantation is consistent with our previously published data that these proteins are elevated in association with inflammatory responses such that occur during reperfusion of a transplanted organ[17] and in patients in intensive with acute respiratory failure.[10] Further data from our studies of short duration vascular interruption of the renal circulation in mice are associated with renal injury which can be decreased significantly if the mice were treated with follistatin.[30] This is consistent with follistatin's capacity to decrease the inflammatory response induced by the rapid elevation of activins A and B levels.

The activin A/follistatin and activin B/follistatin ratios also increase during this perioperative period returning to normal values within 24 hours.[30] The increase in activin levels is also likely to result from the use of heparin since it is well established that the negatively charged heparin displaces follistatin bound to cell surfaces.[13]

Of interest, patients with PGD demonstrated a significantly higher activin A/follistatin ratio up to 2 weeks posttransplant compared with LTx patients demonstrating normal initial graft function. Although 20 patients had PGD, only 7 patients had severe PGD grade 3, with no observable difference seen in active or follistatin levels relating to PGD severity. The persistence of this proinflammatory profile is not unsurprising given that it is recognized that PGD impacts on 30-day mortality[19] and the later development of chronic rejection.[31] Extended follow-up of the current cohort will examine the relationship between proinflammatory activin profiles and the subsequent development of CLAD and its 2 major phenotypes, the bronchiolitis obliterans syndrome and restrictive allograft syndrome. CLAD is the "sum of the parts" but has been assumed to be primarily a chronic rejection alloimmune response. The current study supports

TABLE 2.

Six-month clinical outcomes

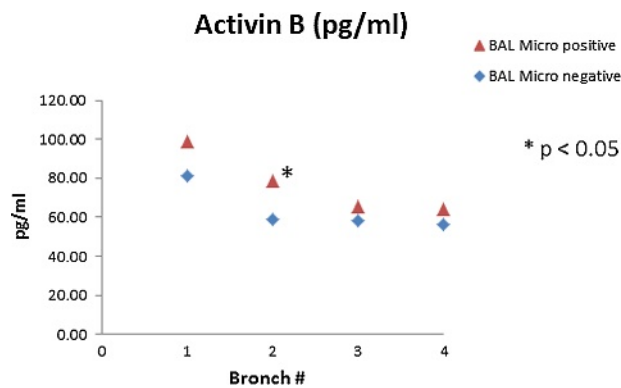| Survival	48 (100%)
Treated acute rejection (≥A2)	8 (17%)
CMV reactivation (viral load > 150 copies)	1 (2%)
Respiratory virus PCR positive (throat swab)	13 (27%)
BAL culture positive	27 (56%)
Aspergillus spp (n = 11)	
Pseudomonas sp (n = 9)	
Other bacteria (n = 16)	

Activin B (pg/ml)

FIGURE 3. Longitudinal changes in the levels of serum activin B in lung transplant patients with positive microbiology in the BAL compared with those without positive microbiological isolates.

the concept that innate immune responses may also be significant drivers of pathological scarring after LTx.

Levels of follistatin were significantly lower than those reported for normal controls[10] at all-time points. Thus, the ratio of activins A and B to follistatin was elevated, suggesting a proinflammatory phenotype and an environment that may contribute to the later development of CLAD. Similar profiles of high activin and low follistatin have been reported in COPD and human bronchial epithelial cells exposed to cigarette smoke.[15] These data raise the possibility that in patients with higher activin to follistatin ratios may benefit from the use of follistatin to diminish the "proinflammatory environment" and decrease the frequency of CLAD.

Furthermore, we could demonstrate that activin B levels were elevated in patients who had positive culture results in the BAL. Neutrophilic infiltration into the lung allograft is a precursor of CLAD, and both airway infection and the elevated activin levels may be contributors to neutrophilic inflammation, as has been described in asthma[12,13] and COPD.[15,16]

Limitations of our study include the relatively small number of subjects studied, and the short 6-month duration of follow up since CLAD typically occurs years after LTx. Multivariate analyses for other recipient, donor and perioperative factors that might influence results including age, sex, body mass index, muscle mass, and underlying disease and the use of heparoids could be considered in future analyses of larger studies. The impact of the trauma of surgery could also be compared by studying, for example, patients having a thoracotomy for lung resection.

In conclusion, activin and follistatin remain mediators of interest in LTx. Longitudinal studies are now required to link these to late LTx clinical outcomes. Theoretically, in future studies, follistatin or other activin antagonists could be administered during the peri- and post-LTx period to abrogate a typically progressive path to CLAD.

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