A Comparison of Intra-Pulmonary Profibrotic Chemokine Levels in Lung Transplant Recipients With and Without Chronic Lung Rejection

David Ferris

A. Study Purpose and Rationale

Chronic lung rejection (CLR), characterized histologically by bronchiolitis obliterans (BO) or physiologically by brochiolitis obliterans syndrome (BOS), is the major factor limiting long term survival after lung transplantation. Almost one third of lung transplant recipients will develop CLR during the first year after surgery, while more than half of patients will progress to CLR within the first two years.[1] Histologically, CLR is defined by patchy scar formation and fibrosis of the small airways, often accompanied by intimal thickening and sclerosis of associated blood vessels. The pathologic lesions consist of an organized inflammatory response. Mature collagen deposits fill or partially fill the bronchioles, and are associated with proliferating fibroblasts, extracellular matrix, and a chronic, mononuclear cell infiltrate.[2]

The pathogenesis of CLR is still hypothetical, but is currently conceptualized as a "response to injury" phenomenon.[3] In this postulated sequence of events, both immunologic and non-specific injury stimulate a stereotyped repair response involving the proliferation and accumulation of smooth muscle cells and fibroblasts and the release of a molecular cascade of fibroproliferative mediators. This initial response to injury then promotes further immune recognition, making additional immunologic injury more likely, until the process becomes self-propagating. The details of what drives this process forward, until the endpoint of CLR is reached, have not yet been fully elucidated.

One critical mediator of fibrogenesis is transforming growth factor-P (TGF- β). Produced by fibroblasts and activated alveolar macrophages, this cytokine induces fibroblast activation and proliferation, as well as collagen and extracellular matrix deposition in the lung.[4] TGF-β increases the transcription of fibronectin and procollagen genes and downregulates the transcription of genes for collagenases and proteases.[5] One small study measuring gene expression of profibrotic mediators in lung transplant recipients showed a trend toward higher levels of TGF-β m-RNA in brochoalveolar lavage (BAL) cells from subjects with CLR compared with subjects without CLR.[6] In another study of TGF-β in lung transplant patients, subjects who went on to CLR were shown to have marked peaks of alveolar

[1] Reichenspurner H, Girgis RE, Robbins RC, et al. Stanford experience with obliterative bronchiolitis after lung and heart-lung transplantation. Ann Tliorac Surg 1996; 62: 1467-72.

[2] Boehler A, Kesten S, Weder W, et al. Bronchiolitis obliterans after lung transplantation. Chest 1998; 114: 1411-26.

[3] Halloran PF, Homik J, Goes N, et al. The "injury response": a concept linking nonspecific injury, acute rejection, and long-tem transplant outcomes. Transplant Proc 1997; 29: 79-81

[4] Roberts ABI McCune BK, Sporn NIB. TGF-P: regulation of extracellular matrix. Kidney Int 1992; 41: 557

[5] Pelton RW, Moses HL. The beta-type transforming growth factor. Mediatiors of cell regulation in the lung. Am Rev Respir Dis D90; 142: S31-35.

[6] Bergmann M, Tiroke A, Schafer H, et al. Gene expression of profibrotic mediators in bronchiolitis obliterans syndrome after lung transplantation. Scand Cardiovasc J 1998-,32: 97-103.

macrophage TGF-β m-RNA expression compared to subjects who did not progress to CLR.[7] Although TGF-β is one of the most potent profibrotic cytokines yet described, its use as a marker for the development of CLR is limited.[8] In addition, for a number of technical reasons, targeting TGF-P in order to interfere with fibrosis has been found to be "fraught with serious problems."[9]

A much more promising group of proteins with a crucial role in the inflammatory process is the chemokine family. Easily measurable in BAL samples by ELISA, chemokines are small proteins with chernotactic properties responsible for the recruitment of inflammatory cells into areas of inflammation. Chemokines work by binding to receptors on leukocytes and tissue cells which are coupled to GTP-binding proteins. Most receptors recognize more than one type of chemokine and several chemokines bind to more than one receptor.[10] A number of therapeutic strategies designed to take advantage ofthe interaction between chemokines and their receptors have been developed. These include interference with chemokine/receptor binding utilizing receptor blocking antibodies and disruption of the intracellular signaling process through the use of receptor antagonists that bind to the chemokine receptor but fail to initiate a signal. Both of these strategies have been implemented in animal models.[11]

Chemokines have recently been an area of active investigation in lung transplantation. For example, one study has shown that neutrophilia in BAL samples from patients with CLR correlates with higher levels of the chemokine IL-8.[12] Another study has demonstrated that alveolar macrophage production of the chemokine RANTES (regulated upon activation normally T expressed and secreted) is increased during both acute rejection (AR) and CMV pneumonitis.[13] Furthermore, in a rat model of lung allograft rejection, neutralization of RANTES with anti-RANTES antibodies has been shown to attenuate mononuclear cell recruitment during episodes of AR.[14]

Numerous clinical studies have demonstrated increased intrapulmonary chemokine levels during various stages of fibrotic lung disease.[15] Fibroblasts have been shown to secrete a number of chemokines, including MCP-1, MIP-lα, and RANTES, and to express a variety of chernokine receptors following cytokine activation.[16] The chemokine MCP- I has been shown to contribute to collagen production by

---

[7] Charpin JM, Valcke J, Kettaneh L, et al. Peaks of transforming growth factor-P mRNA in alveolar cells of lung transplant recipients as an early marker of chronic rejection. Transplantation 1998; 65: 752-755.

[8] Bergmann M, et al.

[9] Hogaboam CM, Steinhauser ML, Chensue Sw, et al. Novel roles for chemokines and fibroblasts in interstitial fibrosis. Kidney International 1998; 54: 2152-2159.

[10] Baggiolini M. Chemokines and leukocyte traffic. Nature 1998; 392: 565-568.

[11] Strieter RM, et al. "The good, the bad, and the ugly": the role of chemokines in models of human disease. J. Imimol 1996; 156: 3583-3586.

[12] DiGiovine B, Lynch JP 3', Martinez FJ, et al. Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. J Immunol 1996; 157: 41944202.

[13] Gianpaola M, Magnam A, Fattal M, et al. Intrapulmonary production of RANTES during rejection and CMV pneurnonitis after lung transplantation. Transplantation 1996; 61: 1757-1762.

[14] Belperio JA, Chen B, Xue YY, et al. Lung transplantation aIlograft rejection is attenuated by in vivo neutraliztion of RANTES. ALA/ATS 1999; Poster: 311 (Abstract).

[15] Hogaboarn CM, et al.

[16] Lukacs NW, Kunkel SL, Allen R, et al. Stimulus and cell-specific expression of C-X-C and C-C chemokines by pulmonary stromal cell populations. Am J Physiol 1995; 268: L856-861.

fibroblasts. MCP-I activated fibroblasts increase TGF- β production.[17] In addition, TGF-β has been shown to alter the pattern of chemokine receptor expression on fibroblasts and other inflammatory cells, downregulating a number of chemokine receptors and upregulating others, including the receptor CCR4 which binds the chemokines MCP-1, MIP-1α, and RANTES. [18]

In an attempt to elucidate a potential driving force underlying the "response to injury" model of CLR, we will measure levels of profibrotic chemokines in serial BAL samples from lung transplant recipients with and without CLR. We expect to find higher post-transplant levels of MCP-1, MIP-1α, and RANTES in patients with CLR. There may higher chemokine levels at different points leading up to the development of CLR and there may be a rise in chernokine levels that precedes the development of CLR. Elevated levels of these chemokines, measured with a simple ELISA test, could potentially serve as predictive markers for the development of CLR. Future therapies might focus on interfering with chemokine/receptor interactions with the goal of slowing the course of this devastating disease.

B. Study Design

This retrospective, case-control study will seek to correlate the development of CLR with elevated levels of the chemokines MCP-1, MIP-1α, and RANTES in BAL samples. The case group will include those subjects meeting criteria for CLR as defined below. The control group will include those patients who remained free of CLR-defining criteria two years after lung transplantation. Chemokine levels in BAL specimens obtained after lung transplantation and up to the development of CLR in the case group will be compared to chemokine levels in BAL specimens obtained over an identical follow-up period after lung transplantation in matched controls. CLR will be defined both histologically and physiologically. Subjects with one or more transbronchial biopsy specimens showing histologic evidence of BO, defined as scarring and fibrosis of small peripheral airways, as determined by a pathologist and noted in the patient's medical record, will be included in the CLR group. Inaddition, those subjects with serial pulmonary function tests (PFTs) demonstrating stage 2 or 3 BOS, defined as a decline in FEV I values to 65% or less of the baseline post-transplantation value, in the absense of acute rejection and infection, as recorded in the patient's medical record, will also be included in the CLR group.

The lung transplantation program at New York-Presbyterian Hospital's Columbia-Presbyterian Campus performs an average of 10 procedures per year. This will provide about 70 potential subjects for the 7 year period of the study, as described below. Slightly more than half of these patients should survive and follow-up appropriately for two years after transplantation. About half of this group should develop CLR within 2 years, thus providing an estimated 15 to 20 patients each for both the case and the control group.

C. Study Subjects

Study participants will include all patients who underwent single or bilateral lung transplantation at the Columbia-Presbyterian Campus of New York-Presbyterian Hospital between June 1990 and June 1997. Study eligibility will be limited to those patients who followed up as described below and who survived for two years after surgery, with the exception of patients with documented CLR who expired before two years. These patients will be included in the study as members of the CLR group. Patients who

---

[17] Gharaee-Kennani M, Denholin EM, Phan SH. Costimulation of fibroblast collagen and tramforming growth factor beta-I gene expression by monocyte chemoattractant protein-I via specific receptors. J Biol Chem 1996; 271: 17779-17784.

[18] Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming, and Th1/Th2-mediated responses. Immunology Today 1999; 19: 568-574.

underwent intrapulmonary stenting procedures, which could interfere with the ability to diagnose CLR on clincal grounds, will be excluded. The study will also exclude those patients with concomitant heart transplantation. Acceptable follow-up will not differ from standard clinical care. The study will include those patients who, as part of a routine clinical surveillance protocol, underwent serial pulmonary function testing (PFT) and regular, periodic bronchoscopies with BAL and transbronchial biopsy for two years after surgery. These evaluations were carried out on a preset schedule of every 2 weeks for the first 2 months after surgery, followed by every month for the next 4 months, then every 3 months for 6 months, and finally at 6 month intervals, for a total of 12 routine testing points for each patient who continued to follow-up for two years after transplantation. Additional testing was carried out as indicated on clinical grounds. For all patients included in the study, the results of the above tests, as well as basic clinical information, were recorded in the patients' medical record and were obtainable for review. BAL specimens obtained from each patient at the time of bronchoscopy were filtered through a single layer of cotton gauze and then centrifuged at 1000 RPM for 10 minutes at 4 degrees C. Supernatants were stored at - 80 degrees C. All eligible subjects gave written informed consent prior to all procedures and previously agreed to allow the use of their tissue as well as access to their medical records for research purposes.

D. Study Procedures

A chart review will be conducted in order to extract and record demographic data and basic clinical information on each subject, including age at time of transplantation, gender, type of lung transplantation (single lung or bilateral lung), and clinical indication for lung transplantation: chronic obstructive lung disease associated with cigarette smoking, other obstructive lung diseases including cystic fibrosis, interstitial lung disease including idiopathic pulmonary fibrosis, and pulmonary hypertension. For each BAL sample to be analyzed, culture results, transbronchial biopsy results, and PFTs from the date of BAL sample collection will be reviewed in order to document the presence of acute rejection, histologic grade 2 or above; infection with CMV, PCP, other viral, or bacterial pneumonia; or CLR by either histologic or physiologic criteria. The elapsed time from the date of transplantation to the date of documented CLR will also be recorded in order to faditate appropriate case-control matching. Based on the results of this record review, patients wiII be divided into case and control groups as described above. Levels of MCP- 1, MIP- I a, and RANTES in BAL samples will be quantified by ELI SA using standard kits. Expected control group chernokine levels, estimated from prior studies, and displayed as mean +/- SD, are as follows: MCP- 1: 2000 +/- 1000 pg/n-d; MIPI a: 50 +/- 25 pg/ml; RANTES: 100 +/- 50 pg/ml.

E. Statistical Analysis

Controls will be paired with cases based on time from transplantation to development of outcome. The area under the curve for each chemokine from all available BAL samples obtained up to and including the development of outcome will be compared between groups using a two-tailed ttest. We will also compare chemokine levels at baseline after lung transplantation and at the time of development of outcome, and compare the change over time between the two groups. In addition, an analysis of co-variance will be utilized to account for possible confounders, including the presence of grade 2 or higher acute rejection and infection with CMV, PCP, other viral organisms, and bacterial pneumonia, all of which have been associated in prior studies with the development of CLR. With an estimated 20 subjects in the case group and 20 matched controls, at 80% power, a two-tailed t-test comparison would detect a significant difference (a = 0.05) of .90 standard deviations for each chemokine. Based on the estimated baseline chemokine levels and standard deviations noted above, the study will therefore have an 80% chance to detect a 45% difference between groups for all three cytokines, ie MCP- I - 2000 vs. 2900, MIP- I a - 50 vs. 72.5, RANTES - 100 vs 145.

F. Confidentiality of Study Data

The identity of all subjects will be kept strictly confidential. A unique code number will be used for all study subjects for the purpose of data collection and analysis. Data will be stored in a secure location, accessible only to the investigators.

G. Other

None of the other sections of the IRB Protocol Sample Format apply to this retrospective, case-control study.