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The Journal of Heart and Lung Transplantation

http://www.jhltonline.org

Altered progenitor cell and cytokine profiles in bronchiolitis obliterans syndrome

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KEYWORDS:
rejection;
fibrocytes;
cytokines;
lung;
transplantation;
progenitor cells

BACKGROUND: Bone marrow–derived progenitor cells may play a key role in both lung repair and in fibrogenesis. The contribution of $CD45^+$collagen-1^+ fibrocytes to fibrosis has been documented elsewhere and recently identified epithelial-like progenitor cells marked by Clara cell secretory protein ($CCSP^+$) may be protective after lung injury. Interplay between these populations has not yet been studied in bronchiolitis obliterans syndrome (BOS) post–lung transplant.

METHODS: In a cross-sectional design, blood samples were analyzed for $CCSP^+$ cells and $CD45^+$collagen-1^+ fibrocytes by flow cytometry. Plasma cytokines were analyzed by multiplex array.

RESULTS: A higher proportion of circulating fibrocytes was measured in patients with BOS Grade $\geq$1 than in those with BOS Grade 0(p). In parallel, a lower proportion of $CCSP^+$ cells was found in BOS $\geq$1 patients compared with BOS 0(p) and non-transplant controls, resulting in an altered cell ratio between the groups. A higher ratio of $CD45^+$collagen-1^+ to $CCSP^+$ cells was associated with greater airflow limitation based on FEV_1 and FEV_1/FVC ratio. No relationship between cell profiles and time post-transplant was found. Plasma analysis showed an increase in key stem cell and inflammatory cytokines in both groups post-transplant, whereas stromal-derived factor-1 and vascular endothelial growth factor were increased in cases of BOS $\geq$1 specifically. Plasma stromal-derived factor-1 levels also correlated with fibrocytes post-transplant.

CONCLUSIONS: Overall, altered progenitor cell profiles were found in patients who developed advanced BOS, which may be mediated by alterations in circulating cytokines. Ultimately, measurement of progenitor cell profiles may lead to further insight into the pathogenesis of airflow obstruction after lung transplantation.

J Heart Lung Transplant 2012;31:222–8

Lung transplantation is the only therapeutic option for patients with end-stage lung diseases such as pulmonary fibrosis, emphysema and cystic fibrosis. The current lack of effective therapy for these diseases places an enormous burden on our health-care system and an uncertain outcome for these patients and their families. Bronchiolitis obliterans syndrome (BOS) after lung transplantation continues to be a serious problem. Due to high rates of BOS, average 5-year survival of lung recipients is reported to be 55%, the worst of all solid-organ transplants except small bowel.[1] The course of bronchiolitis obliterans is progressive and unresponsive to treatment, with approximately 50% and 75% of patients developing BOS by 5 and 10 years after transplantation, respectively.[2] Although much attention has been focused on controlling alloimmune-mediated damage, reparative and fibrotic processes also participate in the pathophysiology of this dysfunction. Currently, BOS is defined as irreversible, obstructive changes in pulmonary function tests.[3] Biomarkers that might antedate the irreversible manifestations of this syndrome are part of an active area of current research.

Circulating fibrocytes were first identified as an infiltrating cell population in murine wound chambers,[4] and are routinely defined by CD45 expression and intracellular collagen-1. Once recruited to sites of injury, fibrocytes differentiate in response to transforming growth factor-beta (TGF-β) to adopt an activated phenotype, expressing collagen-1 as well as CC chemo-

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1053-2498/$ -see front matter
doi:10.1016/j.healun.2011.11.012

kine receptor 3 (CCR3), CCR5, CCR7 and CXC chemokine receptor 4 (CXCR4),[5] and have been shown to adopt a myofibroblast phenotype expressing α-smooth muscle actin in vitro[5] and in vivo.[6] Fibrocytes have been studied in the pathology of pulmonary fibrosis, linking the recruitment of circulating fibrocytes to fibrotic foci development and matrix deposition.[7–9] Increased fibrocyte numbers have been shown to be an independent predictor of mortality in pulmonary fibrosis patients.[10] Fibrocytes have also been implicated in obliterative bronchiolitis after lung transplant. Studies of bronchoalveolar lavage fluid (BALF) cells from human lung allograft recipients have demonstrated the presence of a fibrocyte-like population resident in the lung,[11] whereas others have reported that bronchiolitis obliterans lesions of lung transplant patients contain considerable numbers of recipient-derived fibroblasts,[12] suggesting multiple cell sources may contribute to the fibrogenesis. In addition, tissue sections from lung transplant patients with obliterative bronchiolitis have been shown to have significantly increased numbers of fibrocytes identified by CXCR4/prolyl4-hydroxylase.[13] It was very recently shown that an increase in circulating fibrocyte numbers can be measured in BOS patients after lung transplant,[14] an observation that supports the results to be presented herein.

A novel, recently identified epithelial progenitor cell marked by Clara cell secretory protein (CCSP) expression may also be important for lung tissue homeostasis and regeneration after injury.[15] Increased levels of $CCSP^+$ progenitor cells in both the peripheral blood and bone marrow have been found in mouse models of injury.[15]

Biomarkers to assist in diagnosis of BOS or to further characterize disease development are not currently in clinical use, but are being explored. For example, the level of CCSP, a marker of the bronchiolar epithelium, has been shown to be significantly lower in BOS compared with acute rejection or no rejection. After the first month postoperatively, serum and bronchoalveolar lavage (BAL) CCSP levels were lower in the patients who developed BOS compared with those who did not.[16] Changes in circulating cell populations may be a biomarker that could predict or pre-date conventional changes in spirometric tests in BOS patients, providing a useful tool to monitor graft function.

Using a cross-sectional design, in this study we aimed to quantify the number of circulating $CCSP^+$ epithelial-like progenitor cells and $CD45^+collagen\text{-}1^+$ fibrocytes in patients after lung transplant to determine whether there is an association between BOS status and these combined cell profiles. In addition, plasma cytokine levels were quantified with the aim of identifying key alterations in these cell-recruitment mediators.

Methods

Patient recruitment

Our study population consisted of a cross-section of post-transplant patients. Written consent for participation was obtained during regularly scheduled clinic visits or at the time of transplant re-listing. Inclusion criteria included >1 year and <15 years post–lung transplant and exclusion criteria included acute rejection or infections, as determined by the overall clinical judgment of the responsible physician. In our program, biopsies are only routinely performed beyond the first year at 18 and 24 months after transplantation. Normal control samples were collected from healthy volunteers who were informed of the research project and were not compensated for their participation.

This study was approved by the research ethics board of the University Health Network (Study 07-0598TE).

Sample preparation

Peripheral blood samples (10 ml) were collected in heparinized vacutainers. Equal parts of the blood sample were prepared by Ficoll isolation to obtain the peripheral blood mononuclear cell (PBMC) fraction and by high-speed centrifugation to obtain all peripheral blood leukocytes (PBLs). PBLs were further treated with red cell lysis buffer for 20 minutes prior to analysis. Plasma was collected from the centrifuged aliquot and stored at $-80°C$ until further analysis.

Flow cytometry

Freshly isolated PBMCs were blocked with 10% goat serum and 10% Fc Block (Miltenyi Biotech), then stained with rabbit anti-mouse/human CCSP (1:500; Upstate Labs) or IgG control antibody (R&D Systems), followed by AlexaFluor 488 secondary antibody (1:1,000; Invitrogen).

For fibrocyte staining, samples were blocked as noted earlier, then incubated with surface antibody for CD45 (1:5; BD Biosciences). Cells were then treated with Cytoperm solution (BD Biosciences) and subsequently maintained in Cytoperm/Cytowash solution. Permeabilized cells were stained rabbit–anti-human collagen type 1 primary antibody (1:100; Rockland Immunochemicals) or rabbit IgG isotype control antibody (R&D Systems), followed by goat–anti-rabbit AlexaFluor 488 secondary antibody (1:1,000; Invitrogen).

All samples were analyzed using a 2-laser, 5-color analyzer (FC500; Coulter Cytomics) and data were analyzed using FLOWJO software.

Cytokine arrays

Plasma cytokines were analyzed using a Luminex-based multiplex array (BioPlex System; Bio-Rad). A total of 17 cytokines were analyzed in parallel by two simultaneous multiplex assays using Group 1 and Group 2 bead sets, as defined by the manufacturer. All procedures were performed according to the manufacturer's protocols. Briefly, samples were thawed to 4°C and 1 volume of plasma sample was diluted with 3 volumes of assay diluent. A master mix of the coupled magnetic beads was prepared from the stocks provided, immediately prior to use. The BioPlex Pro II wash station with pre-loaded magnetic programs for the BioPlex Pro flat-bottom filter plates was used. The assay was performed as described in the product literature with no alterations and then analyzed immediately after preparation. BIOPLEX MANAGER software was used for data acquisition.

Statistics

Statistical analysis was performed using GRAPHPAD PRISM software. Normality was tested using the D'Agostino and Pearson