Inhibiting CXCL12 blocks fibrocyte migration and differentiation and attenuates bronchiolitis obliterans in a murine heterotopic tracheal transplant model

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Objectives: Fibrocytes are integral in the development of fibroproliferative disease after lung transplantation. Undifferentiated fibrocytes (CD45+anti-collagen 1+CXCR4+) preferentially traffic by way of the CXCR4/CXCL12 axis and differentiate into smooth muscle actin-producing (CD45+CXCR4+α-smooth muscle actin+) cells. We postulated that an antibody directed against CXCL12 would attenuate fibrocyte migration and fibro-obliteration of heterotopic tracheal transplant allografts.

Methods: A total alloantigenic mismatch murine heterotopic tracheal transplant model of obliterative bronchiolitis was used. The mice were treated with either goat-anti-human CXCL12 $F(ab')_2$ or goat IgG $F(ab')_2$. Buffy coat, bone marrow, and trachea allografts were collected and analyzed using flow cytometry. Tracheal luminal obliteration was assessed using hematoxylin-eosin and Direct Red 80 collagen stain.

Results: Compared with the controls, the anti-CXCL12–treated mice showed a significant decrease in tracheal allograft fibrocyte populations at 7 and 21 days after transplantation. Bone marrow and buffy coat aspirates showed the same trend at 7 days. In the anti-CXCL12–treated mice, there was a 35% decrease in luminal obliteration at 21 days (65% vs 100% obliterated; interquartile range, 38% vs 10%; $P = .010$) and decreased luminal collagen deposition at 21 and 28 days after transplantation ($P = .042$ and $P = .012$, respectively).

Conclusions: Understanding the role of fibrocytes in airway fibrosis after lung transplantation could lead to a paradigm shift in treatment strategy. Anti-CXCL12 antibody afforded protection against infiltrating fibrocytes and reduced the deterioration of the tracheal allografts. Thus, the CXCR4/CXCL12 axis is a novel target for the treatment of fibro-obliteration after lung transplantation, and the quantification of fibrocyte populations could provide clinicians with a biomarker of fibrosis, allowing individualized drug therapy. (J Thorac Cardiovasc Surg 2013;145:854-61)

Bronchiolitis obliterans syndrome (BOS) is a leading cause of morbidity and mortality after lung transplantation.[1] Lung fibroblasts and myofibroblasts are critical for the development of fibrosis and are thought to arise from 3 locations: the resident proliferation of fibroblasts, epithelial to mesenchyme transition, and bone marrow-derived mesenchymal progenitor cells, fibrocytes.[2]

Fibrocytes (CD45+anti-collagen 1 [Col1]+CXCR4+) are bone marrow-derived mesenchymal stem cells that are released into the circulation in response to numerous inflammatory threats and microenvironmental cytokines. They travel to injured tissues by way of the CXCR4/CXCL12 chemokine axis. Fibrocytes differentiate into α-smooth muscle actin (α-SMA+) producing fibroblasts/myofibroblasts.[2,3] Although fibrocytes have been shown to respond to several chemokines and express CCR3, CCR5, and CCR7 chemokines, the CXCR4/CXCL12 biologic axis is the predominate driving force for fibrocyte trafficking after airway injury.[4,5]

Fibrocytes were first described in the context of normal wound healing; however, their importance in the progression of fibrotic and asthmatic lung disease has since been demonstrated.[6] Patients with exacerbation of idiopathic pulmonary fibrosis (IPF) have been shown to have an elevated proportion of peripheral blood fibrocytes compared with patients with stable IPF. Furthermore, the total circulating fibrocyte population in those with IPF was found to be an independent predictor of death.[7]

In a prospective study aimed at quantifying circulating fibrocyte populations (CD45+Col1+) in patients after lung transplantation, we found a statistically significant increase in the circulating fibrocyte number in patients diagnosed with BOS (defined according to the forced expiratory

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Abbreviations and Acronyms

α-SMA = α-smooth muscle actin
BOS = bronchiolitis obliterans syndrome
Col1 = collagen 1
IPF = idiopathic pulmonary fibrosis
mTOR = mammalian target of rapamycin

volume in 1 second) compared with that in patients without BOS (8.9×10^5 cells/mL vs 2.96×10^5 cells/mL, respectively). Furthermore, we found significant incremental increases in circulating fibrocyte numbers with advancing BOS stage, suggesting a role for fibrocytes in BOS progression.[8] This has been corroborated by another study, which found a statistically significant increase in the number of fibrocytes on staining for CXCR4/prolyl 4-hydroxylase in lung biopsy specimens from lung transplant patients with BOS compared with controls.[9]

The associations between fibrocyte numbers and BOS progression in human studies has suggested, but not yet proved, that fibrocytes have detrimental effects in pulmonary fibrosis. Given the correlation of fibrocyte number and clinical BOS stage and the importance of the CXCR4/CXCL12 axis in fibrocyte trafficking, we hypothesized that immunotherapy directed against CXCL12 would attenuate airway fibrosis and obliteration in a well-established murine heterotopic tracheal transplant model of obliterative bronchiolitis.[10,11]

METHODS

Generation and Purification of Anti-CXCL12 IgG $F(ab')_2$ Fragments

Goat anti-human CXCL12 antibodies were purified using the Melon Gel IgG purification Kit (Thermo Scientific, Rockford, Ill). Normal goat IgG and purified goat anti-human CXCL12 IgG were digested with Immobilized Pepsin (Pierce, Rockford, Ill). Fragments were recovered and dialyzed against phosphate-buffered saline. $F(ab')_2$ were purified from Fc fragments using NUNC Protein G columns (Nunc, Rockford, Ill).

Mice

All mice (Jackson Laboratory, Bar Harbor, Maine) received humane care according to the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals." The Animal Care and Use Committee at the University of Virginia approved the study protocol.

Experimental Group Design

We used a heterotopic subcutaneous tracheal transplant model of bronchiolitis obliterans, as previously described.[11] A major histocompatability complex class I and II mismatch was produced by transplanting 4 Balb/c ($H\text{-}2^d$) tracheas into 1 C57BL/6 ($H\text{-}2^b$) recipient. Four donor trachea allografts were used per recipient to ensure adequate tracheal tissue for fibrocyte determination and histologic examination. The mice were divided into 2 groups: the IgG control group [intraperitoneal injections of normal goat IgG $F(ab')_2$] and the anti-CXCL12 group [intraperitoneal injections of anti-CXCL12 $F(ab')_2$]. Each group included 6 recipients for a total of 120 donors and 30 recipients. Thus, there were a total of 240 donors and 60 recipients. All recipients were given intraperitoneal injections at days –1, 0, and 1, and then every other day until the endpoints. The mice were killed, and the allografts were collected on days 3, 7, 12, 21, or 28. Tracheal allografts from 5 of the 6 mice at each point were pooled for flow cytometry analysis, and the tracheal allografts from the sixth mouse were used for histologic examination and Sirius Red staining.

Fluorescence Activated Cell Sorter Analysis

Single cell suspensions isolated from bone marrow, peripheral blood, and trachea allografts were stained with peridinin-chlorophyll-protein complex–labeled CD45, phycoerythrin-labeled CXCR4 (BD Biosciences, San Diego, Calif), or isotype controls. Subsequently, the cells were permeabilized using cytofix/cytoperm for staining with DyLight-488 conjugated anti-Col1, phycoerythrin-labeled α-SMA, or isotype controls. Anti-Col1 and a rabbit IgG isotype control were conjugated using a DyLight-488 conjugation kit (Thermo Scientific, Rockford, Ill). Four-color analysis of the stained cells was performed on a FACSCanto II flow cytometer using FACSDiva, version 6.0, software (BD Biosciences). All analyses were blinded.

Histologic Examination and Measurement of Fibrosis and Luminal Obliteration

The allograft tracheal tissues were fixed, embedded, sectioned, and stained with hematoxylin and eosin. Allograft trachea sections were photographed at 4× magnification. Collagen deposition was quantified according to the percentage of luminal obliteration of the tracheal allografts using ImagePro Plus software. Eight allografts were measured in each group.

Collagen Staining and Densitometry

The tracheal sections were deparaffinized, rehydrated, and stained with Direct Red 80 (Sigma Aldrich, St. Louis, Mo). The images were captured for quantification using Image J software. The collagen signal in luminal fibro-obliteration tissue was semiquantified using the same software but with a different parameter setting.

Statistical Analysis

Fibrocytes from buffy coat, bone marrow, and tracheal single cell suspensions are presented as box and whisker plots. Each box and whisker plot at a given point represents 5 mice. Continuous data are reported as either the mean ± standard deviation or median and interquartile range. Independent sample comparisons were performed using either the Mann-Whitney U test or Kruskal-Wallis test. Statistical significance was set to $P < .05$. Group comparisons were unpaired, and the P values are 2-tailed. The analyses were performed using predictive analytics software (version 18.0.0; IBM Corp, Chicago, Ill).

RESULTS

Attenuation of Fibrocyte Trafficking Using Anti-CXCL12 $F(ab')_2$ Antibody

We first assessed the ability of neutralizing anti-CXCL12 $F(ab')_2$ antibodies to attenuate fibrocyte trafficking and differentiation in the murine model of bronchiolitis obliterans. Bone marrow, buffy coat, and trachea allograft single-cell suspensions from Balb/C mice subcutaneously transplanted with BL6 trachea were analyzed for total undifferentiated (CD45+Col1+CXCR4+) and differentiated fibrocyte (CD45+Col1+α-SMA+) cell populations.

The comparison of buffy coat isolates from mouse whole blood preparations revealed significant decrease in circulating undifferentiated fibrocytes in mice treated with