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Rapamycin Blocks Fibrocyte Migration and Attenuates Bronchiolitis Obliterans in a Murine Model

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Abstract

Background—Fibrocytes are integral in the development of fibroproliferative disease. The CXCL12/CXCR4 chemokine axis has been shown to play a central role in fibrocyte migration and the development of bronchiolitis obliterans post lung transplantation. Inhibition of the mTOR (mammalian target of rapamycin) pathway with rapamycin has been shown to decrease expression of both CXCR4 and its receptor agonist, CXCL12. Thus, we hypothesize that rapamycin treatment would decrease fibrocyte trafficking into tracheal allografts and prevent bronchiolitis obliterans.

Methods—A total alloantigenic mismatch, murine heterotopic tracheal transplant model of bronchiolitis obliterans was used. Animals were either treated with rapamycin or dimethyl sulfoxide (DMSO) for 14 days post tracheal transplant. Fibrocyte levels were assessed via flow cytometry, and allograft neutrophil, CD3+ T-cell, macrophage, and smooth muscle actin levels were assessed via immunohistochemistry. Tracheal luminal obliteration was assessed on hematoxylin and eosin stains.

Results—Compared to DMSO controls, rapamycin-treated mice showed a significant decrease in fibrocyte levels in tracheal allografts. Fibrocytes levels in recipient's blood showed a similar pattern, although not statistically significant. Furthermore, animals treated with rapamycin showed a significant decrease in tracheal allograft luminal obliteration compared to controls. Based on immunohistochemistry analyses, populations of α -SMA positive cells, neutrophils, CD3+ T-cells, and macrophages were all decreased in rapamycin-treated allograft versus DMSO controls.

Conclusions—Rapamycin effectively reduces recruitment of fibrocytes into tracheal allografts and mitigates development of tracheal luminal fibrosis. Further studies are needed to determine the cellular and molecular mechanisms that mediate the protective effect of rapamycin against bronchiolitis obliterans.

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Keywords

bronchiolitis obliterans; lung transplantation; lung rejection

Introduction

Lung transplantation continues to be the preferred treatment strategy for many end-stage pulmonary diseases. Despite its treatment successes, the long-term results of lung transplantation remain limited by chronic allograft rejection, known as bronchiolitis obliterans (BO). The histologic findings in BO are manifested as scarring of the small airways of the transplanted lung. Approximately 50% of lung transplant recipients will develop BO by 5 years post-transplant [1]. Although we know some of the risk factors for BO development, including ischemia-reperfusion injury and episodes of acute rejection, currently we do not have any treatment modalities that effectively prevent BO [2]. Additionally, we have no consistent treatments that halt the progression of BO once lung function begins to decline. Therefore, therapeutic advances that mitigate the development of BO could dramatically impact the long-term viability of lung transplantation.

Our lab has studied the role of fibrocytes in the development of bronchiolitis obliterans. Fibrocytes are bone marrow derived progenitor cells with the ability to differentiate down mesenchymal cell lineages [3–5]. They have been implicated in many pathologic processes of chronic inflammation and fibrosis, including arthritis, chronic kidney disease, scleroderma, asthma, and idiopathic pulmonary fibrosis [3, 4, 6–8]. They originate in bone marrow, mobilize into the circulation in response to inflammatory chemokines, and home to sites of inflammation. Once there, they differentiate into myofibroblasts and begin the process of tissue remodeling and collagen deposition that ultimately culminates with fibrosis [5].

The CXCL12/CXCR4 ligand-receptor pair has been reported to play a vital role in fibrocyte trafficking [9, 10]. Our lab has previously shown that blocking this axis with an anti-CXCL12 antibody reduces fibrocyte levels in the circulation and in transplanted allografts in a murine heterotopic tracheal transplant (HTT) model of BO [11]. Furthermore, anti-CXCL12 treatment limits the allograft fibro-obliteration characteristic of BO. Similarly, drugs that inhibit mammalian target of rapamycin (mTOR) have been shown to downregulate CXCR4 expression in models of pulmonary fibrosis [12]. This downregulation limits the ability of fibrocytes to mobilize and chemotax, consequently inhibiting the development of fibrosis in this model.

In light of these results, we hypothesized that treatment with an mTOR inhibitor (rapamycin) would reduce fibrocyte infiltration into tracheal allografts and prevent the development of airway fibro-obliteration in a well-established murine heterotopic tracheal transplant model of BO.

Material and Methods

Animals

All mice (Jackson Laboratory, Bar Harbor, ME) received humane care in accordance with 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” prepared by the National Academy of Science and published by the National Institutes of Health. The study protocol was reviewed and approved by the Animal Care and Use Committee at the University of Virginia before experiments were initiated.

Murine Heterotopic Tracheal Transplant Model

We utilized a murine HTT model of bronchiolitis obliterans, as previously described [13–15]. Donor mice were anesthetized with an intraperitoneal ketamine/xylocaine solution containing heparin sulfate. Animals were exsanguinated via direct cardiac puncture with a 26-gauge syringe. The trachea was exposed via a midline cervical incision and the tracheal segment distal to the thyroid cartilage and proximal to the sternal notch was explanted.

Recipient mice were anesthetized with inhaled isoflurane. The back was shaved and prepped with topical antiseptic providone-iodine (10%) solution followed by alcohol (70%). Two small vertical skin incisions were made. Two tracheal allografts were implanted through each incision into two separate subcutaneous pockets. A total of four tracheas were transplanted into each recipient animal. The skin incisions were closed with prolene suture.

Experimental Design

Experimental mice were divided into 3 groups. 1) Isograft controls – tracheas from C57BL6 mice were transplanted into genetically identical C57BL6 mice. 2) Allograft controls – Balb/c tracheas were transplanted into C57BL6 mice, producing a major histocompatibility complex class I and II mismatch. These animals were treated with dimethyl sulfoxide (DMSO). 3) Rapamycin treatment – Balb/c tracheas were transplanted into C57BL6 mice, which were then treated with rapamycin (Selleck Chemicals LLC, Houston, TX).

Rapamycin was administered daily (10mg/kg intraperitoneally) in treatment mice up to 14 days post-transplant; allograft controls received daily injections with the same volume of DMSO. Mice were euthanized at days 7, 14, or 28 post transplantation. Blood, bone marrow, and tracheal grafts were collected at this time. Blood and bone marrow were used for flow cytometry. Tracheal isografts and allografts were used for flow cytometry, histologic staining, and immunohistochemistry.

According to our previous studies [11], peak tracheal allograft fibrocyte infiltration occurs 7 days post transplantation. Therefore, flow cytometry was used to assess blood, bone marrow, and tracheal allograft fibrocyte levels in animals sacrificed on day 7. There were 6 recipient mice in each group (6 isograft, 6 allograft + DMSO, 6 allograft + rapamycin). Of the four tracheas transplanted into each recipient in the day 7 mice, 3 were used for flow cytometry and 1 was used for histology. Bone marrow and buffy coat isolations from each animal were also used for flow cytometry.

Histology was performed on tracheal allografts from mice from all 3 time points. In recipient mice sacrificed at 14 and 28 days, there were 2 recipient mice for each group. All four transplanted tracheas were used for histology and IHC (a total of 8 tracheas per group).

Flow Cytometry

Flow cytometry was performed as previously described [11]. Single cell suspensions were isolated from bone marrow, blood, and tracheal grafts. Each sample came from one mouse, which included bone marrow harvested from both femurs, 1mL of blood, and the pooled cells isolated from 3 transplanted tracheas. Cells were stained for the fibrocyte markers CD45, CXCR4, collagen-1 (Col1), and alpha smooth muscle actin (α -SMA) as follows. Cells were stained with peridinin-chlorophyll-protein complex-labeled anti-CD45, phycoerythrin-labeled anti-CXCR4 (BD Biosciences, San Diego, CA), or isotype controls. Next, cells were permeabilized using cytofix/cytoperm and stained with Alex-488 conjugated anti-col1, phycoerythrin-labeled anti- α -SMA, or isotype controls. Anti-col1 and a rabbit IgG isotype control were conjugated using Alex-488 conjugation kit (Invitrogen, Grand Island, NY). Four-color analysis of the stained cells was performed on a Beckman Coulter CyAn ADP LX flow cytometer using FlowJo software (Tree Star Inc, Ashland, OR). All analyses were blinded.

Histology

Harvested tracheas were fixed in 4% zinc-formalin, and after 24 hours, were imbedded in paraffin and sectioned for staining. Tracheal samples were stained with hematoxylin and eosin (H&E).

Immunohistochemical Staining of Macrophages, Neutrophils, and Smooth Muscle Actin

Immunohistochemical staining of macrophages and neutrophils were performed according to our previous publication [15]. Briefly, rat anti-mouse neutrophil (AbD Serotec, Raleigh, NC), rat anti-mouse macrophage (Accurate Chem, Westbury, NY), or AP-conjugated monoclonal anti- α SMA (Sigma, St Louis MO) antibodies were used as primary antibodies. AP-conjugated anti-rat IgG (Sigma, St. Louis, MO) was employed as a secondary antibody. Fast-Red (Sigma, St Louis MO) was used as substrate. Purified normal rat IgG (eBioscience Inc, San Diego, CA) was used as a negative control. The sections were counter-stained lightly with hematoxylin. Macrophage and neutrophil infiltration were semi-quantified using Image-Pro Plus software. The densitometric value of positive staining area of each section was blindly selected and measured using slides viewed at 20 $\times$ magnification. Results are reported in pixels per millimeter.

Immunohistochemical Staining of CD3+ T cells

CD3+ T cell staining was performed according to our previous publications [15, 16]. Briefly, tracheal sections (5 μ m) were dehydrated and endogenous peroxidase activity was quenched with 1% hydrogen peroxide. After antigen retrieval with Unmasking Solution (Vector Laboratories, Burlingame, CA) and blocking with 10% serum, the specimens were stained with goat anti-mouse CD3 antibody (Santa Cruz Biotechnology) as the primary antibody. The immunoreactivity of CD3+ cells was visualized by adding 3,3-

diaminobenzidine tetrahydrochloride (DAKO Corp.). The number of CD3+ T cells per high power field (viewed at 40× magnification) was assessed. At least 4 fields were counted per trachea by blinded observers. The average cell number per slide was used for statistical analysis.

Measurement of Luminal Obliteration

Sections of tracheal graft H&E stains were photographed at 4× magnification. This analysis was performed on tracheas collected at 28 days post transplant, the time at which total luminal obliteration has typically occurred in transplanted allografts. A total of 8 tracheas from the rapamycin group and 8 tracheas from the DMSO group were used. Area of obliterated lumen and total area of tracheal lumen were measured using ImagePro Plus software in a blinded fashion. Percentage of luminal obliteration was calculated by dividing the area of fibrosis by the total area of lumen.

Statistical Analysis

Flow cytometry data was analyzed using pairwise comparisons between study groups (isograft vs. DMSO, DMSO vs. rapamycin, isograft vs. rapamycin) using the Wilcoxon Rank Sums test for non-parametric data. Data results are reported as the median number of fibrocyte cells, with interquartile range [IQR=25th, 75th percentile]. Immunohistochemistry and luminal obliteration analysis data are presented as means ± standard error of the mean. Student's *t*-test was used to compare group cell counts, densitometric analysis, and luminal obliteration. All calculated test statistics were used to derive two-sided *p*-values. A value of *p* < 0.05 was considered significant.

Results

Rapamycin blocks fibrocyte infiltration into tracheal allografts

Table 1 displays fibrocyte levels within transplanted tracheas, blood samples, and bone marrow samples at 7 days post transplantation. Based on flow cytometry, fibrocyte levels (CD45⁺col1⁺CXCR4⁺) within tracheal allografts were significantly lower after rapamycin treatment compared to DMSO controls (*p*=0.041; Table 2). As fibrocytes differentiate toward collagen-depositing lineages, they begin expressing smooth muscle actin (SMA). In our analysis of SMA+ cells (CD45⁺col1⁺CXCR4⁺SMA⁺), there were significantly fewer of this cell type with rapamycin treatment (*p*=0.015; Table 2). When comparing fibrocyte and SMA+ cell counts between the rapamycin treatment group and isograft controls, there were no significant differences in tracheal cell counts between these groups for either subpopulation (*p*=0.70; Table 2).

Immunohistochemistry for smooth muscle actin (SMA) was also performed on tracheas at 14 days post transplantation. Representative stains are shown in Figure 1. With developing luminal fibrosis, tracheas from the DMSO-treated group showed positive SMA staining (red in Fig. 1) within the tracheal lumen, corroborating our flow cytometry data for elevated SMA+ cell numbers. In contrast, tracheas from the rapamycin-treated group did not show SMA staining within the tracheal lumen.

Rapamycin's effect on fibrocyte mobilization

Fibrocyte levels were assessed in the blood and bone marrow of recipient mice at 7 days post tracheal transplantation using flow cytometry. In the blood samples, fibrocyte and SMA⁺ subpopulations were decreased in the rapamycin treatment groups compared to DMSO controls (Table 1). However, these differences were not statistically significant for CD45⁺col1⁺CXCR4⁺ cells ($p=0.13$; Table 2) or CD45⁺col1⁺CXCR4⁺SMA⁺ cells ($p=0.065$; Table 2).

Within the bone marrow samples, there were somewhat lower fibrocyte and SMA⁺ cell counts within the rapamycin treatment group (Table 1). However, these differences were again not statistically significant for CD45⁺col1⁺CXCR4⁺ cells ($p=0.31$; Table 2) or CD45⁺col1⁺CXCR4⁺SMA⁺ cells ($p=0.59$, Table 2).

Rapamycin reduces immune cell infiltration into tracheal allografts

Immunohistochemistry was performed on tracheal sections from recipient mice 14 days post transplant to stain for neutrophils, CD3⁺ T cells, and macrophages. On densitometric analysis, tracheas from the rapamycin treatment group demonstrated fewer neutrophils ($12.0 \times 10^6 \pm 2.33 \times 10^6$ vs $26.5 \times 10^6 \pm 6.49 \times 10^6$, $p=0.039$) and macrophages ($34.9 \times 10^6 \pm 3.2 \times 10^6$ vs $88.3 \times 10^6 \pm 26.2 \times 10^6$, $p<0.001$) compared to the DMSO-treated control group. Additionally, cell counting showed fewer CD3⁺ T cells in tracheas from the rapamycin treatment group compared to DMSO controls (37.9 ± 3.27 vs 57.4 ± 6.18 , $p=0.006$).

Rapamycin reduces allograft luminal obliteration

H&E stains of tracheal grafts were assessed at 28 days post transplantation for obliteration of the tracheal lumen characteristic of BO. In tracheas from mice treated with rapamycin, there was a dramatic reduction in luminal obliteration compared to DMSO controls, with almost no luminal fibrosis in the rapamycin-treated group and virtually uniform fibrosis in the DMSO-treated group (3.0 ± 2.14 vs 97.8 ± 3.63 , $p<0.001$; Fig. 2).

Comment

In this study, we sought to investigate whether treatment with an mTOR inhibitor, rapamycin, would reduce fibrocyte mobilization and invasion into transplanted tracheal grafts and prevent the development of bronchiolitis obliterans. Utilizing a murine heterotopic tracheal transplant (HTT) model of bronchiolitis obliterans, we have demonstrated that treatment with rapamycin reduces fibrocyte infiltration into tracheal allografts compared to DMSO-treated controls. Concordant with a reduction in fibrocyte numbers, there is almost no evidence of tracheal luminal fibrosis at 28 days post-transplant in these allografts. Fibrocyte counts and luminal obliteration in the rapamycin treatment group are comparable to the results seen in the isograft tracheas.

The CXCL12/CXCR4 axis is key in fibrocyte chemotaxis to areas of inflammation. Inhibition of this axis has shown therapeutic potential at reducing fibrocyte-mediated fibrosis in idiopathic pulmonary fibrosis (IPF) and bronchiolitis obliterans (BO). In a

bleomycin-treated murine model of IPF, treatment with an anti-CXCL12 antibody or a direct CXCR4 antagonist have both shown protective effects against the development of fibrosis [10, 17]. Similarly, our lab has demonstrated that treatment with an anti-CXCL12 antibody effectively attenuates allograft fibro-obliteration in a murine HTT model of BO [11]. Xu and colleagues have corroborating data, reporting a significant reduction in luminal obliteration after treatment with a CXCR4 antagonist in the murine HTT model [18].

The anti-fibrotic capacity of mTOR inhibitors is currently being investigated in many disease processes. Studies performed by Mehrad and associates investigated the use of the mTOR inhibitor rapamycin in the treatment of IPF [12]. In their murine model, they reported a decrease in CXCR4 expression with rapamycin treatment. Subsequently, they saw a reduction in circulating fibrocyte levels, fibrocyte infiltration into the lung, and collagen deposition in bleomycin-challenged mice after rapamycin treatment.

Additionally, a German group has studied the mTOR inhibitor everolimus in a rat lung transplant model [19]. They found that treatment with everolimus beginning post-operative day 0 prevented the development of chronic rejection and histologic evidence of BO at 60 days post lung transplant. Interestingly, animals that did not initiate treatment prior to post-operative day 14 uniformly developed BO by day 60. This finding suggests that monotherapy with mTOR inhibitors has a protective effect against the development of chronic rejection but is unable to reverse the cascade towards BO after its initiation by a significant episode of rejection. Once again, this reinforces the idea that preventing episodes of acute rejection is closely tied to the prevention of chronic rejection and BO.

The results of the present study have important implications in our understanding of the pathogenesis of bronchiolitis obliterans and the role of fibrocytes in this process. First, our data provide further evidence for the association between fibrocyte migration to pulmonary allografts and the development of BO. When fibrocyte chemotaxis is blocked via treatment with an mTOR inhibitor, the cascade towards airway obliteration is halted. While previous studies investigating therapies aimed at blocking fibrocyte trafficking have used treatment modalities that are yet to be approved for use in humans, mTOR inhibitors have been FDA approved for human use for more than a decade.

Human studies investigating the association between mTOR inhibitor therapy and bronchiolitis obliterans have shown mixed results. Two randomized, double-blind, multicenter trials have reported that treatment with everolimus more effectively prevented a decline in lung function when compared to azathioprine treatment [20, 21]. In contrast, another randomized, multicenter trial by Bhorade and colleagues showed no difference in graft survival or chronic rejection at one year when treated with sirolimus or azathioprine [22]. However, since less than 10% of lung transplant recipients experience chronic rejection within the first year post-transplant, this study group lacks adequate follow-up to assess the true, long-term benefits of sirolimus treatment in these patients.

Despite the potential therapeutic uses for mTOR inhibitors, this family of medications has several side effects that have limited their widespread utilization. mTOR inhibitors mitigate the development of fibrosis, but they also have a negative impact on wound healing. There

have been reports of an increased incidence of airway anastomosis dehiscence in patients treated with sirolimus immediately after lung transplantation [23, 24]. Other adverse effects of mTOR inhibitors include lipid abnormalities, venous thromboembolism, stomatitis, diarrhea, and less commonly interstitial pneumonitis [25–27].

This study has a few noteworthy limitations. First, although the murine HTT model is technically simple and provides very reproducible obliteration of the tracheal allografts, it is not without its shortcomings. The allograft is not aerated or revascularized, and is a large airway model of a small airway disease. An aerated murine orthotopic tracheal transplant model has been described, but the tracheal anastomosis of this technique is more technically challenging, and this model does not produce histologic luminal obliteration as reliably as the heterotopic model. Secondly, while rapamycin has been shown to block the mTOR pathway, reduce CXCR4 expression, and inhibit fibrocyte trafficking, it also has known immunosuppressive properties. The current study demonstrates this effect, with lower numbers of neutrophils, CD3+ T cells, and macrophages in tracheas from the rapamycin treatment group compared to DMSO controls. Therefore, we are unable to discern whether the observed reduction in fibrocyte chemotaxis is due to direct inhibition of fibrocytes or due to the immunosuppressive activity of rapamycin. If fibrocytes migrate based on the inflammatory chemokines produced by the immune-mediated attack of tracheal allografts, then suppression of the immune response would reduce fibrocyte recruitment. Likely, it is a combination of both effects that limits fibrocyte invasion and protects against BO development.

In conclusion, rapamycin protects against the development of bronchiolitis obliterans in a murine HTT model. This protective effect is associated with a reduction in fibrocyte recruitment to the tracheal allografts, although the impact of fibrocyte blockade versus other pharmacologic mechanisms remains unclear. Further studies are needed to delineate the relative contributions of immunosuppression and fibrocyte inhibition on mTOR inhibitor-mediated mitigation of airway obliteration. With this dual mechanism of action, this family of medications may prove to be the most effective agents in preventing BO development in lung transplant patients.

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Figure 1.

Representative immunohistochemical stains of tracheal grafts for SMA at Day 14 post transplant. DMSO controls demonstrate positive SMA staining (Fast-Red) within the tracheal lumen, indicative of the developing fibrosis. Rapamycin-treated tracheas show no SMA staining within the lumen. Magnification of images is 40 $\times$. BF = bright field. Fast-Red = SMA stain. DAPI = nuclear stain. Merged = Fast-Red + DAPI together.

Figure 2.

Rapamycin treatment reduces luminal obliteration of tracheal allografts at Day 28 post transplant. (A) Images of representative H&E stains of tracheal allografts. DMSO-treated show complete luminal obliteration with fibrosis; rapamycin-treated shows no luminal obliteration (pink substance within lumen is mucus). (B) Bar graph of percent luminal obliteration in each treatment group. $n = 8$ tracheas per group. Data shown are mean $\pm$ standard error of the mean.

Table 1

Fibrocyte cell counts at 7 days post tracheal transplantation.

Sample and fibrocyte population	Isografts (n=6)	DMSO (n=6)	Rapamycin (n=6)
Trachea			
CD45+ col1+ CXCR4+	1,983.5 [700, 2616]	6,099.5 [2131, 8442]	1,306.5 [792, 2104]
CD45+ col1+ CXCR4+ SMA+	799.0 [565, 2557]	1,890.2 [1390, 3167]	732.8 [675, 816]
Blood			
CD45+ col1+ CXCR4+	3,753.0 [493, 7812]	1,601.5 [913, 14648]	475.6 [36.3, 1860]
CD45+ col1+ CXCR4+ SMA+	1122.2 [147, 2147]	732.3 [306, 3934]	147.6 [5.34, 488]
Bone Marrow			
CD45+ col1+ CXCR4+	127,962 [66277, 211057]	143,125 [85282, 173588]	77,648 [37135, 133916]
CD45+ col1+ CXCR4+ SMA+	66,617 [3045, 151201]	71,380 [19287, 123228]	25,642 [7825, 91320]

All results are reported as median [interquartile range].

Table 2

Comparison of fibrocyte levels between treatment groups.

Sample and fibrocyte population	Rapamycin vs DMSO <i>p</i> value	Rapamycin vs Isograft <i>p</i> value	DMSO vs Isograft <i>p</i> value
Trachea			
CD45+ col1+ CXCR4+	0.04	0.59	0.07
CD45+ col1+ CXCR4+ SMA+	0.02	0.70	0.24
Blood			
CD45+ col1+ CXCR4+	0.13	0.07	0.82
CD45+ col1+ CXCR4+ SMA+	0.07	0.07	0.70
Bone Marrow			
CD45+ col1+ CXCR4+	0.31	0.24	0.70
CD45+ col1+ CXCR4+ SMA+	0.59	0.94	0.82

p values < 0.05 are statistically significant.