Published in final edited form as:

Ann Thorac Surg. 2013 August ; 96(2): . doi:10.1016/j.athoracsur.2013.04.068.

Short-course Rapamycin Treatment Preserves Airway Epithelium and Protects Against Bronchiolitis Obliterans

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Abstract

Background—Damage to airway epithelium is closely related to the development of bronchiolitis obliterans (BO) in pulmonary transplantation. Rapamycin protects against BO development in a murine model, but its use in lung transplant patients is limited by its side effects. We hypothesized that short-course rapamycin dosing could be used to prevent airway epithelium loss and protect against BO development in a murine model.

Methods—A total alloantigenic mismatch, murine, heterotopic tracheal transplant model of BO was used. Animals were treated with either rapamycin or dimethyl sulfoxide (controls) according to 1 of 3 treatment regimens: 1) day 1 through 14 post-transplant, 2) day 3 through 7 post-transplant, or 3) day 14 through 28 post-transplant. Epithelial loss was assessed via H&E stains at 14 and 28 days post-transplant. Tracheal luminal obliteration was assessed at 28 days.

Results—Early rapamycin treatment was protective against epithelial loss at 14 days post-transplant compared to controls ($p < 0.001$). Rapamycin treatment from days 1-14 was more effective at epithelial preservation ($p = 0.002$) and reducing luminal obliteration ($p < 0.001$) at 28 days compared to rapamycin treatment from days 3-7. Late rapamycin treatment (days 14-28) allowed for recovery of the previously denuded epithelium at 28 days (92.5% epithelial loss to 35.6%) and a reduction in BO ($p < 0.001$).

Conclusions—Short-course rapamycin treatment protects against airway epithelium loss and subsequent development of bronchiolitis obliterans in a murine model. Because of its immunosuppressive and anti-fibrotic effects, rapamycin may prove to be the ideal medication to prevent chronic rejection and BO in lung transplant patients.

Keywords

bronchiolitis obliterans; lung transplantation; lung rejection

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Presented at the 49th Annual Meeting of the Society of Thoracic Surgeons, January 28, 2013, Los Angeles, CA, USA

Introduction

Lung transplantation is the therapeutic endpoint of many chronic progressive pulmonary diseases. However, the long-term success of this treatment strategy remains suboptimal, with median post-transplant survival of about 5 years [1]. The main factor contributing to graft failure is chronic rejection, manifested histopathologically as bronchiolitis obliterans (BO), or scarring of the small airways.

Repeated insults to the transplanted lung, often in the form of infections or episodes of acute rejection, cause inflammation and tissue damage [2, 3]. Effective repair and regeneration following these insults usually ensures maintenance of pulmonary function; however, when these insults initiate a profibrotic cascade, BO develops and lung function declines. Therefore, both the inflammatory cycle and the pro-fibrotic cascade are potential therapeutic targets to mitigate the development of BO.

We have studied the involvement of fibrocytes in BO pathogenesis. Fibrocytes are a unique progenitor cell involved in many processes of chronic inflammation and fibrosis [4-8]. They originate from the bone marrow from hematopoietic cells, but they are able to differentiate down several mesenchymal cell lineages [6, 7, 9]. We have shown that blocking fibrocyte migration slows the progression of BO in the murine heterotopic tracheal transplant (HTT) model [10]. [Note: Obliterative airway disease (OAD) is the histologic correlate of BO in the tracheal transplant model; these terms will be used interchangeably.]

In the HTT model, the tracheal epithelium cycles through predictable phases of loss and regeneration [11-14]. During the initial phase (day 0-3), the graft suffers ischemic injury during the heterotopic transplantation, and thus, the luminal epithelial cell layer is lost. This is followed by a period of neovascularization and reperfusion (day 3-7), which allows for graft epithelial regeneration. However, reperfusion also allows for infiltration of circulating immune cells and fibrocytes at this time. Based on our previous studies, fibrocyte levels typically peak around day 7 post tracheal transplantation [10]. Next, recognition and attack of the foreign allograft by these immune cells initiates a secondary loss of luminal epithelial cells (day 7-14). Without an intact epithelial cell layer, fibrocytes are allowed to infiltrate into the tracheal lumen and differentiate into collagen-producing cells, eventually leading to the luminal fibro-obliteration characteristic of BO (day 14-28).

The secondary loss of epithelium necessarily precedes the development of luminal obliteration in this model [11]. Therefore, therapeutic options that prevent this epithelial cell loss could be used to prevent the development of BO. One such therapeutic option is treatment with rapamycin. Rapamycin has both immunosuppressive and anti-fibrotic properties [15]. We have shown that rapamycin treatment prevents secondary loss of the epithelium and mitigates the development of BO in the HTT model [16]. Rapamycin treatment also reduces intra-tracheal levels of fibrocytes [16].

Rapamycin belongs to a family of medications that inhibit the mTOR (mammalian target of rapamycin) pathway. These medications are currently used for immunosuppression in transplant patients, but their use is limited by several side effects, including impaired wound healing, lipid abnormalities, and diarrhea [17-20]. Therefore, short-course dosing could be a potential strategy to maximize rapamycin's ability to prevent BO while minimizing unwanted side effects.

In this study, we chose to investigate further the relationship between short-course rapamycin treatment, epithelial integrity, and the development of BO. We also sought to compare the relative effectiveness of different rapamycin treatment durations as well as early versus late initiation of rapamycin treatment on the development of BO. We

hypothesized that a short-course, as opposed to continuous, rapamycin treatment would be as effective in prevention of secondary graft epithelial loss and would subsequently protect against BO development. Furthermore, we hypothesize that early initiation of treatment would be more effective at preventing BO than delayed initiation of treatment.

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 Animal Care and Use Committee at the University of Virginia reviewed and approved this protocol.

Murine Heterotopic Tracheal Transplant Model

We utilized a murine HTT model of bronchiolitis obliterans, as previously described [16, 21-23]. Briefly, donor mice were anesthetized and their trachea were removed via a midline cervical incision. Donor mice were humanely sacrificed. Next, recipient mice were anesthetized and four tracheal allografts were implanted through two small vertical incisions made along the back. Time from harvest to implantation was 5 to 20 minutes.

Experimental Design

Balb/c tracheal allografts were transplanted into C57BL6 recipients, producing a major histocompatibility complex class I and II mismatch. These mice were then treated with either rapamycin (10mg/kg intraperitoneally once daily) or, in the control group, with an equivalent volume of dimethyl sulfoxide (DMSO). Three different intermittent dosing regimens were tested: 1) treatment on days 1 through 14 post-transplant, 2) treatment on days 3 through 7 post-transplant, and 3) treatment on days 14 through 28 post-transplant. Each regimen had a rapamycin treatment group and a DMSO control group.

The first regimen (day 1-14) tested intermittent rapamycin dosing that covered both the primary and secondary loss of epithelium, as well as the time of reperfusion with immune cell and fibrocyte infiltration. The second regimen (day 3-7) covered only the key period of reperfusion and epithelial regeneration. The third regimen (day 14-28) allowed for reperfusion, epithelial regeneration, immune cell infiltration, fibrocyte infiltration, and secondary loss of epithelium before initiating treatment. This regimen tested whether epithelium could be regenerated after its loss and whether epithelial regeneration would lead to prevention against BO development.

In untreated mice, transplanted tracheas typically exhibit loss of luminal epithelium by 14 days post-transplant and show luminal obliteration by 28 days post-transplant [11-14]. Therefore, we assessed for epithelial integrity at 14 and 28 days post-transplant and for luminal obliteration at 28 days post-transplant. Mice were sacrificed on these days and tracheal allografts were harvested for H&E and immunohistochemistry.

For a given treatment (eg. rapamycin) with a given treatment regimen (eg. day 1-14) and a given timepoint (eg. sacrifice at 14 days), two recipient mice received 4 transplanted tracheas each, for a total of 8 tracheas per group. In total, 96 tracheas were transplanted into 24 mice.

Histology

Tracheal allografts were fixed in 4% zinc-formalin, imbedded in paraffin, and sectioned for staining with hematoxylin and eosin (H&E).

Immunohistochemical Staining of Basal Epithelial Cells

Immunohistochemical staining for basal epithelial cells (epithelial progenitor cells) was performed as previously described [24, 25]. The tracheal sections (5 μ m) were dehydrated. Antigen retrieval was performed using Unmasking Solution (Vector) according to manufacturer's instructions. Immunostaining was performed with rabbit anti-mouse p63 antibody and Guinea pig anti-cytokeratin 14 antibody (Lifespan Biosciences, Seattle WA) using Vectastain ABC kit (Vector Laboratories, Burlingame, CA). After incubation with an avidin-biotin complex, immunoreactivity was visualized by incubating the sections with 3, 3'-diaminobenzidine tetrahydrochloride (DAKO Corp, Carpinteria, CA) to produce a brown precipitate, and then counterstained with hematoxylin. Images were taken with an Olympus BX51 high magnification microscope equipped with an Olympus DP70 digital camera (Center Valley, PA).

Assessment of Epithelial Loss

After harvesting tracheal allografts at 14 and 28 days post-transplantation, cross-sectional cuts were made through the central portion of the specimens. The specimens were then stained with H&E and photographed at 4X magnification using ImagePro Plus software. Approximately four sections were taken from each trachea. Percent epithelial loss was calculated on all sections and then averaged. Percent epithelial loss was determined in a blinded fashion by dividing the length of denuded internal luminal circumference from the total luminal circumference. Measurements were obtained using ImagePro Plus software.

Measurement of Luminal Obliteration

Tracheal allografts at 28 days post-transplantation were sectioned as described above, stained with H&E, and imaged at 4X magnification with ImagePro Plus software. Approximately four sections were taken from each trachea. Percent luminal obliteration was calculated on each section and then averaged. Percent luminal obliteration was determined in a blinded fashion by dividing the area of luminal fibrosis by the total luminal area.

Statistical Analysis

Differences in epithelial loss and luminal obliteration were analyzed using single factor analysis of variance (ANOVA) with a Bonferroni correction factor for multiple pairwise comparisons. Comparisons were made between different treatment regimens for a given therapy and between different therapies for a given regimen. Data are presented as means $\pm$ standard deviation. All calculated test statistics were used to derive two-sided *p*-values. A value of *p* < 0.05 was considered significant.

Results

Early rapamycin treatment prevents epithelial loss

Figure 1 presents epithelial loss at 14 days post-transplantation. Mice treated with rapamycin for 14 days showed almost no loss of the tracheal epithelium, while DMSO controls showed near total loss ($0.75 \pm 1.75\%$ vs $92.50 \pm 10.37\%$ epithelial loss, $p < 0.001$). Representative day 14 H&E stains of tracheal allografts are shown in **Figure 2**, clearly demonstrating epithelial preservation in the rapamycin group and epithelial loss in the DMSO group. Representative allograft immunohistochemical stains at day 14 are shown in

Figure 3. CK14 and p63 staining of basal epithelial cells demonstrates epithelial preservation in the rapamycin group and loss in the DMSO group.

A treatment regimen of rapamycin on days 3 through 7 post-transplantation showed similar protection against epithelial loss at 14 days compared to rapamycin treatment from days 1 through 14 ($0.71 \pm 1.89\%$ vs $0.75 \pm 1.75\%$ epithelial loss, $p=1.00$; Fig 1).

Figure 4 depicts epithelial loss at 28 days post-transplant with each treatment regimen. Mice treated with rapamycin from days 1 through 14 showed continued preservation of tracheal epithelium, while DMSO controls again showed complete loss of the epithelium ($2.50 \pm 3.78\%$ vs $100.0 \pm 0\%$ epithelial loss, $p<0.001$). In comparison, treatment with rapamycin from days 3 through 7 showed varying amounts of epithelial loss at 28 days that was significantly greater than epithelial loss after rapamycin treatment from days 1 through 14 ($50.00 \pm 35.59\%$ vs $2.50 \pm 3.78\%$ epithelial loss, $p=0.001$; Fig 4). However, rapamycin treatment from day 3 through 7 did show significantly less epithelial loss compared to DMSO controls ($50.00 \pm 35.59\%$ vs $100.0 \pm 0\%$ epithelial loss, $p<0.001$).

Late rapamycin treatment allows for regeneration of epithelium

Mice in the 14 to 28 day rapamycin treatment group did not receive therapy prior to day 14 and as a result, they demonstrated almost complete epithelial loss at 14 days (Fig 1). Interestingly, at 28 days post-transplantation, there is only partial loss of luminal epithelial integrity ($35.63 \pm 31.33\%$; Fig 4), demonstrating that rapamycin treatment is associated with regeneration of the damaged epithelium. The completeness of this regeneration varied however, ranging from 10% to 90% across tracheal allografts. In contrast, all DMSO controls treated from days 14 through 28 showed complete loss of epithelium at 28 days post-transplant, which was significantly more than the rapamycin treatment group ($100.0 \pm 0\%$ vs $35.63 \pm 31.33\%$ epithelial loss, $p=0.001$).

Rapamycin treatment reduces allograft luminal obliteration

Percent tracheal luminal obliteration at 28 days post-transplantation is graphically displayed in **Figure 5**. Tracheal allografts from all DMSO controls suffered almost uniform luminal obliteration. In contrast, tracheal allografts from animals treated with rapamycin from day 1 through 14 showed almost no luminal obliteration ($2.50 \pm 3.78\%$ vs $93.13 \pm 7.99\%$, $p<0.001$). Representative allograft H&E stains in the day 1 through 14 treatment groups are shown in **Figure 6**. Furthermore, this rapamycin treatment regimen showed significantly less luminal obliteration compared to rapamycin treatment from days 14 through 28 ($20.63 \pm 16.13\%$, $p=0.005$) and similar luminal obliteration compared to rapamycin treatment from days 3 through 7 ($8.57 \pm 8.52\%$, $p=1.00$). Rapamycin treatment from days 3 through 7 and from 14 through 28 both showed less luminal obliteration than DMSO controls (both $p<0.001$).

Comment

This study demonstrates that short-course dosing of rapamycin prevents the secondary loss of tracheal allograft luminal epithelium and subsequent development of bronchiolitis obliterans in a murine model. A rapamycin course covering the critical period of reperfusion and epithelial regeneration (day 3-7) protects against epithelium loss and BO, but not as robustly as the 14-day treatment regimen. Dosing initiated after reperfusion, immune cell and fibrocyte infiltration, and secondary epithelial loss (day 14-28 dosing) allows for some epithelial regeneration and recovery, but we still observe progression towards luminal obliteration in some of these tracheas.

As described in this study and others, secondary epithelial loss in the HTT model is a critical step in the development of airway obliteration [11, 26, 27]. Therefore, therapies targeting the epithelium have been investigated as a strategy for BO prevention. For example, Adams and colleagues heterotopically transplanted isograft (genetically identical) tracheas into recipient mice after tracheal epithelial damage by protease digestion [26]. One group of tracheas was reseeded with airway epithelial cells, while the control group was not. The control tracheas developed luminal obliteration, demonstrating the key role of epithelial damage in BO development, as the donor tracheas were genetically identical to the recipient. In contrast, tracheas in the epithelial cell treatment group showed significant reduction in luminal obliteration. Similarly, we have demonstrated that treatment of allograft tracheas with recipient epithelial cells prevents BO development in the HTT model [11].

Because secondary epithelial loss is predicated on immune-cell invasion, targeting this immune response has been shown to delay or prevent epithelial loss. Higuchi and colleagues demonstrate that RAG1 knockout (RAG1-KO) mice, which lack both B and T cell populations, are protected against secondary graft epithelial loss [28]. Similarly our lab has shown histologically that rapamycin treatment reduces neutrophil, macrophage, and CD3+ T cell infiltration into recipient allografts, as compared to controls [16]. We believe that through mitigation of immune cell infiltration into tracheal allografts, rapamycin is protective against epithelial loss.

Cells and pathways involved in collagen deposition and fibrosis are another potential therapeutic target to prevent BO. In addition to its immunosuppressive effects, rapamycin also has anti-fibrotic properties, evidenced clinically by its negative impact on wound healing [20]. Fibrocyte trafficking is largely dependent on the CXCR4-CXCL12 receptor-ligand pair [29, 30]. By reducing CXCR4 expression, rapamycin block fibrocyte mobilization to areas of inflammation [15]. Our lab has shown that treatment with a 14-day course of rapamycin (day 1-14 post-transplant) decreased fibrocyte infiltration, preserved luminal epithelial integrity, and prevented BO at 28 days post-transplantation in heterotopically transplanted trachea [16]. The current study builds upon these findings, investigating the effects of several rapamycin dosing regimens on epithelial preservation and BO development. Therefore, because rapamycin has the ability to suppress the immune response and the pro-fibrotic cascade, it holds unique potential as a therapeutic agent designed to prevent BO.

The present study has notable clinical implications. Short-course rapamycin dosing at times of peak immune cell and fibrocyte infiltration appears to temporarily halt the inflammatory cycle and progression towards airway obliteration. As would be expected, a longer treatment course has a more robust effect on preventing BO. Additionally, in some cases, initiation of rapamycin treatment after immune cell infiltration, epithelial damage and loss, and fibrocyte infiltration allows for the regeneration of airway epithelium and prevention against BO development. However, some allografts still develop luminal obliteration despite rapamycin treatment, suggesting that, in some cases, the epithelial damage cannot be reversed and the progression to BO may not be preventable.

These findings suggest that the ideal time to initiate rapamycin treatment would be prior to immune cell and fibrocyte infiltration in response to an acute insult. However, in lung transplant patients, it will likely be difficult, if not impossible, to predict or determine when these insults occur. Therefore, the results from the delayed-dosing regimen suggest that treatment after the initial insult could still provide substantial benefits by affecting the inflammatory and pro-fibrotic cascades that lead towards BO.

The early dosing regimens in this study suggest that a longer treatment course of rapamycin more effectively prevents BO. Whether a 5-day or 14-day course would have the same effect in humans cannot be extrapolated from the results in this model. Further studies will need to be performed in lung transplant patients to investigate the optimal dosing regimen and duration for rapamycin, attempting to maximize BO prevention while minimizing medication side effects. Of note, the mice in our experiments tolerated the rapamycin treatment well and showed no signs of impaired wound healing or surgical infections.

This study has limitations. The heterotopic tracheal transplant model of bronchiolitis obliterans is a large airway model of a small airway disease in humans. The transplanted tracheas are neither aerated nor surgically revascularized. Other murine models of BO exist, such as the orthotopic tracheal transplant model and the extremely technically-challenging lung transplant model [31, 32]. However, these other models do not generate airway obliteration as consistently as the HTT model, and are more difficult to reproduce technically. Therefore, the HTT model remains the most reliable model of BO. Additionally, because rapamycin has effects on the immune response and on the pro-fibrotic pathways involving fibrocytes, the relative importance of these two modes of action cannot be delineated by the current set of experiments.

In conclusion, short-course treatment with rapamycin protects against the loss of airway epithelium and subsequent development of bronchiolitis obliterans in a murine model. Rapamycin treatment timed to prevent immune cell and fibrocyte infiltration produces a more robust effect on airway protection, but late treatment still allows for some recovery of airway epithelium. Because of its immunosuppressive and anti-fibrotic effects, rapamycin may prove to be the ideal medication to prevent chronic rejection and BO in human lung transplant patients.

Discussion

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Paper presented by Jacob Gillen, M.D., Charlottesville, Virginia. jacob.gillen@gmail.com

Discussion by Ross M. Bremner, M.D., Ph.D., Phoenix, Arizona. ross.bremner@chw.edu
Dr. R. Bremner (Phoenix, Arizona): A very nice study, thank you.

I just wondered if you tried any other agents besides rapamycin versus DMSO. Have you used any other agents? What about steroids, FK, and other mTOR inhibitors that you might have used to see if this is an mTOR-specific event?

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Response by Jacob Gillen, M.D., Charlottesville, Virginia.

Dr. Gillen: That's a very good question. We have not used any yet. We have plans to do what you are proposing. We chose rapamycin because of its mTOR inhibition and antifibrocyte effects, but with the current study we are not able to delineate how much of the effects are the immunosuppressive properties versus the antifibrocyte properties of rapamycin. Therefore, we plan on performing head-to-head, rapamycin versus cyclosporine versus other immunosuppressive regimen to try to tease out exactly how much of it is the mTOR inhibition versus the general immunosuppression.

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Paper presented by Jacob Gillen, M.D., Charlottesville, Virginia. jacob.gillen@gmail.com

Discussion by Norihisa Shigemura, M.D., Ph.D., Pittsburgh, Pennsylvania. shigemuran@upmc.edu Dr. N. Shigemura (Pittsburgh, PA): I have a question about this loss of epithelial cells. Was this due to the apoptotic changes or the changes in just the cell turnover? Have you ever seen the turnover studies or something?

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Response by Jacob Gillen, M.D., Charlottesville, Virginia.

Dr. Gillen: Excuse me, I'm having trouble hearing you.

Dr. Shigemura: So this cause of the epithelial cell loss, was this coming from the apoptosis or these cell turnover changes?

Dr. Gillen: I am unable to comment on that differentiation. We did not specifically investigate apoptosis versus cell turnover.

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Paper presented by Jacob Gillen, M.D., Charlottesville, Virginia. jacob.gillen@gmail.com

Discussion by Ankit Bharat, M.D., St. Louis, Missouri. bharata@wudosis.wustl.edu

Dr. A. Bharat (St. Louis, Missouri): Let me build on that question a little bit more. So the cell loss of the epithelium that you're seeing being lost in the mice that were treated with DMSO, to me it seems very fast and very early. You're seeing epithelial loss on day 7 and day 14, and also the fibrocyte infiltration that you're seeing at day 7 is somewhat puzzling to me because these are recipients that have never seen the donor before. So these are naive mice, correct?

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Response by Jacob Gillen, M.D., Charlottesville, Virginia.

Dr. Gillen: Yes.

Dr. Bharat: And for naive mice to develop a fibrocytic response in 14 days or 7 days is too robust to me. And a naive immune system doesn't produce, at least to my knowledge, fibrocytes in the graft at day 7.

So I was a little puzzled on that, and I was wondering if you had any thoughts about that.

Dr. Gillen: We see a peak in fibrocyte numbers within the tracheal graft at day 7, but we do not see developing fibrosis until after day 14. We see complete epithelial loss in the control grafts around day 14, which is consistent with results reported in the literature. I cannot speak specifically to whether it is the antigen-specific immune response mounted against the trachea or innate immune response that is recruiting the fibrocytes in.

Dr. Bharat: I agree with you. That's certainly one possibility. But we've actually used this model pretty extensively in our lab at Wash U, and what we've seen actually is that rapamycin has a pretty strong protective effect on cell apoptosis.

So these heterotopic tracheas that you are transplanting, they are ischemic. It's not a vascularized transplant.

Dr. Gillen: Correct.

Dr. Bharat: So the epithelial cells, which are the most vulnerable cells in that graft, they are very sensitive to apoptosis. And rapamycin, actually, in some of the early studies we're seeing actually is protected against that apoptosis.

So I think the early response that you're seeing is not really immune mediated. It's probably some other effect that rapamycin is creating. That's why Dr. Bremner's question is very relevant. We need to look at some other drugs to see if you can sort of look into the mechanisms of how this is happening.

Dr. Gillen: Yes, that makes sense. I'll look into that after this talk and discuss it with Dr. Lau in planning our future experiments.

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Paper presented by Jacob Gillen, M.D., Charlottesville, Virginia. jacob.gillen@gmail.com

Discussion by Robert Duane Davis, Jr., M.D., Durham, North Carolina. davis053@mc.duke.edu

Dr. R.D. Davis (Durham, North Carolina): Could I ask you a question? On your delayed rapamycin, did you check to see if it was donor or recipient epithelial cells that had populated the graft?

52. Intermittent mTOR Inhibition Prevents Development of Bronchiolitis Obliterans by Preservation of the Airway Epithelium. Response by Jacob Gillen, M.D., Charlottesville, Virginia.

Dr. Gillen: In the delayed group?

Dr. Davis: So you demonstrate that you lose the epithelium at day 14. At day 28 you come back and you're able to say it's populated by an epithelial population. Is it donor or is it recipient that's actually repopulated?

Dr. Gillen: We didn't look at that specifically in the day 14 through 28 group. We looked at it in the loss due to the ischemia reperfusion injury, and in that initial regeneration it is from the donor. But we didn't specifically look for that in our delayed group.

Dr. Davis: You may want to look at that.

Dr. Gillen: Yes, agreed.

Dr. Davis: The other caution would be this is more of an OAD model than an OB model, and I would always be hesitant when you're using this to refer it straight to OB because there are different mechanisms involved.

Acknowledgments

C.L.L. is supported by a grant sponsored by the National Heart, Lung, and Blood Institute (1K08HL094704) as well as matching funds from the Thoracic Surgery Foundation for Research and Education. I.L.K. is supported by a grant sponsored by the National Institute of Health (5R01HL092953). J.R.G. and is supported by a training grant under I.L.K. sponsored by the National Institute of Health (NIH T32HL007849).

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Figure 1. Loss of tracheal luminal epithelium at 14 days post-transplant. Early rapamycin treatment shows protection against epithelial loss. n=8 tracheas per group. Data shown are mean percent epithelial loss for each group.

Figure 2. Representative H&E stains of tracheal allografts at 14 days post-transplantation. Treatment with rapamycin shows preservation of tracheal luminal epithelium (40X image). In contrast, DMSO controls show complete loss of tracheal epithelium (40X image).

Figure 3. Representative immunohistochemical staining of tracheal allografts at 14 days post-transplantation. p63 and CK14 both stain for basal epithelial cells. Rapamycin treatment prevents loss of this epithelial cell population compared to DMSO controls. Magnification is 40x.

Figure 4.

Loss of tracheal luminal epithelium at 28 days post-transplant. Rapamycin treatment shows protection against epithelial loss, with treatment from days 1 through 14 as the most effective regimen. n=8 tracheas per group. Data shown are mean percent epithelial loss for each group.

Figure 5.

Tracheal luminal obliteration at 28 days post-transplant. Rapamycin treatment shows protection against luminal obliteration, with treatment from days 1 through 14 and days 3 through 7 as the most effective regimens. n=8 tracheas per group. Data shown are mean percent luminal obliteration for each group.

Figure 6.

Representative H&E stains of trachea at 28 days post-transplant. Treatment with rapamycin shows no luminal obliteration (4X image) and preservation of luminal epithelium (20X image). In contrast, DMSO controls show complete luminal fibro-obliteration (4X image) and complete loss of tracheal epithelium (20X image).