Effects of azithromycin and tanomastat on experimental bronchiolitis obliterans

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Objective: Azithromycin has become a standard of care in therapy of bronchiolitis obliterans following lung transplantation. Matrix metalloprotease-9 broncho-alveolar lavage levels increase in airway neutrophilia and bronchiolitis obliterans. Interleukin-17 may play a role in lung allograft rejection, and interleukin-12 is downregulated in bronchiolitis obliterans. Whether these mechanisms can be targeted by azithromycin remains unclear.

Methods: Bronchiolitis obliterans was induced by transplantation of Fischer F344 rat left lungs to Wistar Kyoto rats. Allografts with azithromycin therapy from day 1 to 28 or 56 and mono- or combination therapy with the broad-spectrum matrix metalloprotease inhibitor tanomastat from day 1 to 56 were compared to control allografts and isografts. Graft histology was assessed, and tissue cytokine expression studied using Western blotting and immunofluorescence.

Results: The chronic airway rejection score in the azithromycin group did not change between 4 and 8 weeks after transplantation, whereas it significantly worsened in control allografts ($P = .041$). Azithromycin+tanomastat prevented complete allograft fibrosis, which occurred in 40% of control allografts. Azithromycin reduced interleukin-17 expression ($P = .049$) and the number of IL-17^+/$CD8^+$ lymphocytes at 4 weeks, and active matrix metalloprotease-9 at 8 weeks ($P = .017$), and increased interleukin-12 expression ($P = .025$) at 8 weeks following transplantation versus control allografts.

Conclusions: The expression of interleukin-17 and matrix metalloprotease-9 in bronchiolitis obliterans may be attenuated by azithromycin, and the decrease in interleukin-12 expression was prevented by azithromycin. Combination of azithromycin with a matrix metalloprotease inhibitor is worth studying further because it prevented complete allograft fibrosis in this study. (J Thorac Cardiovasc Surg 2015;149:1194-202)

See related commentary on pages 1203-4.

Bronchiolitis obliterans (BO) following lung transplantation is one of the major factors compromising long-term survival. It is the leading cause of death between 3 and 5 years after lung transplantation in adults, and its clinical correlate BO syndrome (BOS) is prevalent in about 50% of recipients surviving 5 years.[1] Risk factors of BO include nonspecific alloimmune-independent and alloimmune-dependent mechanisms[2] that are already triggered by ischemia-reperfusion injury. Proinflammatory cytokines that are upregulated in primary graft dysfunction can increase HLA-II antigen expression in the graft, thus triggering donor-specific alloimmunity.[3] Each additional insult, including acute rejection, infection, or gastroesophageal reflux disease, further increases the risk of the development of a persistent self-promoting inflammatory reaction that may lead to maladaptive excessive fibroproliferation in the small airways, resulting in the histologic picture of BO. In the development of BO, inflammatory reactions and tissue remodeling are intricately linked by the interaction of recipient immunity and structural lung cells, which may also promote inflammation.[4] The overall result is progressive loss of graft function with airflow obstruction.

Matrix metalloproteases (MMPs) are produced by immunologically active cells and involved in matrix degradation in fibrotic tissue remodeling. MMP-9 is produced by neutrophils and macrophages, and its levels are increased in broncho-alveolar lavage (BAL) fluid of patients with airway neutrophilia that is associated, for example, with BOS or infection.[5,6] Interleukin (IL)-17 is upregulated in pulmonary allograft rejection[7,8] and co-regulates MMP-9 expression.[9] The IL-17 antagonist IL-12 has been reported to be downregulated in BAL fluid of BOS patients.[10] In addition, patients with BOS had increased numbers of T cells secreting IL-17 and interferon (IFN)-γ,[11] versus stable lung transplant recipients.

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Abbreviations and Acronyms

Allo	= allograft
AZI	= azithromycin
AZI+tano	= azithromycin+tanomastat
BAL	= broncho-alveolar lavage
BALT	= bronchus-associated lymphoid tissue
BO	= bronchiolitis obliterans
BOS	= BO syndrome
CINC	= cytokine-induced neutrophil chemoattractant
DAPI	= 4′,6-diamidino-2-phenylindole
EVG	= elastica van Gieson
HE	= hematoxylin-eosin
IFN	= interferon
IL	= interleukin
Iso	= isograft
MMP	= matrix metalloprotease
SD	= standard deviation
TNF-α	= tumor necrosis factor alpha
WKY	= Wistar Kyoto

The macrolide antibiotic azithromycin (AZI) has become an important therapeutic option for patients with BOS and improves their survival[12]; in particular, those with airway neutrophilia benefit from AZI.[13] A recent meta-analysis of 10 studies on AZI therapy for BOS showed a significant improvement in lung function over an average observation period of 7 months.[14] A clinical trial of prophylactic AZI treatment demonstrated a decreased incidence of BOS.[15] Multiple studies in vitro and in vivo have shown immunomodulatory effects of AZI and its ability to counteract bacterial biofilm generation and gastroesophageal reflux.[16] Recently, BAL samples of lung transplant recipients have been studied before and after initiation of AZI, and a differential regulation of a number of cytokines by AZI was shown.[5] In contrast to BAL, a human lung tissue sample representative of BO is difficult to obtain, so a model system may help to further understand the action of AZI in vivo. As human BO develops in distal airways, we chose an orthotopic left lung transplantation model in rats for our study instead of a tracheal allograft model. Transplantation of Fischer F344 lungs to Wistar Kyoto (WKY) rats leads to acute rejection and subsequent chronic changes with predominant vascular rejection, but BO-like lesions develop in about 70% to 80% of the animals at 60 days following transplantation.[17-19] In the current study, we investigated the effects of prophylactic AZI on BO in this model at 4 and 8 weeks posttransplant, corresponding to acute inflammatory and chronic stages of allograft dysfunction. No immunosuppression other than a single dose of prednisolone at transplantation to avoid hyperacute rejection on the first day was given to determine the immunomodulatory effects of AZI alone. To investigate whether the combination of AZI with a broad-spectrum MMP inhibitor could be beneficial in preventing BO, a combination of AZI and tanomastat was evaluated prophylactically for 8 weeks following transplantation.

MATERIAL AND METHODS

Animals

Pathogen-free, major histocompatibility complex RT1-mismatched male rats, that is, F344 and WKY obtained from Harlan Winkelmann (Borchen, Germany; 250-300 g at transplantation), were utilized in this study.[20] This study was approved by the Institutional Biomedical Ethics Committee and the rats were housed in the Center for Biomedical Research at the Medical University of Vienna in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The following groups were studied: F344-WKY allografts (4 weeks n = 5, 8 weeks n = 5), WKY-WKY isografts (4 weeks n = 4, 8 weeks n = 4), azithromycin in allografts (AZI group; 4 weeks n = 4, 8 weeks n = 4), and broad-spectrum MMP inhibitor tanomastat (BAY-12-9566, kindly gifted by Bayer Healthcare Austria, Vienna) alone or in combination with AZI for 8 weeks in allografts (Tano group, n = 5; AZI+tano group, n = 4). AZI was started intraperitoneally (i.p.) on the first day following transplantation, repeated once daily for 3 days at a dose of 50 mg/kg body weight, and then continued i.p. cycled 3 times per week (Monday-Wednesday-Friday). Tanomastat was administered orally at a dose of 15 mg/kg body weight[21] once per day starting on the first day following transplantation.

Transplantation Procedure

The orthotopic transplantation of left lung isografts (WKY-WKY) or allografts (F344-WKY) was performed as described.[17,22] The heart and lungs were perfused with Perfadex (Vitrolife, Gothenburg, Sweden) with addition of heparin, prednisolone (Solu-Dacortin; Merck, Vienna, Austria), and epoprostenol (Flolan; Glaxo Smith Kline, Uxbridge, United Kingdom) via the right-ventricular outflow tract. A cuff technique was employed for the anastomoses.[23] The recipients received a single steroid dose (50 mg prednisolone) and single-shot antibiotic prophylaxis with 60 mg piperacillin/tazobactam i.p. immediately before transplantation. No other immunosuppressive therapy was given at any time.

Histologic Analysis

The transplanted lung was equally divided in an upper half that was fixed in 5% paraformaldehyde and a lower half that was frozen in liquid nitrogen for protein extraction. Histologic slides were stained with hematoxylin-eosin and elastica van Gieson (EVG) and examined by a pathologist (G.D.) in a blinded fashion. Histologic findings were attributed to groups of absent, low-grade, high-grade, and fibrotic chronic vascular and airway changes as described.[18] The categories of chronic vascular rejection were as follows: low-grade chronic vascular rejection: occlusion of small vessels with mononuclear infiltrate and fibrous tissue; high grade: vascular sclerosis of small and medium-sized arteries and veins with fibrointimal thickening; and fibrotic chronic vascular changes: complete allograft fibrosis. The categories of chronic airway rejection were as follows: low-grade chronic airway rejection: initial signs of intraluminal granulation tissue polyps in more than one terminal bronchiole or loose subepithelial fibrin structures around terminal bronchioles; high grade: BO with fibrosis of the submucosa of terminal bronchioles with partial or complete luminal occlusion; and chronic fibrotic airway changes: complete allograft fibrosis.

Immunofluorescence Staining

Immunofluorescence was performed on formalin-fixed paraffin-embedded lung tissue sections. After deparaffinization, antigen retrieval was performed in citric acid (pH 6.0) for 20 minutes at 80°C. Then the