Experimental and Toxicologic Pathology 65 (2013) 383–389

Contents lists available at SciVerse ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Suppression of bronchiolitis obliterans in allogeneic rat lung transplantation—Effectiveness of everolimus☆

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ARTICLE INFO

Article history:
Received 1 August 2011
Accepted 4 December 2011

Keywords:
Bronchiolitis obliterans
Vasculopathy
Everolimus
Animal model
Chronic rejection

ABSTRACT

Background: Chronic rejection (CR) after lung transplantation (LTX) manifests pathologically by fibrotic airway remodelling and bronchiolitis obliterans (BO). The role of the mammalian target of rapamycin inhibitor everolimus in preventing this process is poorly understood.
Methods: A rat model of left lung allo-transplantation (Fisher 344 to Wistar Kyoto) was used to analyze the effect of everolimus (2.5 mg/kg/day) on the development of CR. Drug therapy began on postoperative day (POD) 0, 7 and 14 characterizing different grade of acute rejection (AR) of the allograft before drug treatment.
Results: Non-treated recipients developed severe acute rejection (AR) and first signs of CR on POD 20 and a pronounced CR on POD 60. On POD 20, only application of everolimus from POD 0 to 60 significantly reduced acute inflammatory infiltration ($p < 0.001$). Independent of treatment scheme, everolimus suppressed the development of early signs of chronic alterations (POD 20). However, neither early (POD 7–60) nor late (POD 14–60) application of everolimus affected the progression of CR (POD 60). Only its initial treatment (POD 0–60) inhibited the development of BO and vasculopathy ($p < 0.001$). An additional finding was a decrease in body weight after drug application.
Conclusion: The effectiveness of everolimus after rat LTX depended on the grade of inflammation of the allograft before initiation of drug treatment. Only allografts with no or low grade AR benefit from long-term treatment with everolilmus in the prevention of BO after LTX. It could be speculated that conversion to an everolimus-based immunosuppression after LTX might only be successful in patients free of BO.

1. Introduction

Long-term outcome after lung transplantation (LTX) depended on the development of bronchiolitis obliterans (BO), a manifestation of chronic allograft rejection that affects more than 50% of recipients who survive the early post-transplant period (Trulock et al., 2007; Gourishankar and Halloran, 2002). The underlying mechanism includes repeated injury and inflammation of graft epithelial cells and subepithelial structures of small airways leading to extensive fibroproliferation due to ineffective epithelial regeneration and aberrant tissue repair resulting in partial or complete occlusion of the bronchioles (Stewart et al., 2007; Neuringer et al., 2005). Acute rejection (AR) and lymphocytic bronchiolitis are the major risk factors for chronic rejection (CR) (Hachem, 2009). In a retrospective study on 259 adult LTX recipients it was shown that already a single episode of minimal AR without recurrence or subsequent progression is a significant predictor of BO (Hachem et al., 2005). However, early diagnosis of AR is often difficult because of low sensitivity of lung biopsies including insufficient tissue collection and irregular sample collection. As a consequence, few AR episodes remained undetected and untreated. Moreover, only rejections ≥2 were assessed to be dangerous (Martinu et al., 2010). This aggravates an estimation of the allograft damage. This might be one reason for doubtful effectiveness of new immunosuppressive drugs after LTX. So far no treatment has reliably prevented the development or slowed the progression of BO.

The immunosuppressive and antiproliferative properties of the mammalian target of rapamycin (mTOR) inhibitor everolimus might be a promising therapeutic strategy after LTX (Nashan, 2002). Everolimus inhibited growth factor-driven lymphocyte proliferation, proliferation of nonhematopoietic cells (Nashan, 2002)

☆ SH, TP were supported by a grant from the Deutsche Forschungsgemeinschaft (No. HI-1333). MvS was founded by a grant from University of Regensburg (No. I101-03).
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doi:10.1016/j.etp.2011.12.002

and human lung fibroblasts in vitro (Azzola et al., 2004), and attenuates collagen deposition in experimental pulmonary fibrosis. The proliferation signal inhibitor effectively prevented graft rejection in rat models of allotransplantation (Schuler et al., 1997; Schuurman et al., 1997). The in vivo effects after LTX were shown in two different animal models. Preventive and continuous triple-drug immunosuppression (everolimus, cyclosporine, methylprednisolone) inhibited epithelial destruction and luminal obliteration in a heterotopic swine lung allograft model (Salminen et al., 2000). However, this model is non-physiological. The subcutaneous implantation of donor lung sections caused severe initial ischaemia, inadequate drug supply, non-physiological ventilation, missing anatomical airway structures, and short graft implantation time. The other model is a unilateral LTX rat model that benefits from a full organ transplantation allowing assessment of acute bronchial, vascular and parenchymal rejection. Only co-application of everolimus and cyclosporine prevented AR (Hausen et al., 2000). Monotherapy with everolimus had no immunosuppressive effect (Hausen et al., 1999). However, due to the usage of major histocompatibility complex (MHC)-mismatched strains of inbred rats this model could only be used to verify AR but not CR processes. The therapeutic range based on experiences with rat kidney and heart allotransplantation models (Schuler et al., 1997; Schuurman et al., 1997). The effective dosage ranged between 0.5 and 5 mg/kg/day. Hausen et al. (1999, 2000) used 2.5 mg/kg/day. The effectiveness of the drug was limited by side effects such as weight loss using continuous application of high doses of everolimus (Hausen et al., 1999; Schuurman et al., 1997). In the present study, we preferred the full organ model in a non-MHC allogeneic rat model (F344-to-WKY) to verify the impact of everolimus on the development of chronic allograft rejection (Matsumura et al., 1995; Hirt et al., 1999). This study included the initial state of the allografts defining different extent of premature damage of epithelial tissue and grading of AR.

2. Materials and methods

2.1. Animals

Male Wister Kyoto (WKY) and Fischer (F344) rats were purchased from Charles-River (Sulzfeld, Germany; 286 ± 30 g initial body weight) and kept under conventional conditions. All animals received human care in compliance with the Principles of Laboratory Animal Care formulated by the European Union Guide for the Care and Use of Laboratory Animals (publication No. 86/609/EWG). Approval was granted by the institutional ethical committee at the University of Regensburg.

2.2. Lung transplantation and follow-up

Orthotopic left LTX was performed in a moderate allogeneic F344-to-WKY ($n = 51$) rat strain combination and in the syngeneic WKY-to-WKY ($n = 20$) combination (Matsumura et al., 1995; Hirt et al., 1998). Briefly, donor lungs were removed and immersed in cold saline. The recipient animals were intubated and anesthetized. A left thoracotomy was performed, and the left lung was removed (ischaemia, 1 h). The left donor lung was placed into the recipient chest and pulmonary artery, pulmonary vein as well as the bronchus was anastomosed.

The general health status of the recipients was assessed by daily weight measurement and intermittent observation of grooming behavior and feces. Individual blood levels of everolimus were determined 20 and 40 days after initiation of drug therapy (24 h after drug administration) to estimate the stability of the blood levels. Blood was taken of the tail vein (0.5 ml) and analyzed by high performance liquid chromatography in the Department of Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg. Drug blood levels between 17 and 24 μg/l were measured.

2.3. Study design

Due to the difficulties to verify the degree of acute inflammation of the lung allograft in humans we selected different initiation times for our drug therapy to satisfy different acute rejection grades of the allograft. Rats in group 1 ($n = 15$) were not treated. In groups 2–4 animals received everolimus (RAD001, Novartis Pharma, Basel, Switzerland) (2.5 mg/kg body weight, intragastral). Drug treatment was in group 2 ($n = 15$) from postoperative day (POD) 14 to 60, in group 3 ($n = 9$) from POD 7 to 60 and in group 4 ($n = 12$) from POD 0 to 60. Microemulsion formulation of Everolimus for oral administration was provided to the investigators by Novartis Pharma (Basel, Switzerland). The drug was given in a single daily dose throughout the study. Dose was based on the individual daily weight of each rat. Rats were killed on POD 20 (group 1: 9; group 2: 5; group 3: 3; group 4: 6) and POD 60 (group 1: 6; group 2: 10; group 3: 6; group 4: 6). In addition, in each group, five syngeneic transplants were performed and euthanized on POD 60. Right lungs were used as an internal control.

2.4. Grading of acute and chronic rejection

For histological analysis of transplant rejection right native and transplanted left lungs from recipients rats were harvested, and fixed in 5% paraformaldehyde (Merck, Darmstadt, Germany). Three paraffin sections of 5 μm were prepared and stained with hematoxylin and eosin (HE) and two sections were stained with Masson-Goldner tricrome staining (MG) for grading rejection. The acute vascular rejection (A0–A4: degree of acute vascular rejection) and acute bronchiolar rejection (B0, B1R, B2R, BX: degree of acute airway inflammation) was graded according to the working formulation of The International Society for Heart and Lung Transplantation (ISHLT) (Stewart et al., 2007). For a detailed diagnosis of chronic lung rejection, the working formulation of the ISHLT was modified. C0 and D0 described lung sections with normal pulmonary parenchyma. Low grade chronic bronchiolar rejection classified allografts with first signs of intraluminal polyps of granulation tissue or loose subepithelial fibrin structures around ≥1 terminal bronchioles. C1/BO described dense fibrosis in the submucosa of terminal bronchioles with destruction of the smooth muscle cell layers of the airway wall. As a consequence the entire lumen could be a distored (constrictive bronchiolitis) or completely obliterated by scar tissue. An extension of scar tissue into the peribronchiolar interstitium was possible. Accordingly, low grade chronic vascular rejection was introduced to describe an obstruction of small vessels. Histological sections presented leukocytes adhered to the endothelium as well as luminal appearance of isolated fibroblasts occluding the small vessel lumen. D1 classified distinct perivascular fibrosis/fibrointimal thickening of the majority of small and medium sized vessels including an extension of perivascular fibrosis into adjacent interstitium.

2.5. Statistical analysis

Histological scoring was performed by a single investigator (blind fashion). For a statistical analysis, we used the proportion of high grade AR (ISHLT-A4, ISHLT-B2R) to compare Everolimus-treated groups vs. the non-treated group (group 1). To verify chronic alterations, we compared the proportion of animals free of CR (= no CR) from group 1 vs. groups 2 to 4 with first signs of CR and a manifest BO and vasculopathy. We used the test of hypothesis for the difference between population proportions (www.morris.umn.edu/~sungurea/statlets/free/tstesthypotpropstatlets.html). 95%