Transplant Immunology 35 (2016) 12–17

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Effects of FTY720 on peripheral blood lymphocytes and graft infiltrating cells in a rat model of chronic renal allograft rejection☆

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ARTICLE INFO

Article history:
Received 6 October 2015
Received in revised form 15 January 2016
Accepted 18 January 2016
Available online 20 January 2016

Keywords:
FTY720
Kidney transplantation
Rat model
Graft infiltrating cells
Chronic rejection

ABSTRACT

Aims: Chronic renal allograft loss is still an unsolved problem in kidney transplantation. We evaluated the impact of FTY720, a S1P receptor agonist, known to deplete lymphocytes from the peripheral blood by sequestering them into lymph nodes and Peyer's patches, on blood lymphocytes and graft infiltrating cells in a rat model of chronic real allograft rejection.
Methods: LEW rats served as recipients for LEW.1U7B kidney grafts. All animals were treated with CsA (5 mg/kg) for 10 days after renal transplantation and monitored for kidney function, peripheral blood lymphocytes and graft infiltrating cells.
In the intervention group (n = 7) FTY720 therapy was started 7 weeks post-KTx in a dose of 0.5 mg/kg p. o. three times a week.
Results: In the control group the survival of the rats was 9, 11, 18 and 4 × 24 weeks, in the intervention group 2 × 8, 9, 2 × 11, 18 and 20 weeks. While in the intervention group the number of T- and B-lymphocytes in the peripheral blood was successfully reduced during FTY720 treatment, both groups showed significant amounts of T- and B-lymphocytes in the kidney grafts. Animals in both groups developed donor specific antibodies, extensive albuminuria and severe chronic changes in the grafts.
Conclusions: FTY720 was highly effective to reduce T- and B-lymphocytes in the peripheral blood, but not effective in clearing their infiltration in the graft. Graft survival was not prolonged by FTY720 treatment starting late after kidney transplantation.

1. Introduction

Chronic renal allograft failure caused by immunological as well as non immunological reasons is still a major obstacle in clinical kidney transplantation (KTx). Due to spare therapeutic options to treat chronic graft loss the search for new immunosuppressive drugs is ongoing. FTY720 (fingolimod) is one of these drugs and, as a prodrug of a sphingosine-1-phosphate (S1P) receptor agonist, it has a unique mechanism of action, compared to common immunosuppressive medications.

FTY720 does not prevent lymphocyte activation or proliferation, but causes homing of lymphocytes to the primary and secondary lymphoid organs and reduces egress from these organs [1–4]. Nephrotoxic side effects as known for calcineurin inhibitors (CNI) are not described for FTY720, therefore it was evaluated in the clinical organ transplantation and autoimmune diseases. FTY720 is now approved for the treatment of multiple sclerosis [5].

Regarding its potential use in organ transplantation FTY720 has been shown in preclinical studies to reduce fibrosis after ischemia reperfusion injury in heart transplantation [6], 5/6 nephrectomie [7] and to prolong allograft survival in different animal studies [1,8,9]. Clinical studies in renal transplanted patients combining FTY720 with CNI demonstrate adequate protection from acute rejection, but a less favorable profile of adverse events, resulting in a withdrawal of FTY720 as an immunosuppressive agent in solid organ transplantation [10,11].

In this study we addressed the effects of FTY720 therapy that started in the late course after KTx in a rat model of mixed cellular and humoral chronic rejection [12,13]. We monitored the impact of the drug on peripheral blood lymphocytes (PBL) and on graft infiltrating cells. Furthermore we monitored alloantibody development and allograft function.

2. Objectives

Due to its ability to reduce the egress of lymphocytes from the secondary lymphoid organs and to home lymphocytes to the secondary

Abbreviations: CNI, calcineurin inhibitor; CsA, cyclosporine A; hpf, high power field; IF/TA, interstitial fibrosis/tubular atrophy; KTx, kidney transplantation; MHC, major histocompatibility complex; MFI, mean fluorescence intensity; PBL, peripheral blood lymphocytes; S1P, sphingosine-1-phosphate; SD, standard deviation.

☆ This study was supported by Novartis Pharmaceuticals Corporation, Basel, Switzerland and Novartis Pharmaceuticals Corporation, Nuernberg, Germany.

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http://dx.doi.org/10.1016/j.trim.2016.01.003
0966-3274/

lymphoid organs FTY720 might lead to a reduced cellular infiltration of transplanted organs and result in the modulation of allograft rejection.

We used a MHC mismatched rat model of chronic cellular and humoral renal allograft rejection to analyze the impact of FTY720 treatment on peripheral blood lymphocytes and graft infiltrating cells.

3. Animals, material and methods

3.1. Kidney transplantation

Male inbred Lewis rats (LEW) served as recipients for male inbred LEW.1U7B rats (kindly provided by K. Wonigeit, Hannover, Germany). Donors and recipients were weight matched and 3–4 month old. Genetically, there is a complete MHC incompatibility between donor and recipient on the LEW background. Additionally there is a difference in the common leukocyte antigen RT7.

Renal transplantation was carried out as described previously [12,13]. In short kidney transplantation was performed heterotopically to the recipients' aorta and vena cava with end to end ureter anastomosis without stenting. Ischemia time was kept less than 30 min. The left native kidney of the recipient was excised simultaneously during transplantation, whereas the right kidney was removed five to seven days after transplantation.

All animals were bred and maintained at the Zentralinstitut fuer Versuchstierzucht, Medizinische Hochschule Hannover, Germany. They were housed under standard conditions and fed with water and rat chow ad libitum. All animal experiments were carried out according to the principles of laboratory animal care.

3.2. Immunosuppression

To prevent acute rejection in this complete MHC mismatched model cyclosporine A (CsA, Sandimmune, Novartis, Basel, Switzerland) was administered for 10 days in a dose of 5 mg/kg bodyweight s.c. in all animals [13].

In the control group ($n = 7$, group 1) no further treatment was carried out. In the FTY720 group ($n = 7$, group 2) rats received FTY720 orally three times a week in a dose of 0.5 mg/kg bodyweight starting seven weeks after transplantation in rats with stable renal function.

3.3. Immunological monitoring

To analyze the changes in PBL during FTY720 therapy blood samples were taken every other week for flow cytometry. PBL were obtained by treatment of EDTA blood samples with erythrocyte lysis buffer (Ortho Diagnostics, Neckargemund, Germany). Samples of $0.5–1.0 \times 10^6$ cells were incubated with primary mAb, washed twice and stained with the secondary antibody (fluorescein (DTAF)-conjugated F(ab′)2 fragment goat-anti-mouse IgG + IgM (Dianova, Hamburg, Germany)). For double labeling, cells were incubated with normal mouse serum to prevent cross-reaction before using the second biotin coupled primary antibody followed by incubation with PE-streptavidin conjugate (BD, Heidelberg, Germany). Fluorescence analysis was performed on a Becton Dickinson FACScan or FACScalibur. 1×10^4 cells measured with a standardized lymphocyte live gate were accumulated on logarithmic scales and analyzed using a cell quest computer program (BD, San Jose, USA).

Differential blood counts were performed to calculate the number of lymphocytes per μl.

The following antibodies were used for flow cytometry: NK-cells (3.2.3)/CD4 (W3/25Bio), CD4 (W3/25)/CD3 (G8.14Bio, BD Heidelberg, Germany), and CD3 (G8.14Bio)/CD8 (MRC OX-8) for double labeling and B-cells (Ki-B1R) for single staining.

Four representative kidneys of each group were analyzed by immunohistochemistry for graft infiltrating cells as described previously [14] by using the following antibodies: R73 (TCR constant determinant; BioLegend, Germany), ED1 (CD68, pan-macrophage marker from Serotec, Germany), Ki-B1R (pan B-cell marker; Dianova, Germany) and 3.2.3 for NK-cell staining (BD Heidelberg, Germany).

3.4. Renal function

Serum creatinine (s-creatinine) was determined weekly colorimetrically with a Beckman Creatinine Analyzer (Beckmann Instruments, Inc., Galway, Ireland). For determination of 24 h urinary albumin (u-albumin) excretion, the rats were placed into metabolic cages every other week. U-albumin concentrations were quantified by an enzyme-linked immunosorbent assay (ELISA) specific for rat albumin (Nephrat II, Exocell, Inc., Philadelphia, USA) with a range of 1–100 mg/dl. Urine samples were diluted to receive a diagnostic range up to 500 mg/dl. To evaluate clinical status of health of the animals, all rats were weighed weekly. The experiment was terminated 24 weeks after transplantation or in case of severe weight loss (>20%), serum creatinine above 400 μmol/l (not reached during this study) or overall impaired condition of the animals in accordance with the principles of laboratory animal care.

3.5. Histopathology

For light microscopy renal tissue samples were fixed in 4% buffered formalin, embedded in paraffin and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) and Masson-Trichome. All grafts from the FTY720 group and 6/7 graft of the control group were analyzed.

3.6. Measurement of alloantibodies in sera

Antibodies were detected as described previously [13]. In short PBL from LEW (recipient, negative control) and LEW.1 U (donor MHC) rats served as antigen carriers for flow cytometric analysis. LEW.1 U ($RT1^{uuu}$) rats were bred on a LEW background and share the MHC with the kidney donor (LEW.1U7B), but all other characteristics including RT7 with the kidney recipient (LEW).

Serial samples of recipient sera were analyzed including pretransplant sera as additional negative control. Antibodies bound to the indicator cells were marked with a fluorescein (DTAF)-conjugated goat-anti-rat IgG (H + L) antibody (Dianova, Hamburg, Germany). Binding of donor specific antibodies was evaluated by comparing the mean relative fluorescence intensity of the fluorescein-conjugated anti-rat IgG mAb on non antibody presenting cells (T-lymphocytes) of the pretransplant sera with posttransplant samples.

3.7. Statistical analysis

Graft survival was analyzed using a Kaplan–Meier survival curve and the Log-rank test using GraphPad Prism version 6.04 for Windows (GraphPad Software, USA). Unpaired two-tailed *t*-test was used to compare creatinine and albumin values, PBL numbers and graft infiltrating cells between both groups. P values ≤ 0.05 were given as statistically significant.

4. Results

4.1. Renal function and survival

Weight gain of the animals was comparable in both groups until week 7 post-KTx. After the start of FTY720 treatment animals lost weight while control animals continued to gain weight. Rats sacrificed before the programmed end of the experiment (24 weeks) suffered from weight loss, increased s-creatinine and impaired general condition. The mean s-creatinine at six weeks posttransplantation in the FTY720 group was 90 μmol/l (range 60–120) and in the control group 81 μmol/l (range 50–130). At time of death animals in the FTY720 group had a s-creatinine of 133 μmol/l (range 120–160) and the