Impact of the severity of acute rejection in the early phase after rat lung transplantation on the effectiveness of mycophenolate mofetil to treat chronic allograft rejection

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INTRODUCTION

Although lung transplantation (LTX) is a life-saving intervention for patients with end-stage lung disease, survival rates are still limited by chronic rejection (CR) [1]. CR after LTX is characterized by pulmonary artery intimal proliferation, interstitial fibrosis and most notably, bronchiolitis obliterans (BO) [1], an inflammatory/fibrotic process affecting the small non-cartilagenous airways [2]. BO affects ~50% of recipients within 5 years of LTX and accounts for 29% of deaths between years 3 and 5 post-LTX [1]. Despite improved management strategies to reduce early complications, there are no effective therapies for BO [1]. Optimization of the immunosuppressive regimen might be crucial for improving the long-term outcome [3]. However, to date no consensus has been reached concerning a standardized immunosuppressive regimen, and none of the contemporarily employed immunosuppressive drugs in pulmonary transplantation could prove its superiority [3]. Besides, none of the clinical trials regarded the relevance of the severity of acute rejection (AR) of the allografts in the early phase after LTX. Owing to the difficulties in the early diagnosis of AR in lung biopsies, few AR episodes remained undetected, untreated and increased the risk of allograft damage [2].

Immunosuppressive strategies included minimizing/avoiding AR as well as the development of fibroproliferative responses [2]. Mycophenolate mofetil (MMF) is approved for the prophylaxis of AR after renal, cardiac or liver transplantation [4]. In addition, it appeared to be able to delay the onset of cardiac allograft vasculopathy and to reduce its progression, especially by their inhibitory effects on smooth muscle cell migration and proliferation [5]. As a reversible inhibitor of inosine-5-monophosphate...
dehydrogenase MMF depletes guanosine nucleotides in T- and B-lymphocytes and inhibits their cell cycle, thereby suppressing cell-mediated immune responses and antibody formation [4]. In addition, MMF is a potent anti-fibrolroliferative drug which is effective at clinically relevant concentrations [6], supporting their use for the treatment of patients with early BO.

Only a few clinical trials have been undertaken, but without an unambiguous proof of the superiority of MMF to prevent the development of BO [7–9]. We hypothesized that the effectiveness of MMF in the treatment of chronic lung allograft rejection depended on the severity of AR at the time of drug initiation. An orthotopic rat LTX model using moderately histoincompatible strains of Fisher (F344) and Wistar Kyoto (WKY) rats [10, 11] allows the evaluation of the efficacy of MMF to reduce/avoid the development of BO. Our data indicated that early treatment with the nucleotide-blocking agent reduced the extent of AR and delayed the development of CR after rat LTX. However, the efficacy of MMF failed in recipients with first signs of chronic airway rejection after LTX.

MATERIALS AND METHODS

Study groups

Allogeneic [F344(RT1lvl)-to-WKY(RT1l)] and syngeneic [WKY-to-WKY] rat orthotopic left LTX were performed as described previously [10, 11]. All rats were purchased from Charles-River (Sulzfeld, Germany; 280 ± 20 g initial body weight) and received human care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for Care and Use of Laboratory Animals (National Institutes of Health, No. 85-23, 1985).

In Group 1 (n = 15) rats were without treatment. The dosage of MMF (Novartis Pharma, Basel, Switzerland) was based on own experiences and other animal studies (6–40 mg/kg/day) [12, 13]. Application of 40 mg/kg/day in our rats increased early side effects (early anaemia, worse grooming behaviour, diminished appetite). Owing to ethical aspects, we stopped this drug regimen and reduced dosage to 30 mg/kg/day. Animals of Group 2 (n = 10) received MMF [intraperitoneally (i.p.); daily] from postoperative day (POD) 14 to 60. In Group 3 (n = 9), animals were treated with MMF from POD 7 to 60 and in Group 4 (n = 14) animals received MMF from POD 0 to 60. To ensure that all animals in each treatment group received the designated amount of drug per body weight, the volume of drug administered was determined by each animal’s daily weight. Rats were killed on POD 20 (Group 1: n = 9; Group 2: n = 5; Group 3: n = 3; Group 4: n = 6) and POD 60 (Group 1: n = 6; Group 2: n = 5; Group 3: n = 6; Group 4: n = 8). In addition, in each group, five syngeneic transplants were performed up to POD 60.

Therapeutic drug monitoring

On POD 12, 20 and 60, 24 h after the preceding drug administration, blood samples (0.5 ml) were taken from blood vessels of the tail and were collected in ethylenediaminetetraacetic acid containing tubes. These samples were used to determine the individual blood concentrations of mycophenolic acid (MPA), the active metabolite of MMF. Analysis was performed by high performance liquid chromatography in the Department of Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg.

Acute and CR

For histological analysis, right and left lungs from recipients were harvested. The tissue was fixed in 5% paraformaldehyde (Merck, Darmstadt, Germany) to prepare paraffin sections (5–6 μm) and to stain with haematoxylin and eosin (HE) and Masson-Goldner tricine staining (MG) for grading rejection according to the working formulation of The International Society for Heart and Lung Transplantation (ISHLT) [14] [A0–A4: degree of acute vascular rejection; B0, B1R, B2R, BX: degree of acute airway inflammation; C0, C1: chronic airway rejection (BO); D0, D1: chronic vascular rejection]. To describe the development of first signs of chronic alterations after LTX, we modified the chronic characterization from Stewart et al. [14] as mentioned in the legend of Fig. 3.

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 MMF-treated groups vs the non-treated group (Group 1). To verify chronic alterations, we compared the proportion of animals free of CR (= no and low-grade CR) from Group 1 vs Groups 2–4. We used the test of hypothesis for the difference between population proportions (www.morris.umn.edu/~sungurea/statlets/free/tstesthypotpropstatlets.html). 95% confidence intervals for the difference between group proportions were used to approximate statistical significance (P < 0.05). Unpaired Mann–Whitney rank sum test was used to evaluate statistical differences of body weights. Statistical software SPSS 16.0.2 (SPSS Inc., Chicago, IL, USA) was used. Data are presented as median and interquartile range (25th/75th percentiles). P < 0.05 was considered statistically significant.

RESULTS

Body weight and side effects

The body weight of all animals decreased significantly after transplantation (Fig. 1). While in non-treated animals (Group 1) maximum weight loss was achieved after 2.4 ± 1.4 days [9.1%...
(7.5/10.0) from initial body weight], rats with initial MMF treatment (Group 4) showed a maximum weight loss after 5.7 ± 1.8 days \((P < 0.001)\) [12.2% (10.5/15.6) from initial body weight, \(P = 0.008\)]. Rats from Groups 1 and 2 regained their initial body weight within 8.3 ± 2.9 days. The same was observed for rats from Group 3 (8.4 ± 3.5 days). Initial MMF treatment (Group 4) prolonged this lag of time significantly (20.2 ± 6.4 days, \(P < 0.001)\). Independent of application start, MMF treatment delayed weight gain. Long-term treatment with MMF (from POD 50 to 60) significantly reduced body weight of transplanted rats [(median (25th/75th percentiles); Group 1: 141% (134/155); Group 2: 122% (119/131), \(P = 0.016\); Group 3: 118% (115/124), \(P = 0.002\); Group 4: 118% (117/122), \(P < 0.001\)] (Fig. 1). There was no statistical difference comparing MMF-treated rats from Groups 2–4. The same weight loss was documented of MMF-treated syngeneic rats (data not shown). All animals survived the transplantation procedure independent of drug treatment. Prolongation of drug treatment (>POD 60) increased the side effects of MMF: All MMF-treated animals experienced diminished appetite, compromised food intake or weight loss greater than 5% of their body weight (within 1 week). Ten to twenty percent of MMF-treated animals had diarrhoea. Eighty percent of the rats developed anaemia after POD 60 [erythrocytes, 3.5 ± 2.4 \text{pl}^{-1} (reference, 7–10); haemoglobin, 7.8 ± 4.8 g/dl (reference, 11–18)]. However, the amount of leucocytes remained in a normal range [10.9 ± 11.0 \text{nl}^{-1} (reference, 6–17)].

MPA trough levels

Rats were treated with 30 mg MMF per kg body weight once a day i.p. After a mean application time of 11.8 ± 1.5 days, the blood levels were 0.6 (0.5/0.7) \mu g/ml MPA. Over time, trough levels of MPA remained unchanged [POD 20: 0.35 (0.2/0.6) \mu g/ml, \(P = 0.190\); POD 60: 0.8 (0.8/1.3) \mu g/ml, \(P = 0.111\)].

**Effect of MMF in the early phase after LTX**

Table 1 summarized the degree of acute vascular and airway rejection in the early phase after LTX (POD 20). Figure 2 shows

![Figure 2: Representative histopathology of acute vascular rejection and airway inflammation (POD 20, HE-staining). Allografts from Group 1 (A) and Group 2 (B) show diffuse perivascular (long arrow), interstitial and air-space infiltration of mononuclear cells associated with prominent alveolar pneumocyte damage (stars) (A4). Terminal bronchioles (tB) show an intense mucosal and peribronchiolar mononuclear cell inflammatory infiltrate involving the epithelium with a distinct epithelial damage (short arrow) (B2R). Allografts from Group 3 (C) demonstrate moderate acute vascular rejection (A3), cuffing of mononuclear cells around small vessels (V, long arrow), extension of inflammatory cells into perivascular and peribronchiolar alveolar septa/airsaces (AS) (short arrow) and not-affected respiratory epithelium. Allografts from Group 4 (D) present perivascular cuffing (long arrow) without spreading into adjacent AS (A2) and scattered mononuclear cells surrounding tB (B1R).](https://academic.oup.com/ejcts/article-abstract/42/1/142/356444)
Effect of MMF on the development of BO

Figures 3 and 4 presented the data of chronic vascular and airway rejection on POD 60. Non-treated rats (Group 1) developed pronounced chronic airway rejection with evidence of BO. In addition, all allografts presented chronic vascular rejection with accelerated graft vascular and interstitial fibrosis (Fig. 3). As shown in a representative histology in Fig. 4A, transplanted lungs developed partial complete occlusion of respiratory bronchioles, extensive fibrointimal thickening of small vessels and advanced interstitial changes (proliferative phase of DAD). In >50% of the animals, persisting inflammatory infiltrations were present. However, the extent of inflammatory infiltration decreased with aggravated fibrosis. Late application of MMF (Group 2) did not improve long-term outcome. As shown in Fig. 3, only 20% of the allografts were without BO (non-significant), but these lungs developed a pronounced vasculopathy. Most of the remaining allografts developed BO and vasculopathy as well as persisting mononuclear cell infiltration (Fig. 4B) as seen in non-treated rats. Early application of MMF delayed the development of chronic alterations. Treatment with MMF from POD 7 to 60 (Group 3) already reduced the proportion of animals with high grade chronic vascular alterations (P = 0.003) as well as chronic bronchiolar rejection (P < 0.001) and interstitial destructions (Fig. 3). In addition, these allografts were graded with ISHLT-A 2-3 and ISHLT-B1R-2R (Fig. 4C). LTX with concurrent immunosuppression (Group 4) completely prevented the development of BO and chronic vascular rejection (each P < 0.001) (Fig. 4D). The respiratory epithelium in the small bronchioles was preserved. In addition, there was only minimal acute inflammation.

DISCUSSION

To date, there were no data on the primary effect of MMF on the development of BO. Therefore, we used the F344-to-WKY rat LTX model, the only available model of CR after LTX to study the impact of MMF on the development of BO [10, 11]. The...
immunosuppressive activity of MMF depended on the severity of AR in the early time after LTX. While MMF therapy did not improve the outcome of allografts with high grade acute inflammation and first signs of chronic alterations (Group 2), drug treatment of allografts with low grade vascular and airway damage delayed/prevented the development of BO (Groups 3 and 4).

In the present study, we showed that a monotherapy of MMF reduced the acute inflammatory rejection after allogeneic LTX in rats. The degree of inhibition of AR depended on the time point of drug initiation. The classification of our study groups was based on histopathological observations from Matsumura et al. They described a progression of histological signs of AR and peribroncholar lymphocytic infiltrations within 21 days after LTX. On POD 7 the mean pathological stage of AR was diagnosed with ISHLT-A2/-B1R reaching maximum rejection on POD 14 (ISHLT-A3-4/-B2R) which persisted up to POD 21. Based on these histopathological alterations, we analysed the immunosuppressive activity of MMF and initiated drug therapy on POD 0, POD 7 and POD 14. In our non-treated rats (Group 1), maximum AR was diagnosed on POD 20. The effectiveness of MMF depended on the time point of drug initiation and on the presence of the first signs of chronic alterations of the allograft. The presence of maximum AR on POD 14 could not be reduced by MMF application (Group 2). However, a lower grade of acute vascular and airway rejection on POD 7 might be a prerequisite for promising drug treatment. Despite a low animal size (n = 3), the proportion of animals with high grade acute airway inflammation decreased (compared with Groups 1 and 2). Maximum immunosuppressive activity was shown in Group 4. However, there was no complete suppression of the acute inflammatory reaction. The primary immunosuppressive activity of MMF benefits from the inhibition of the de novo synthesis of guanine nucleotides, a prerequisite for inhibition of lymphocyte proliferation. However, all allografts from Group 4 developed low grade airway inflammation and mild to moderate acute vascular rejection. We supposed that a monotherapy of MMF is an insufficient immunosuppressive therapy to prevent AR after rat LTX. Other pathways such as inhibition of calcineurin and mammalian target of rapamycin (mTOR) became more relevant. Triple drug immunosuppression is the standard therapy after human LTX. Our rat model has a potential drawback: While our selected concentration of MMF agrees with the clinical dosage of 2 g/day (=30 mg/kg/day of a 70 kg patient), the trough levels of MPA were just about half the concentration of that in humans. This might be the reason for insufficient inhibition of AR and the development of first signs of chronic alterations in the long-term follow-up. One consequence would be the increase in the dosage of MMF in our rat model. This strategy failed because of an aggravation of toxic side effects. Already at a concentration of 30 mg/kg/day, side effects manifested in a decreased weight gain and development of anaemia and worsening of grooming behaviour after long-term application (>60 days). The good tolerability of high concentrations of MMF in other animal studies might result from lower application times (<30 days). Similar side effects of MMF were also identified in few patients: gastrointestinal disorders (13–64% of patients), leucopenia (10–35% of patients) and anaemia (9–15% of patients).

The benefit of MMF on the development of BO after LTX was also debatable. Additional mechanisms of MMF include the inhibition of post-transplant proliferation of fibroblasts and smooth muscle cells, inhibition of antibody production affecting acute humoral rejection, inhibiting adhesion molecules that...
facilitate the migration of immune cells towards an allograft [17] and inhibition of nitric oxide synthase that affects processes injurious to an allograft via interactions with superoxides [18]. Furthermore, in vitro studies proved that MMF can trigger an epithelial-mesenchymal-transition response in bronchial epithelial cells [19]. All of these reactions were speculated to be part of the multifactorial process of chronic allograft rejection in form of BO [2]. Under in vivo conditions using a heterotopic tracheal transplantation model in the rat [20] in time, application of MMF prevented the development of obliterative airway disease (OAD). In this model, OAD represents a histological equivalent to BO in the lung. Implantation of tracheal segments into the rat and treatment with 40 mg/kg/day MMF (POD 0–27) resulted in inconsistent and varied luminal obliteration with a mean obliteration of 47%. Epithelium was flattened cuboidal, sporadic and interrupted in coverage, and only seen lining tracheas with minimal obliteration. Delayed application aggravated obliteration to 93% and epithelium disappeared. In contrast, treatment with cyclosporine or rapamycin virtually prevented obliteration and preserved luminal coverage [20]. This model advanced our understanding of the possible underlying molecular mechanisms of BO. However, this model is non-physiological and limits its comparability to clinical reality. Problems with this model are initial ischaemia, diffusion restriction, missing physiological ventilation, no difference between large and small airways, no adequate vascularization to allow optimal drug supply and finally, a short span of time to develop OAD. Therefore, orthotopic LTX models were used to simulate the surgical procedure of human LTX [21]. We approved data from Adams et al. [20]. However, the usage of the F344-to-WKY LTX model allows insights into the development of CR using histopathological diagnosis of whole lungs. In the early phase after LTX (POD 20), non-treated animals presented the first signs of chronic alterations. The progression of the underlying processes might be responsible for the fatal outcome on POD 60 with mostly complete vascular, interstitial and bronchiolar fibrosis of all allografts. The immunosuppressive activity of MMF on the development of BO was shown in Group 4. Initial treatment of transplanted rats with MMF (Group 4) not only reduced the extent of airway inflammation, but also prevented the development of BO and chronic vascular rejection. Up to POD 60, only minimal to moderate acute vascular rejection persisted. In particular, bronchial structures remained unchanged and functional. Similar results were diagnosed after delayed MMF application (Group 3). In this group, MMF could not stop the progression of low grade chronic vascular as well as airway alterations. However, up to POD 60, none of the animals developed a BO. Late application of MMF (Group 2) aggravated the development of BO and accelerated graft vascular sclerosis. We concluded that the presence of the first signs of chronic allograft alterations might be the reason for non-effectiveness of MMF. These alterations were mostly not detectable in the clinical situation. The validity of lung biopsies (transbronchial or surgical) is critical [22]. A high amount of biopsy specimens per procedure is required for a high diagnostic yield. This is associated with significant incidence of complications [22]. There is only one randomized prospective clinical trial to elucidate the value of MMF in pulmonary transplantation [8]. This international multicenter study with a follow-up over 3 years in 320 patients did not show a significant difference between MMF and azathioprine for incidence of BO and survival. However, the study protocol did not include any control of the pharmacokinetics of MMF (missing drug level control). As discussed above, the risk for insufficient immunosuppression might also be responsible for non-effectiveness of MMF in the development of BO. The non-effectiveness of MMF was also shown in the treatment of lung transplant recipients with diagnosed BO. Conversion to MMF can stabilize graft function only in a small percentage of the converted patients [8]. These authors speculated that earlier administration of mTOR-based immunosuppression might be a more promising approach. In this context, we speculated that the additional application of other immunosuppressive drugs such as calcineurin inhibitors or steroids might be only successful on allografts with a low grade of acute inflammatory rejection. Owing to the difficulties in the early diagnosis of AR after LTX few AR episodes remained undetected and untreated. Moreover, only ARs ≥2 were assessed to be dangerous [23]. This aggravates an estimation of the allograft damage in the early phase after LTX. In addition, a closer examination of pulmonary function seems to be helpful in the clinical situation. One might argue that BO still develops because of its many pathogenic factors, and that, therefore, single therapeutic interventions will fail to detect significant differences with respect to this outcome parameter [2].

The application of a monotherapy of MMF in our rat model limited the clinical transferability. In future studies, we will analyse the impact of multiple immunosuppressive combinations. The underlying animal model (F344-to-WKY) was frequently discussed in the literature [21]. Similar to most scientific groups, our present data provided striking evidence of this model to reliably demonstrate the development of BO without a further stimulus. The partial inhibition of AR in the early time after rat LTX allowed us to verify different stages in the development of CR. Especially, timely application of MMF increased the prevalence of early chronic vascular and airway alterations as relevant precursors in the development of BO.

In conclusion, MMF significantly reduced AR and CR after orthotopic rat LTX. However, only allografts with no or mild AR at the time of drug initiation benefited from MMF treatment. In addition, the appearance of early signs of fibroproliferative alterations in the airway structures might prevent a successful long-term outcome.

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REFERENCES


[18] Senda M, DeLustro B, Eguíl E, Natsumeda Y. Mycophenolic acid, an inhibitor of IMP dehydrogenase that is also an immunosuppressive agent, suppresses the cytokine-induced nitric oxide production in mouse and rat vascular endothelial cells. Transplantation 1995;60:1143–8.


