Multiglycosidorum tripterygii versus Tacrolimus for rat tracheal allografts

Ryoichi Nakanishi*, Kosei Yasumoto

Second Department of Surgery, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan

Received 2 December 2004; received in revised form 14 April 2005; accepted 19 April 2005; Available online 29 August 2005

Abstract

Objective: Several other immunosuppressive agents still need to be found for rejection as alternatives to Tacrolimus in lung transplantation. We tried to elucidate the treatment effect of Multiglycosidorum tripterygii on tracheal allografts in comparison to that of Tacrolimus. Methods: Treatment effect of agents on tracheal allografts, undergoing incomplete immunosuppression for 12 weeks after transplantation, was investigated using a heterotopic rat tracheal transplantation model. Treatments with Tacrolimus (1.0 or 1.5 mg/kg per day), Multiglycosidorum tripterygii (150 or 225 mg/kg per day) and a combination of Tacrolimus (1.0 mg/kg per day) and Multiglycosidorum tripterygii (150 mg/kg per day) were applied as a therapy for allografts. Four weeks after administering this therapy, the effect of each treatment was investigated by the morphologic assessment of transplants. Results: Treatment group with high doses of Multiglycosidorum tripterygii demonstrated a significantly better graft patency and lower cartilage dislocation than that without any treatment and tended to show better morphological findings than the other treatment groups, in addition to being safe. Some of allografts with high doses of Tacrolimus or Multiglycosidorum tripterygii therapy had a viable epithelium and viable tracheal glands in part, whereas the allografts with other treatments showed almost a completely denuded epithelium. High doses of Multiglycosidorum tripterygii therapy demonstrated less infiltration of mononuclear cells into the allografts, whereas other therapies showed a higher infiltration of such cells. Conclusions: We conclude that high doses of Multiglycosidorum tripterygii may be a useful alternative to Tacrolimus as an immunosuppressant for rat tracheal allografts.

Keywords: Allograft; Animal model; Lung transplantation; Rejection; Trachea

1. Introduction

Obliterative bronchiolitis (OB) as a form of chronic graft rejection affects approximately 30% of all lung transplant recipients and remains the leading cause of death after lung transplantation [1]. Persistent or recurrent acute rejection is the primary risk factor for OB, although a cytomegalovirus infection and airway ischemia may also play a role in this disease. In the management of such refractory rejection, Tacrolimus has been effective and a useful alternative to Cyclosporine as an immunosuppressant [2]. Currently, the Tacrolimus is used as one of the essential agents of a standard maintenance immunosuppression for clinical lung transplantation.

We have studied the immunosuppressive effect of Multiglycosidorum tripterygii (MT; Taizou Pharmaceutical Factory, Jiangsu Province, China) on rat tracheal allografts since Hachida et al. reported a successful treatment effect of MT on cardiac allografts [3]. We demonstrated the efficacy of MT acceptable for rat tracheal allografts in our previous experiments [4]. On this occasion, we investigated the treatment effect of MT in comparison to that of Tacrolimus on tracheal allografts, undergoing incomplete immunosuppression after transplantation to make an experimental model closer to a clinical situation.

2. Materials and methods

2.1. Animals and anesthesia

Forty-two male Lewis rats and 54 male Brown Norway rats which were 5 weeks old and weighed approximately 120 g were used for experiment. All were premedicated and anesthetized with the intraperitoneal administration of sodium pentobarbital (10 mg/kg). Either harvesting or transplantation was performed with the animals breathing spontaneously without an endotracheal tube after the animals were placed in the supine position. All animals received humane care in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Harvesting of tracheas

The donor animals (42 Lewis rats and six Brown Norway rats) were put to death with an intraperitoneal overdose of...
pentobarbital and were left at room temperature (24 °C). After the donor animals were killed, the whole trachea was identified through a midline cervical to sternal incision and it was excised in continuity. The harvested trachea was trimmed to five-ring segments. Forty-eight tracheal segments in total were retrieved.

2.3. Grafting

The heterotopic tracheal transplantation model implanting in the omentum was carried out as previously described [4]. One tracheal segment was implanted in each recipient rat.

2.4. Experimental design

The model of tracheal allografts, without undergoing any steady immunosuppressive therapies for 12 weeks after transplantation, was designed and made first. After two cycles of immunosuppression designed to administer 1.5 mg/kg per day of Tacrolimus for 3 consecutive days both immediately after and 4 weeks after transplantation, only a one-day administration of 1.5 mg/kg of Tacrolimus was performed at the eighth week after the start of experiment (Fig. 1). Four weeks after the last medication, a second laparotomy was performed to retrieve the transplants for a histopathologic study (group 1: n = 6). After an evaluation of the characteristic histopathology of rejection on this preliminary experiment, several therapies for this model were assessed. In group 2, isogeneic transplantation without any other treatment was performed (Fig. 1). After undergoing the same cycles of immunosuppression as that of group 1, the remaining 36 animals who underwent an allogeneic transplantation were randomly allocated into six treatment groups as follows: group 3, no immunosuppression; group 4a, 1.0 mg/kg per day of Tacrolimus; group 4b, 1.5 mg/kg per day of Tacrolimus; group 5a, 150 mg/kg per day of MT; group 5b, 225 mg/kg per day of MT; and group 6, 1.0 mg/kg per day of Tacrolimus and 150 mg/kg per day of MT. These agents were administered for only 3 consecutive days from 4 weeks after the last medication (Fig. 1). Tacrolimus diluted by saline solution was administered intramuscularly. MT was given orally by gavage in a suspension form. The suspension was made from the powder and 0.5% hydroxy-propyl-methyl-cellulose diluted at a concentration of 10 mg/ml before application. The method for both producing and then feeding the MT suspension to rats was similar to that of Hachida et al. [3]. At 16 weeks after surgery, we performed a second laparotomy to retrieve the transplants for a histopathologic study in groups 2–6 (n = 6, respectively).

2.5. Morphologic assessment

Morphologic assessment was performed in a blinded fashion as previously described. Briefly, we attempted to quantify the viability of the heterotopically grafted trachea by objectively calculating the percent patency of the cross-sectional area (CSA) of the middle part of the graft and by calculating the ratio of chondrocytes possessing a viable nucleus in the cartilage, and by subjectively evaluating the morphology of the epithelium and cartilage. Infiltration by mononuclear cells was also assessed, because this is closely associated with allograft rejection [4–7].

2.6. Statistical analysis

All data were presented as the mean ± SD of the mean. The Wilcoxon signed-rank test was used for the analysis of all parameters. A P-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Animals

All animals survived for the entire experimental protocol period. The average weight of the animals increased steadily between before and after experiments (Table 1). No problems regarding the weight of the animals were observed in any groups and there was no difference in the weight of the animals among all groups. The weight of the animals receiving MT treatment slightly increased in comparison to that of other groups, but the increase was not caused by edema. The animals receiving MT treatment (groups 5a, 5b and 6) showed a larger amount of fat than the other groups.

3.2. Macroscopic findings

All tracheal grafts were wrapped firmly by the omentum. Grossly, the lumen of the allografts in group 1 showed a severe narrowing because of fibrosis (Table 1). All free tracheal isografts (group 2) appeared normal and also preserved their luminal rigidity, whereas the allografts without immunosuppression 12 weeks after transplantation (group 3) demonstrated a narrowed lumen (Fig. 2). The percent patency of the allografts assessed 16 weeks after transplantation (group 3) was slightly worse than that of allografts assessed 12 weeks after transplantation (group 1) because of progressive submucosal thickening (Table 1). The allografts with single agent immunosuppression using high doses of Tacrolimus (group 4b) and high doses of MT (group 5b) preserved their luminal rigidity, but showed a slightly narrowed lumen (Fig. 3A) (Table 1). On the other hand, one of six allografts with low doses Tacrolimus and MT (group 6)
could not preserve the lumen structure, and thus the mean patency was less than 50% (Table 1). The patency of the allografts in group 5b was significantly better than that in group 5a (using low dose MT), whereas there was no difference between groups 4a (using low dose Tacrolimus) and 4b (Table 1). No mucoid fluid filled the lumen of any allografts except for groups 4b and 5b.

### 3.3. Microscopic findings

The epithelium and tracheal glands of the allografts in groups 3-6 were almost completely obliterated and was significantly worse than that of isografts in group 2, whereas some of allografts in groups 4b and 5b (using high doses immunosuppression) had a normal pseudostratified ciliated or nonciliated epithelium and tracheal glands in part (Fig. 3B) (Table 2). The cartilage of the allografts except group 2 showed little degeneration and their viable chondrocyte ratio was almost equivalent to that of isografts in group 2 (Table 2). On the other hand, the allografts in group 5b alone showed little dislocation of the cartilage, whereas those except groups 2 and 5b demonstrated moderate grade dislocation. The score of cartilage dislocation in groups 4a, 4b and 5a was significantly higher and worse than that in group 2, whereas this score in group 5b was significantly lower and better than that in group 3 (Table 2). The mononuclear cells often infiltrated

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Graft status</th>
<th>Agent*</th>
<th>Dosage (mg/kg per day)</th>
<th>Average weight before ex. (g)</th>
<th>Average weight after ex. (g)</th>
<th>Patency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cf.</td>
<td>Allo</td>
<td>None</td>
<td>0</td>
<td>119.4</td>
<td>314.5</td>
<td>29.08±21.32</td>
</tr>
<tr>
<td>2</td>
<td>Iso</td>
<td>None</td>
<td>0</td>
<td>121.4</td>
<td>333.2</td>
<td>92.43±6.41</td>
</tr>
<tr>
<td>3</td>
<td>Allo</td>
<td>None</td>
<td>0</td>
<td>121.4</td>
<td>333.2</td>
<td>21.75±18.37*</td>
</tr>
<tr>
<td>4a</td>
<td>Allo</td>
<td>Tac</td>
<td>1.0</td>
<td>118.2</td>
<td>337.9</td>
<td>25.33±18.04*</td>
</tr>
<tr>
<td>4b</td>
<td>Allo</td>
<td>Tac</td>
<td>1.5</td>
<td>116.6</td>
<td>334.6</td>
<td>51.27±28.13*</td>
</tr>
<tr>
<td>5a</td>
<td>Allo</td>
<td>MT</td>
<td>150</td>
<td>120.7</td>
<td>347.1</td>
<td>26.68±17.30*</td>
</tr>
<tr>
<td>5b</td>
<td>Allo</td>
<td>MT</td>
<td>225</td>
<td>120.2</td>
<td>349.1</td>
<td>62.53±17.92*</td>
</tr>
<tr>
<td>6</td>
<td>Allo</td>
<td>Tac</td>
<td>1.0</td>
<td>116.4</td>
<td>346.4</td>
<td>45.80±26.45*</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SD of the mean. Ex., experiment; cf., a preliminary group in which tracheal allografts without undergoing steady immunosuppression were assessed 12 weeks after transplantation; Iso, isogeneic; Allo, allogeneic; Tac, Tacrolimus; MT, Multiglycosidorum tripterygii. *P<0.05 vs isografts without any treatment (group 2). †P<0.05 vs allografts without receiving immunosuppression (group 3). ‡P<0.05 vs allografts with low dosage of MT (group 5a).

*Agent, an immunosuppressive agent which was used 12 weeks after transplantation.

Fig. 2. Gross findings of tracheal allografts, undergoing no medication after incomplete immunosuppression, 16 weeks after transplantation (group 3). The cartilage is dislocated moderately and the graft is severely stenotic because of progressive submucosal thickening (Hematoxylin and eosin; original magnification, ×25).

Fig. 3. Gross (A) and histologic (B) findings of tracheal allografts, undergoing one cycle of immunosuppression with 225 mg/kg per day of multiglycosidorum tripterygii after incomplete immunosuppression, 16 weeks after transplantation (group 5b). (A) The graft shows patent and has a little mucoid fluid in the lumen. The cartilage is little dislocated (Hematoxylin and eosin; original magnification, ×25). (B) Normal pseudostratified ciliated epithelium is recognized. The cartilage has viable chondrocyte nuclei and new chondrocytes are often seen under the perichondrium. Mononuclear cell infiltration is chiefly seen in the submucosal layer (Hematoxylin and eosin; original magnification, ×200).
the perichondrium of the allografts in groups 3-6, whereas those almost infiltrated around the small vessels in group 1. The mononuclear cell infiltration score in group 5b or 6 tended to be lower than that in group 3 (Table 2).

### 4. Discussion

Recently, a murine heterotopic tracheal transplantation model has often been applied to the study of OB, since the heterotopic allograft reproduced the characteristic histopathology of human OB [8]. We have also used this model to investigate orthotopic tracheal transplantation in large animals, since heterotopic tracheal grafts implanted in the omentum showed the same healing course as orthotopic grafts with omental wrapping [5,6]. In addition, an experimental study using this heterotopic model is reproducible, simple and easy. Therefore, this model is widely applied to the experimental study of lung and airway transplantation. However, several potential limitations of this model were also evident in this study. This experimental model using a large airway eliminated the contact between fresh air and the epithelium, since the tracheal allografts were located in the omentum. Furthermore, the allografts were not surgically revascularized. Although the tracheal graft is a large-airway graft, epithelial injury, submucosal fibrosis, and bronchiectasis have been found in the large airways of patients with known OB [9]. Such factors may alter the immunopathogenesis of rejection in this model. However, since the model reproduces some of the histologic features of rejection reaction in human lung transplants, these shortcomings do not preclude the use of this model in our study [10].

A short course and high doses of medication of immunosuppressive agent are only rarely used in clinical situations, but they seem to induce immunological unresponsiveness in experimental studies, and have the additional advantage of being easily reproducible in experimental studies [11]. We used the strategy of short course and high doses of immunosuppression in this study, because there were some successful reports concerning similar immunosuppression using Tacrolimus [12]. We administered immunosuppressive drugs every fourth week for the allografts, since one course of treatment consisting of 1.5 mg/kg per day of Tacrolimus for 3 consecutive days maintained the allograft viability for 4 weeks in our previous experiments [13]. In preliminary experiments, this administration method using Tacrolimus every fourth week allowed the allografts to avoid a rejection probably due to the induction of immunological unresponsiveness [14]. Regardless adverse effects, these dosages of Tacrolimus may be tolerable for the health of the rat recipient, as demonstrated by the fact of all animals survived for the entire experimental protocol period and only minimal weight changes were observed in the animals in Table 1.

A rejection response in fresh allografts is often recognized as a dislocation and/or destruction of the cartilage due to cellular infiltration and proliferating granulation, despite the fact that chondrocytes are viable [5–7]. The decreased chondrocyte viability as well as progressive cartilage dislocation may be due to the occurrence of gradually progressing ischemia followed by rejection [5]. However, the allografts in group 3, without immunosuppression for 4 more weeks than those in group 1, showed neither progression of the cartilage dislocation nor decrease of the chondrocyte viability. This fact thus suggests that the pre-treatment consisting of two courses plus one-day medication possibly delay or reduce the rejection of allografts. On the other hand, the tracheal allografts in group 3 showed slightly progressive stenosis of the lumen due to fibrosis in comparison to those in group 1, and the infiltration area of mononuclear cells changed from around the small vessels in group 1 to around the perichondrium in group 3. Therefore, a histologic reaction similar to OB in allografts with two courses plus one-day treatment may slowly develop.

MT is extracted from the plant of *Tripterygium wilfordii* Hook-F (TW) and refined repeatedly. MT chiefly contains glycosides with a stable quality and has fewer side effects than other crude extracts of TW. Radix TW is a Chinese herbal medicine, which has been reported to be useful in the treatment of several immune-related diseases such as rheumatoid arthritis, systemic lupus erythematosus, and Behcet’s disease. A number of compounds have been previously isolated from crude aqueous or ethanol extracts of TW root or cultured plant cell lines derived from TW. These compounds include diterpenes, alkaloids, glycosides, dulcitol, wilfordine, quinone methiditerpenes, other

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Graft status</th>
<th>Agent*</th>
<th>Dosage (mg/kg per day)</th>
<th>Epithelial score</th>
<th>Viable chondrocyte ratio</th>
<th>Cartilage dislocation score</th>
<th>Mononuclear cell infiltration score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cf.</td>
<td>Allo</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0.58±0.12</td>
<td>1.17±0.75</td>
<td>1.33±0.52</td>
</tr>
<tr>
<td>2</td>
<td>Iso</td>
<td>None</td>
<td>0</td>
<td>1.97±0.38</td>
<td>0.70±0.13</td>
<td>0</td>
<td>0.33±0.52</td>
</tr>
<tr>
<td>3</td>
<td>Allo</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0.63±0.13</td>
<td>1.50±1.05*</td>
<td>1.50±0.55*</td>
</tr>
<tr>
<td>4a</td>
<td>Allo</td>
<td>Tac</td>
<td>1.0</td>
<td>0</td>
<td>0.64±0.07</td>
<td>1.17±0.41*</td>
<td>1.33±0.52</td>
</tr>
<tr>
<td>4b</td>
<td>Allo</td>
<td>Tac</td>
<td>1.5</td>
<td>0.52±1.13*</td>
<td>0.69±0.06</td>
<td>1.00±0.63*</td>
<td>1.33±0.52</td>
</tr>
<tr>
<td>5a</td>
<td>Allo</td>
<td>MT</td>
<td>150</td>
<td>0</td>
<td>0.63±0.05</td>
<td>1.00±0.63*</td>
<td>1.00±0.89</td>
</tr>
<tr>
<td>5b</td>
<td>Allo</td>
<td>MT</td>
<td>225</td>
<td>0.32±0.73*</td>
<td>0.67±0.07</td>
<td>0.17±0.41†</td>
<td>0.67±0.82</td>
</tr>
<tr>
<td>6</td>
<td>Allo</td>
<td>Tac</td>
<td>1.0</td>
<td>0</td>
<td>0.65±0.08</td>
<td>0.83±0.75</td>
<td>0.67±0.82</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SD of the mean. Ex., experiment; cf., a preliminary group in which tracheal allografts without undergoing steady immunosuppression were assessed 12 weeks after transplantation; Iso, isogeneic; Allo, allogeneic; Tac, Tacrolimus; MT, Multiglycosidorum tripterygi. †P<0.05 vs isografts without any treatment (group 2). ‡P<0.05 vs allografts without receiving immunosuppression (group 3).
triterpenes, B-sitosterol and triptoloides [15]. Two major compounds as triptolide and tripdiolide, isolated from crude extracts of TW, have been reported to represent a novel class of immunosuppressive agents [16]. Although the crude extracts of TW have been suggested to inhibit humoral immunologic reaction, the detailed mechanism of this inhibition remains to be elucidated. Li and Weir have described the ability of TW to suppress antibody synthesis, decrease E-rosette formation of lymphocytes, and reduce lymphocyte response in vitro to both mitogens and alloantigens. They also have reported that TW was more potent than 5-fluorouracil, azathioprine, or dexamethasone in suppressing lymphocyte transformation. The location of the inhibitory effect on activation is unknown, but is likely at the level of blocking DNA replication as evidenced by the minimal number of cells moving from G0/G1 into S and G2+M [17]. TW has been shown to inhibit several afferent immune functions and the generation of cytotoxic T cells by means of reducing both the production and responsiveness of interleukin-2 [17].

The epithelial morphology is reported to be more closely associated with the rejection reaction than the cartilage morphology inasmuch as the epithelium has a strong antigenicity. The epithelium of the allograft disappears after incomplete immunosuppression [6,7]. The high doses of Tacrolimus or MT treatment is considered to be effective, because the epithelium remained in tracheal allografts of groups 4b and 5b. Moreover, the cartilage dislocation score of the allografts receiving high doses of MT treatment was prominently better than that of any other treatments. In comparison to the allografts receiving combination of Tacrolimus and MT treatments in group 6, those receiving high doses of MT treatment revealed better tendencies of graft patency and a cartilage dislocation score, despite of equivalent infiltration of mononuclear cells. High doses of MT therefore seem to inhibit fibrosis as well as immune reaction. On the other hand, no synergistic effect of both Tacrolimus and MT was seen, as evidenced by graft patency and epithelial score. As both drugs have a similar action, they may thus not be able to compliment each other. The detailed pharmacological effect of MT still needs to be elucidated in the future.

The treatment effect of MT on tracheal allografts undergoing incomplete immunosuppression after transplantation seemed to be superior to that of Tacrolimus in this study. This difference in the treatment effects is possibly due to the dosage of each immunosuppressant. The effect of 150 mg/kg per day of MT was acceptable for tracheal allografts and it was comparable to that of 1.0 mg/kg per day of Tacrolimus in our previous studies [4]. A dosage of more than 150 mg/kg per day of MT seems to be desirable, because a dosage of 1.5 mg/kg per day of Tacrolimus was most effective for the treatment of tracheal allografts [13,14]. The immunosuppressive effect of MT has been found to be concentration-dependent [18]. This experimental results may also show a dose-related effect of MT, although we tested MT at only double doses. Regarding adverse effect, these dosages of MT may be tolerable for the health of the rat recipient, as demonstrated by the survival and the minimal weight changes of animals in Table 1. Regarding the mechanism of acceptable results induced by the short course treatment of MT in the rat, MT may also induce immunological unresponsiveness. MT potentially blocks the in vivo generation of antigen-specific cytotoxic T cells, because the action of MT is similar to that of Tacrolimus [19].

In conclusion, high doses of MT monotherapy tended to show better morphological findings of allografts than the other treatments, although there was no significant difference of treatment effect between Tacrolimus and MT. MT may thus be a useful alternative to Tacrolimus as an immunosuppressant for rejection in the heterotopic rat tracheal allotransplantation model. Further studies are needed to investigate MT treatment in animal models.

Acknowledgements

The authors would like to thank Mitsuto Hachida MD for provision of Multiglycosidorum tripterigy and Mrs Miki Shimada, Mrs Yumiko Hase and Miss Kahoru Noda for their skillful technical assistance.

References


