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SHORT COMMUNICATION

Topical pimecrolimus does not prolong clear graft survival in a rat keratoplasty model

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Abstract
Background Long-term application of topical steroids following penetrating keratoplasty is disadvantageous due to side effects (steroid response, cataract, surface disorders). In this study we investigated the efficacy of topical pimecrolimus regarding clear graft survival following allogeneic orthotopic keratoplasty in rats.
Methods A total of 46 penetrating keratoplasties were performed using Fisher rats (allogeneic groups) and Lewis rats (syngeneic group) as donors and Lewis rats as recipients: group 1 (n=11), allogeneic control without therapy; group 2 (n=12), syngeneic control; group 3 (n=11), mycophenolate mofetil (MMF) 40 mg/kg body weight; group 4 (n=12), pimecrolimus 1% ointment twice daily. Four animals of each group were sacrificed for immunohistological evaluation on day 14. Therapy was administered for 18 days. The grafts were evaluated once every 3 days regarding opacity, oedema and vascularisation. Graft rejection was defined as total graft opacity.
Results Mean rejection-free graft survival was 11.4 days in group 1 (allogeneic control), 100 days (total follow-up time) in group 2 (syngeneic control), 24.0 days in group 3 (MMF 40 mg/kg) and 11.6 days in group 4 (topical pimecrolimus). The immunohistological evaluation showed no statistically significant difference in cell infiltration of the grafts comparing groups 1 and 4.
Conclusions Topical immunosuppression with pimecrolimus does not prolong graft survival in the allogeneic keratoplasty rat model.

Keywords Keratoplasty · Immunosuppression · Pimecrolimus

Introduction

Penetrating keratoplasty is the only tissue transplantation for which topical immunosuppressive therapy is post-operatively feasible. To date, only steroids have been routinely used as topical treatment following penetrating keratoplasty at a dose of 3–5 drops per day. Systemic steroids are routinely used in many centres for the first few weeks post-operatively, and in high-risk situations systemic cyclosporin A (CsA) or mycophenolate mofetil (MMF) are additionally administered for the first 6 months [1]. Due to the known side effects of topically administered steroids (steroid response glaucoma, cataract, surface disorders, infections) their use is limited during post-operative therapy; thus, new immunosuppressive agents for topical use are desirable, with lower risk for side effects and potential for long-term treatment.

Pimecrolimus, a new calcineurin inhibitor developed for the topical therapy of inflammatory skin diseases, e.g. atopic dermatitis, easily permeates the skin; it offers a very low risk of systemic exposure and subsequent systemic side effects [4].

Topical pimecrolimus showed good efficacy and safety in dogs for the treatment of keratoconjunctivitis sicca and chronic superficial keratitis [10].

For the topical or systemic treatment with pimecrolimus following penetrating keratoplasty there exist neither clinical nor experimental data.

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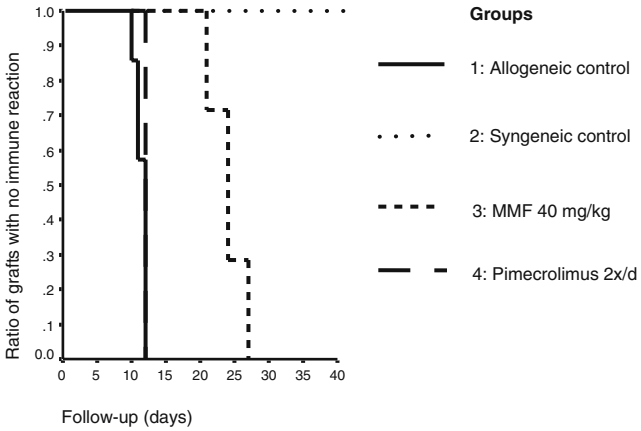

Fig. 1 Kaplan-Meier analysis of rejection-free graft survival

In this study we investigated the efficacy of topical pimecrolimus regarding clear graft survival following allogeneic orthotopic keratoplasty in rats. Systemic MMF has demonstrated a strong immunosuppressive effect in several studies and was used as control [9, 14, 15].

Materials and methods

A total of 46 orthotopic penetrating keratoplasties were performed using Fisher rats (Rtl-l^{vl}) as donors in the allogeneic groups and Lewis rats (Rtl-l^{le}) as donors in the syngeneic control group. Lewis rats were used as recipients in all groups. All animals were female, weighing 150–200 g. They were obtained and cared for in accordance with the European Community Directives and according to the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, NIH Publication Number 85–23 (revised 1985).

Groups were divided up as follows: group 1 (n=11), allogeneic control without therapy; group 2 (n=12), syngeneic control; group 3 (n=11), systemic MMF 40 mg/kg body weight; group 4 (n=12), topical pimecrolimus 1% ointment twice daily. Four animals of each group were sacrificed for immunohistological evaluation on day 14 (CD4, CD8, CD25, CD45, CD161, CD163 and dendritic cells).

Orthotopic penetrating keratoplasties were performed according to the technique of Herbort et al. [5].

Pimecrolimus was applied twice daily under isoflurane anaesthesia for 18 days post-operatively. The 1% ointment (Elidel, Novartis Pharma, Nürnberg, Germany) for the treatment of skin diseases was used. There are no studies available about the differences in the permeability of pimecrolimus in human skin or cornea.

Medication in the MMF control group was given orally with a stomach tube, starting on the day of surgery and continuing daily for 18 days. MMF was given as a suspension in water.

The animals were closely monitored for signs of toxic side effects (such as weight loss) during the entire follow-up.

The grafts were evaluated once every 3 days with the surgery microscope regarding opacity, oedema and vascularisation. Graft rejection was defined as total graft opacity.

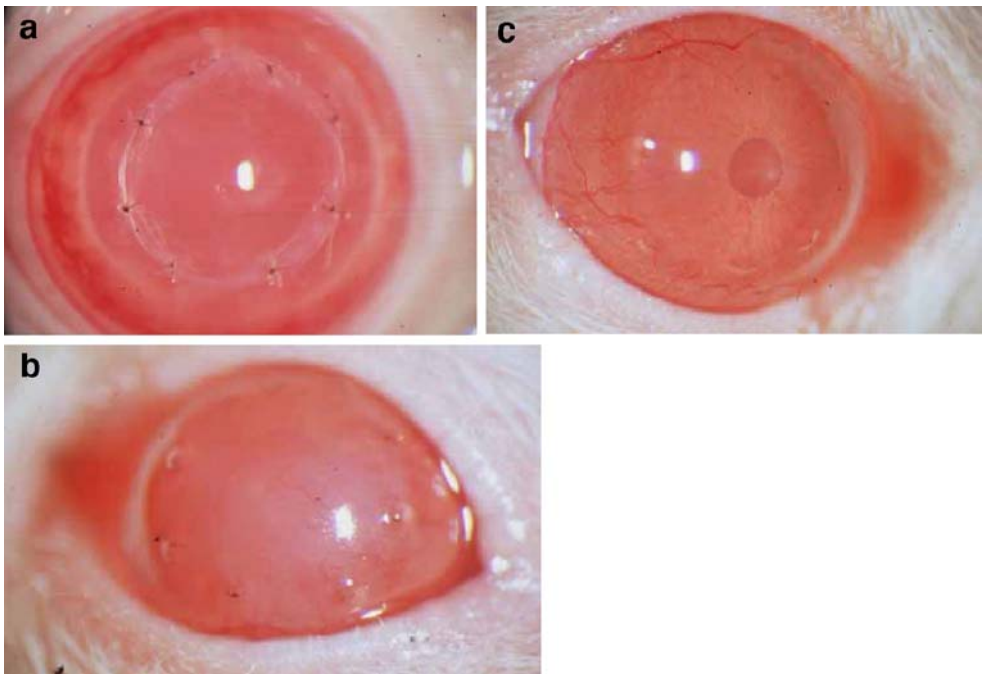

Fig. 2 Corneal grafts. **a** Graft immediately post-operatively. **b** Clear syngeneic graft 100 days post-operatively, sutures lost spontaneously. **c** Allogeneic graft of group 2 with total opacity, 12 days post-operatively

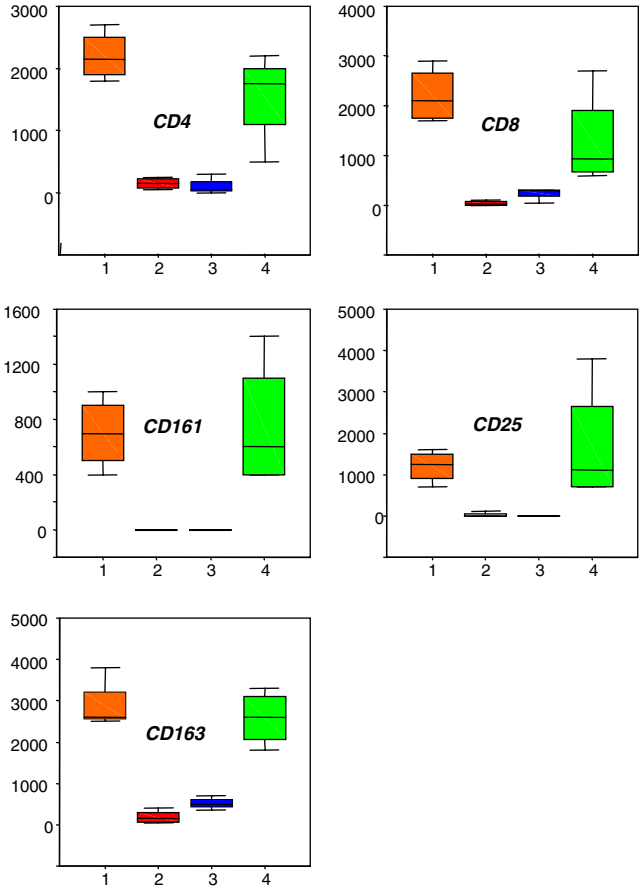

Fig. 3 Box plot graphs of immunohistology (x-axis: groups; y-axis: positive staining cells per mm^2). Groups: 1 (allogeneic control); 2 (ayngeneic control); 3 (MMF 40 mg/kg); 4 (pimecrolimus 1%). Immunohistology: CD4: T-helper cells; CD8: cytotoxic T cells; CD161: NK cells; CD25: IL-2 receptor; CD163: macrophages

We scored opacity as follows: 0=no opacity; 1=slight opacity, details of iris clearly visible; 2=moderate opacity; some details of iris no longer visible; 3=pronounced opacity, pupil still recognizable; 4=total opacity.

Between days 9 and 12, the rats were examined daily to identify the exact day of rejection. To enhance the accuracy of graft evaluation, each animal was evaluated by two examiners (FB and CS). Not all of the grafts experienced total opacification in the MMF group. Some of them reached only opacity level 3 and cleared up afterwards to opacity level 2 or even 1. In such cases, we defined the day of reaching opacity level 3 as the day of rejection.

Statistical analysis

Time to rejection was analysed with the Kaplan-Meier survival analysis [7] and compared with the log-rank test. The densities of infiltrating immune cells were compared statistically using the non-parametric Mann-Whitney test.

All statistical evaluations were performed using SPSS Windows 12.0 (Microsoft Corp., Redmond, WA, USA).

Results

Efficacy

Mean rejection-free graft survival was 11.4 days in group 1 (allogeneic control), 100 days (total follow-up time) in group 2 (syngeneic control), 24.0 days in group 3 (systemic MMF 40 mg/kg) and 11.6 days in group 4

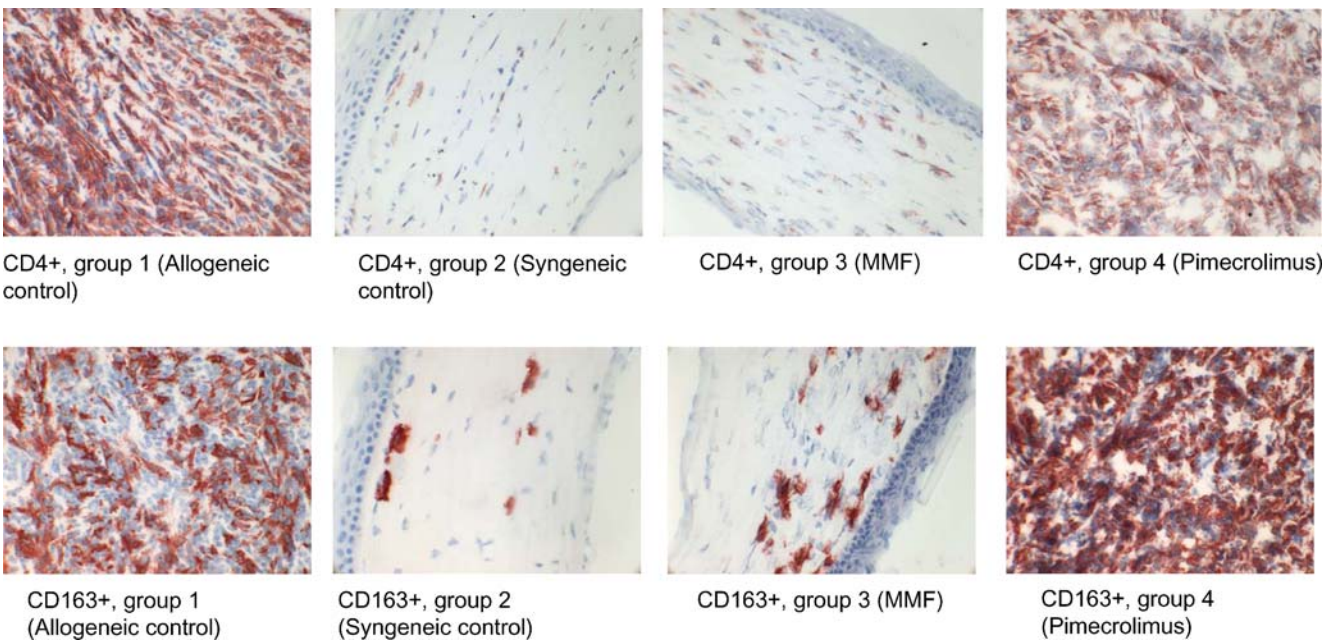

Fig. 4 Immunohistology of corneal grafts; CD4+ (T-helper cells) and CD163+ (macrophages) staining cells

(topical pimecrolimus) (Figs. 1 and 2). Pimecrolimus caused no statistically significant effect in prolonging graft survival. Systemic immunosuppression with MMF prolonged rejection-free graft survival statistically significantly ($p<0.05$).

Immunohistological evaluation

Four animals of each group were sacrificed for immunohistological evaluation (CD4, CD8, CD25, CD45, CD161, CD163 and dendritic cells).

We found dense cell infiltration in the allogeneic grafts in group 1 with CD4+ (T-helper cells; mean 22 cells per 1/100 mm^2), CD8+ (cytotoxic T cells; mean 22 cells per 1/100 mm^2), CD25+ [interleukin (IL)-2 receptor; mean 12 cells per 1/100 mm^2], CD161+ [natural killer (NK) cells; mean 7 cells per 1/100 mm^2] and CD163+ (macrophages; mean 29 cells per 1/100 mm^2) cells (Figs. 3 and 4).

The syngeneic grafts were nearly free of immunologic cell infiltration. Group 3's grafts (MMF) showed a statistically significantly decreased infiltration of CD4+, CD8+, CD25+, CD161+ and CD163+ cells [$p<0.002$ analysis of variance (ANOVA)]. No CD45RA+ (B cells) were detected in any of the grafts.

Only very few dendritic cells (mean 0.5–2 cells per 1/100 mm^2) were found in groups 1, 3 and 4, and no dendritic cells were found in group 2. Due to the low cell count, this difference is not statistically significant.

The decrease in immune cell infiltration in the grafts showed no statistically significant difference between groups 1 (allogeneic control) and 4 (topical pimecrolimus).

Safety

No signs of corneal infection or epithelial defects were observed in any of the groups. One animal treated with MMF died on day 17 of emaciation. No systemic side effects were noted in the three other groups.

Discussion

There is a broad spectrum of systemic immunosuppressive drugs available for treatment in organ transplantation. Especially in high-risk situations, systemic immunosuppression with MMF or CsA is recommended for about 6 months post-operatively [2, 13].

The possibility of a topical immunosuppression is a major advantage of the corneal transplantation compared to solid organ transplantation. Yet only steroids are routinely used topically following penetrating keratoplasty. A broader armamentarium of topical immunosuppressive agents would be desirable in both normal and high-risk keratoplasties [3].

CsA is a very potent immunomodulating calcineurin inhibiting drug administered for a broad spectrum of chronic inflammatory diseases of the ocular surface; however, the corneal permeability of topical CsA is insufficient for endothelial graft rejection prophylaxis. Topical FK506, a potent calcineurin inhibitor as well, showed some efficacy in the rat keratoplasty model in combination with systemic FK506 [6]. However, FK506 used in inflammatory corneal and conjunctival diseases and following penetrating keratoplasty caused problems regarding tolerance in two pilot studies [11, 12].

To date no studies have been performed dealing with topical or systemic treatment with pimecrolimus following penetrating keratoplasty.

Topical pimecrolimus showed good efficacy and safety in dogs for the treatment of keratoconjunctivitis sicca and chronic superficial keratitis [10].

In this study, topical immunosuppression with pimecrolimus failed to prolong graft survival statistically significantly in the allogeneic keratoplasty rat model. Furthermore, pimecrolimus caused no reduction of graft cell infiltration compared to the allogeneic group.

There are different explanations for the lack of immunosuppressive efficacy of topical pimecrolimus.

Rats treated with topical pimecrolimus started rubbing their eyes after awaking from anaesthesia. This could be explained by a burning sensation, which is known to be a side effect of topical pimecrolimus. On the one hand eye rubbing could have led to damage of the grafts and a subsequent enhanced rejection and on the other hand the ointment was removed too quickly to develop an immunosuppressive effect. We applied pimecrolimus ointment twice daily. An application of pimecrolimus four times a day was not beneficial in the treatment of dermatological diseases [8]. However, a more frequent application could be advantageous due to different pharmacokinetic properties of pimecrolimus in the cornea. In this study we used the 1% pimecrolimus ointment for skin diseases. There are no data available about the permeability of this ointment in the cornea.

In addition, this rat keratoplasty model could be inappropriate for the investigation of topical immunosuppressants due to the very strong immune reaction. The efficacy of pimecrolimus should be tested in future studies in a low rejection model in mice or at a higher dosage.

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