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Evaluation of a topical cyclosporine A prodrug on corneal graft rejection in rats

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Purpose: To assess the efficacy of a topical cyclosporine A (CsA), water-soluble prodrug, for promoting the survival of allogenic rat corneal grafts after penetrating keratoplasty (PKP).

Methods: Corneas of Brown-Norway rats (donors) were transplanted to Lewis rats (recipients). Transplanted rats were divided in three treatment groups: group I (PBS) and group II (0.26% Debio088) received drops five times per day. Group III received a daily intramuscular CsA injection (10 mg/kg/day). Blood CsA concentrations were measured on days 2 and 14. On day 4, 10, 13 after PKP, grafts were scored for corneal transparency, edema and extent of neovascularization. An opacity score of greater than or equal to 3 was considered as a nonreversible graft rejection process. On day 14, the experimental eyes were processed for histology.

Results: On day 13, 12 of the 18 corneal transplants (67%) in group I showed irreversible graft rejection. Three of 18 transplants (19%) in group II and 5 of 16 transplants (28%) in group III showed irreversible graft rejection (p=0.013/ p=0.019, OR=0.14/0.06 versus vehicle). Each mean clinical score for edema, opacity, and neovessels in group II were significantly lower than those of the grafts in group I (respectively p=0.010, p=0.013, p=0.024) and III except for neovessels (respectively p=0.002, p=0.001, p=0.057). Histology confirmed the clinical results. The mean CsA blood levels for groups II and III were, respectively 54±141 μg/l and 755±319 μg/l on day 2 and 14±34 μg/l and 1318±463 μg/l on day 14.

Conclusions: Debio088 CsA prodrug drops given five times daily are as effective as intramuscular injection of 10 mg/kg/ day for the prevention of acute corneal graft rejection in rats.

Cyclosporin A (CsA) is a cyclic undecapeptide produced by *Tolypocladium inflattum Gams* and other *fungi imperfecti*. It acts mainly by inhibiting interleukin-2 activation in T helper lymphocytes and subsequently causes suppression of T cells activation and proliferation [1,2] that is reversible when the treatment is stopped. CsA has been widely used for the prevention of kidney, heart, liver, and lung transplantation in humans and has been also introduced for the prevention of acute corneal graft rejection after penetrating keratoplasty (PKP) [3-5]. In rats, intramuscular injections (IM) of CsA (10 mg/kg/ day) increased the survival rate of corneal grafts [6,7]. Systemic administration of CsA is, however, associated with unavoidable side effects. Therefore, local administration of CsA to the eye, if effective, is desired. Unfortunately, due to the lipophilic characteristics of CsA and its poor water solubility, topical formulations do not penetrate the ocular tissues and are not well tolerated by the patients [8-10]. Oily formulations of 2% CsA demonstrated beneficial influences on immune diseases of the ocular surface as vernal keratoconjunctivitis (VKC) but their beneficial effect on the prevention of corneal graft rejection is disputable [11,12].

To increase CsA bioavailability, collagen shield [13], polymeric particles [14,15], liposomes [16], and chitosan nanoparticles [17] have been evaluated with some success [14,18].

Another strategy to enhance the bioavailability of the lipophilic CsA is the synthesis of an inactive hydrophilic chemically modified molecule, which can be converted, within the tissues, into the active form after enzymatic transformation. Debio088 (DeBiopharm, Switzerland) is a water soluble prodrug of CsA (Figure 1A), which releases active CsA within minutes by ester hydrolysis (Figure 1B) [19].

The purpose of this study was to evaluate the potential of a topically instilled CsA hydrosoluble prodrug to delay corneal graft rejection in the rat and to compare it with the effect of a systemically administered CsA.

METHODS

Animals: Lewis rats were recipient for corneal graft from Brown Norway (BN) rats. In this model, the rejection process is clinically detectable from day 5-6 after transplantation and is complete, in most cases, on the fourteenth day after PKP [20].

Seventy-eight 8-week-old female rats (52 Lewis and 26 BN; Iffa-Credo, Bruxelles, Belgium), weighing 200-250 g were used. The animals were cared for in accordance with the Directives of the European Community as well as with the Association for Research in Vision and Ophthalmology resolution on the use of animals in ophthalmic and vision research. At the end of the experiments, the rats were sacrificed by intraperitoneal injection of a lethal dose of pentobarbital.

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Reagents: A 0.26% aqueous solution of prodrug (Debio088), equivalent to 0.2% CsA, was aseptically prepared every day in five aliquots. Each aliquot was opened and used for instillation of the group of rats on the same day. Treated rats received an IM injection of 10 mg/kg/day of CsA Sandimmun® (Novartis, Basel, Switzerland) [7]. Phosphate buffered saline (PBS), pH=7 (isotonic), was used as a vehicle of Debio088 in the control group.

Experimental design: Corneal grafts were performed by two corneal surgeons (J.L.B. and F.B.C.). Central corneal buttons of both eyes from 26 BN rats were prepared using a 3.0 mm trephine (biopsy punch; Stiefel, Rueil-Malmaison, France). These buttons were grafted into a 3.0 mm corneal bed in the right eyes of 52 Lewis rat recipients. The day of surgery was day 0. For corneal grafting, Lewis rats were anesthetized with a mixture of 125 mg/kg ketamine chlorhydrate (UVA, Ivry sur Seine, France) and 5 mg/kg chlorpromazine (Specia Rhône Poulenc, Paris, France). Maximal pupil dilation was obtained by repeated instillation of 0.5% tropicamide every 10 min, 1/2 h prior to surgery. A paracentesis was performed at 0.5 mm from the limbus before trephination, and the anterior chamber was immediately filled with viscoelastic (Healonid, Pharmacia, Uppsala, Sweden). A 3.0 mm trephination was performed with a biopsy punch and completed with vanas scissors. The BN corneal button was sutured with a 10-0 prolene (Ethicon, Saint Stevens-Woluwe, Belgium) continuous 8 points suture. Gentamycin ointment was applied at the end of surgery. No corticosteroids were used.

Transplanted eyes with immediate postoperative complications (suture rupture or cataract) were not included in the study.

Treatment protocols: The Lewis rats with transplanted corneas were randomly assigned to the three different treatment groups: group I was treated five times/day with PBS instillations; group II was treated five times/day with 0.26% Debio088 instillations; and group III was given 10 mg/kg/day CsA IM injections.

In previous studies, CsA in oil formulations was applied topically three to five times per day [16,18,21]. The contact time being reduced with a hydrosoluble compounds, we have chosen to administer the prodrug five times daily.

Fifty-two successful grafts were segregated within the three groups as follows: group I=18 eyes, group II=18 eyes, and group III=16 eyes.

Clinical evaluation of graft failure: Biomicroscopic examination and photographs were performed on days 4, 10, and 13 after surgery. The evaluation of transplants was performed using the established scoring system for transparence of the transplanted graft, edema, and neovascularization [22]. Clinical scoring of the grafted corneas was made by two masked examiners as follows: Corneal transparency: 0 (clear cornea), 1 (slight opacity), 2 (mild opacity with iris details visible), 3 (moderate, iris details not visible), and 4 (white cornea); edema: 0 (no edema), 1 (slight edema), 2 (diffuse and moderate stromal edema), and 3 (diffuse marked stromal edema); neovascularization: 0 (no observable growth of new vessels), 1 (new vessels invading less than 1/3 of the recipient

bed), 2 (new vessels invading less than 2/3 of the recipient bed), 3 (new vessels growing up to the limiting ring of the graft), and 4 (new vessels invading the graft).

All parameters were recorded, but graft rejection evaluation was based on opacity alone since graft opacity was shown to be the more accurate rejection parameter [22]. Rejection was defined as graft opacity greater than or equal to 3, which was greater than the opacity seen in isografts at any time[23].

Histological examination: On day 14, the rats were sacrificed, the grafted eyes enucleated, and fixed in 4% paraformaldehyde. Six μm thick sections were prepared and stained with hematoxylin and eosin.

Blood CsA levels: In animals receiving drops of Debio088, blood was taken two days after surgery, between the fourth and the fifth administration of the day and on the fourteenth day, at the time of sacrifice. In the CsA IM group, blood was taken on days 2 and 14 after surgery, 23 h after the last IM injection of CsA. Blood samples were transferred into tubes containing EDTA and frozen at -20 °C until analyzed.

CsA concentration was measured in whole blood by using a commercially available validated enzyme multiplied immunoassay technique (EMIT) with a MIRA Plus analyzer (Roche Diagnostics, Basel, Switzerland) according to the Dade
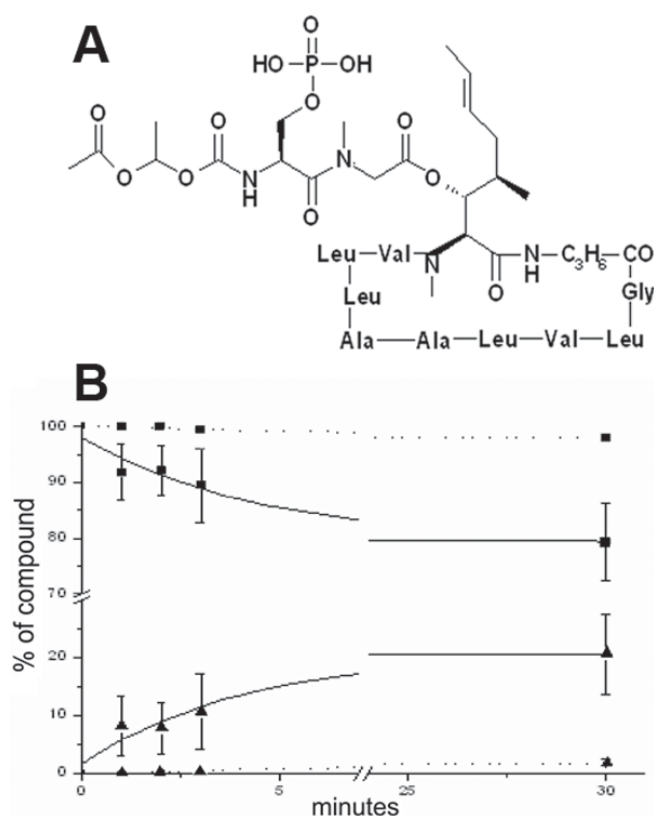

Figure 1. **A**: Debio088 formula prodrug consisting in a water-solubilizing moiety grafted on a free hydroxyl function of CsA. M=1570. **B**: Ex vivo conversion kinetics of Debio088 (closed square; 0.2% w/v in isotonic PBS solution) into CsA (closed triangle) incubated with rabbit tears (solid line) and isotonic PBS pH 7.4 (discontinueous line), mean±S.D., n=3.

Boehring manufacturer's instructions. Debio088 does not cross-react with CsA in this EMIT assay (EMIT 2000 Cyclosporine Specific Assay. catalog number 6R019UL). The limit of quantification of the assay was 40 μg/l.

Statistical analysis: Rejection rates were compared using the χ^2 test with the Yates correction or Fisher exact test when required. Mean clinical scores for rat transplants were expressed as means ±SEM and compared using the nonparametric Kruskal-Wallis ANOVA test and Dunn's post-test. A $p<0.05$ was considered significant.

RESULTS

Body weight: Upon initiation of the study, the mean body weight for the rats was 152.5 g, 159.3 g, and 155 g, respectively, for groups I, II, and III (p>0.1). The mean weight did not change significantly between the treated groups during the period of study.

Tolerance: The frequent instillation of Debio088 prodrug did not induce any toxic clinical reactions on the eyelids or the ocular surface of the treated eyes.

Clinical scores: Opacity, edema, neovascularization, and total scores recorded on days 4, 10 and 13 after surgery are illustrated in Figure 2. On day 4, the mean edema score was not significantly different between treatment group I (PBS control) and groups II (Debio088) and III (CsA IM; p=0.4 and 0.2, respectively). Also, it was not different between group II and group III (p=0.73; Figure 2A). The mean opacification scores were not significantly different between group I and groups II and III (p=0.4 and 0.3, respectively) and not significantly different between group II and group III (p=0.8; Figure 2B). The mean neovessel scores were not different between group I and groups II or III (p=0.1 and 0.4, respectively) and not different between group II and group III (p=0.52; Figure 2C).

On day 10, the mean edema score was significantly higher in group I compared to group III (p=0.01) but not to group II (p=0.07; Figure 2A). The mean opacification score was significantly higher in group I compared to group II (p=0.008), but there was no significant difference when compared to group III (Figure 2B). The mean neovessel score were not significantly different between group I and groups II or III (p=0.11 and 0.47, respectively) and not different between groups II and III (p=0.42; Figure 2C).

On day 13, the mean edema score was significantly higher in the control group I when compared to group II (Debio088; p= 0.010) or to group III (CsA IM; p=0.0002). No significant differences were observed in the mean edema score between groups II and III (p=0.13; Figure 2A). The mean corneal opacity score was significantly higher in the control group I when compared to either group II (p=0.01) or to group III (p=0.001). No significant difference was found between groups II and III regarding the corneal opacity score (p=0.40; Figure 2B).

The mean neovessel score was not significantly higher in the control group when compared to either group II or group III and no significant differences were observed between group II and III (Figure 2C).

Therefore, when irreversible corneal rejection processes are clinically observed in the majority of the grafts in the control group, prevention of the rejection processes are observed in the group of rats treated either with systemic CsA or with topical Debio088. The treatment benefits of systemic CsA treatment appear clinically comparable to the benefits observed with the use of the topical formulation of CsA prodrug.
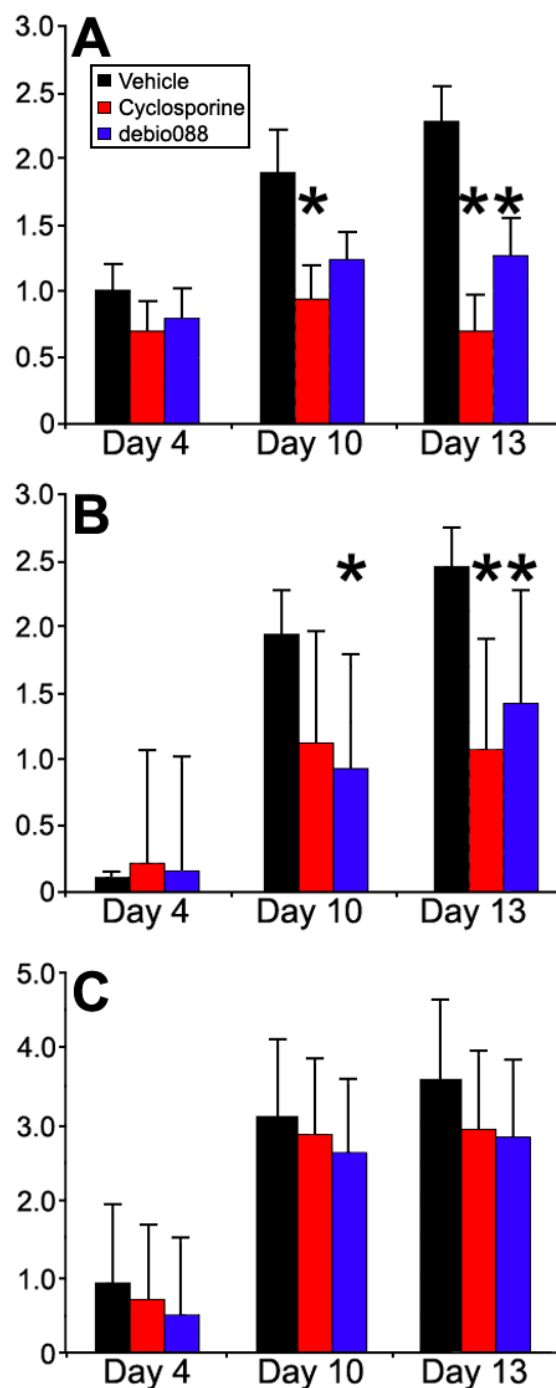

Figure 2. Mean clinical scores (±SEM) as a function of time. **A**: Edema scores, **B**: Opacification scores, **C**: Neovessels scores. The asterisk represents a significant difference compared to the vehicle group with a p<0.05.

Percentage of rats with corneal graft rejection: According to the predetermined criteria, the percentage of corneas with irreversible graft rejection on day 13, opacity score of greater than or equal to 3, was 12/18 (67%) in group I, 5/18 (28%) in group II, and 3/16 (19%) in group III. At this time point, the percentage of rejected grafts was significantly higher in group I when compared to either group II (p=0.013, odds ratio=0.12) or to group III (p=0.019 odds ratio=0.19). Figure 3 shows representative cases of each treatment group.

Histology: Histology observations paralleled the findings obtained by clinical scoring among groups. In the vehicle-treated (control) eyes, rejected grafts showed that the prominent inflammatory cell infiltrate along with the formation of granulomas were accompanied by a marked increase of the graft thickness (Figure 4A,B). New vessels are invading all the graft corneal layers in this case (Figure 4B, thick arrows).

The nonrejected grafts within the CsA-IM treated eyes showed only minimal inflammatory cell infiltrates at the subepithelial and stroma levels (Figure 4C,D). However, new vessels were clearly identified in the anterior stroma of the corneal graft (Figure 4D, small arrow). The thickness of the graft remained unchanged compared to the recipient bed. When the graft in this group of rats was rejected, a marked inflam-
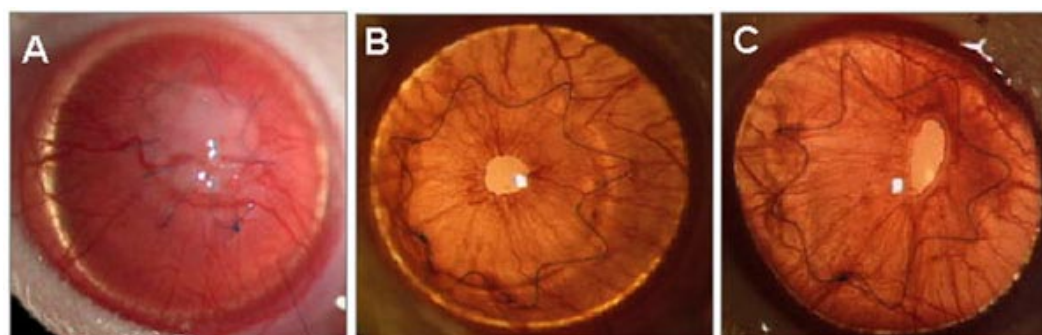

Figure 3. Representative cases of each treatment group on slit-lamp examination at the thirteenth postoperative day showing a rejected corneal graft (**A**) with a maximal clinical score and two clear grafts from the systemic CsA (**B**) and Debio088 (**C**) treated groups.
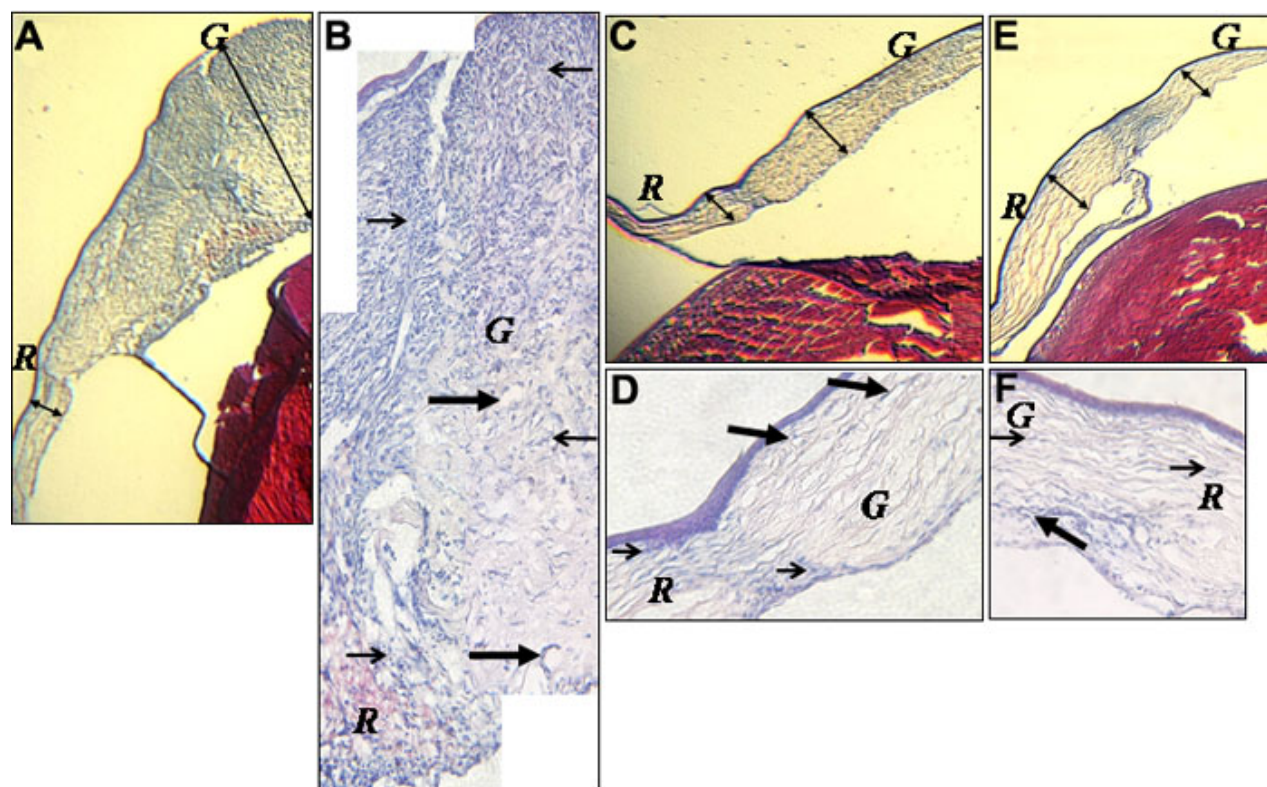

Figure 4. Corneal histology (Hematoxylin-eosin staining) showing **A**: Control rejected cornea (2.5x) showing an important corneal edema. **B**: Inflammatory cell infiltration (arrows) and neo-vessels (thick arrows) are observed at higher magnification (25x). **C**: IM CsA non-rejected graft showing no corneal graft edema (2.5x). **D**: Minimal cell infiltrate (arrow) and neo-vessels (thick arrows) are observed within the graft at higher magnification (25x). **E**: Debio088 non-rejected graft showing no corneal graft edema (2.5x). **F**: Minimal cell infiltrate (arrow) at higher magnification (25x). In all panels, "R" indicates recipient and "G" indicates graft.

matory cell infiltrate as well as neovessels were observed in the subepithelial layer and in the anterior corneal stroma. These corneas showed an increased thickness of 15-25% over the nonrejected corneal thickness (not shown).

In the Debio088-treated corneas, both the graft and the recipient junction had a normal structure with a thickness in the normal range with a moderate inflammatory cell infiltrate observed within the stroma (Figure 4E,F).

CsA concentration in whole blood: The mean CsA blood levels, measured at trough level, for groups II and III were, respectively 54±141 µg/l and 755±319 µg/l on day 2 and 14±34 µg/l and 1,318±463 µg/l on day 14. All rats showed a blood level >300 µg/l on day 2 and >600 µg/l on day 14 in group III. In the Debio088 group, the mean CsA blood levels were below the threshold of detection on day 14.

DISCUSSION

Thirteen days after surgery, 12 of the 18 grafts (67%) treated with PBS (vehicle of Debio088) eye drops showed irreversible corneal graft rejection processes. Rejection rates of 67-100% were reported in control groups of similar studies [11,16,24,25]. When administered systemically at immunosuppressive doses (7.5-10 mg/kg), CsA has been shown to significantly delay corneal graft rejection in animal models [7,26]. In high risk PKP patients, systemic CsA also had beneficial effects on graft rejection [3,5]. In our study, systemic daily IM injections of 10 mg/kg CsA inhibited the rejection in 81% of the grafts, despite CsA blood levels above 600 µg/l on day 14 in all rats. In preclinical studies, the efficacy of systemic CsA differs, depending on the experimental conditions [7,26-28]. The reason for this phenomenon may be multiplex. It may be associated with factors related to the host graft interactions and the resulting regional, local (and systemic) immunological cellular and humoral processes playing a crucial role on the individual graft opacity and rejection final endpoint. Recently, Vallelado et al. [27] showed that sustained release of CsA from subcutaneously injected microspheres was more efficient than systemic daily injections of CsA in the prevention of graft rejection in the rat, with microspheres resulting in lower CsA blood levels.

In our experiments, the local instillation of the prodrug, five times daily, significantly decreased the percentage of grafts demonstrating irreversible rejection processes (28% versus 67%) at day 13. The efficacy of Debio088 was similar to that of IM 10 mg/kg CsA, but without the potential side effects of systemic therapy. The immunosuppressive action of the locally instilled CsA prodrug on corneal graft rejection is not the result of a systemic immunosuppression, as no detectable CsA blood levels were found in the Debio088-treated rats. However, a loco-regional action of CsA in draining nodes cannot be ruled out and could explain the therapeutic immunosuppressive effect of locally administered CsA.

Both systemic and locally administered CsA induced a limited effect on the growth of neovessels, particularly at the early phase of the rejection process. No differences in neovessels scores were observed between the control and the CsA-treated groups. CsA may have pro- or antiangiogenic

activities depending on the type of VEGF receptors expressed by endothelial cells [29]. The CsA-induced gingival vascularization and overgrowth has been associated to the decreased expression of the angiostatic thrombospondin-2 and not to an increased expression of angiogenic genes [30]. On the other hand, the inhibitory effects of CsA on VEGF transduction pathways on intestinal microvascular endothelial cells have been associated to vasculopathy during chronic rejection processes [31]. In corneal graft rejection, the roles of VEGF and VEGF receptors have recently been demonstrated [32,33], but the effect of CsA on corneal vessels as well as the lymphatic growth during the rejection process has not yet been explored. CsA mechanisms of action on the different graft rejection processes still remain to be explored.

From a clinical point of view, prolonged CsA administration is required for the prevention of graft rejection [34] and may help to reduce the use of long-term steroid therapy. Unavoidable systemic side effects make the local delivery of CsA preferable in these cases.

The effect of 0.5 or 2% topical CsA in oily suspensions or as a liposome formulation had been reported as having beneficial graft rejection prevention outcomes [11,16,18,21]. Other delivery systems or formulations have been evaluated to improve the bioavailability and tolerance of locally administrated CsA. Subconjunctival biodegradable implants of CsA or topically applied CsA-loaded nanocapsules have also been shown to prolong graft survival without systemic diffusion of CsA [18,35]. Another strategy has explored the possibility to implant a CsA-loaded polymeric solid implant in the anterior chamber after PKP [21].

The possible use of a low concentration of CsA prodrug in an aqueous solution has evident theoretical advantages including a good tolerance and higher capability of corneal barriers crossing.

In conclusion, this study shows that repeated local administrations of a hydrosoluble CsA prodrug are as efficient as systemic CsA to delay the corneal graft rejection processes occurring after allogeneic corneal grafts in rats, without detectable CsA systemic levels. Further studies are needed to understand the mechanism of CsA delivery, its kinetics within the eye compartments, and to determine the optimal concentration to be used potentially in humans.

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