Effect of Metalloprotease Inhibitors on Corneal Allograft Survival

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Purpose. The expression of Fas ligand (FasL) in the cornea is essential for corneal allograft acceptance in mice. Because the expression of FasL on the surface of cells is sensitive to cleavage with matrix metalloproteases (MMPs), this study examined whether inhibitors of MMPs would lead to increased FasL expression and improved corneal allograft survival.

Methods. Corneal endothelia derived from mice and humans were treated with MMP inhibitors, and FasL expression was examined. BALB/c mice were engrafted with C57BL/6 or C57BL/6-*gld* corneas and treated with an ointment containing the MMP inhibitor, doxycycline. Corneal allograft survival was monitored for 50 days.

Results. Corneal endothelial cells from both mice and humans displayed increased surface expression of FasL after treatment with MMP inhibitors. The increase in surface expression was further evidenced by the ability of these cells to kill Fas-expressing target cells. Mice treated with doxycycline after corneal allograft transplantation showed significantly prolonged allograft survival and an increase in the overall acceptance rate.

Conclusions. MMP inhibitor treatment of cornea-derived endothelial cells results in increased FasL expression and function. MMP inhibitor treatment prolongs corneal allograft survival and results in a modest increase in corneal allograft acceptance. (*Invest Ophthalmol Vis Sci.* 2004;45:1169–1173) DOI:10.1167/iovs.03-0932

Corneal graft transplantation is a common surgical procedure, with more than 46,000 grafts performed last year (http://www.restoresight.org/eye_banks/eye_banks.htm; provided by the Eye Bank Association of America, Washington, DC). Corneal transplantation is also one of the most successful types of transplantation performed, with 1-year failure rates that range from 10% to 15%.[1,2] When these grafts are monitored for 5 years, the failure rate has been estimated to be between 20% and 30%.[2–5] Numerous mechanisms have been proposed to account for such a high rate of success, and several of these have received experimental support in the literature. Graft acceptance relies on the immune privileged nature of the anterior chamber (AC) of the eye. Factors known to contribute to immune privilege are the avascular and alymphatic nature of the graft bed, the presence of immunosuppressive factors,[6] and the expression of FasL by ocular tissue.[7,8] Other factors thought to contribute to immune privilege include the relative absence of antigen-presenting cells, low expression of major histocompatibility complex (MHC) molecules,[9] and the induction of immune deviation to corneal antigens.[10]

FasL expression in the eye also prevents unwanted blood vessel growth into the retina[11] and the cornea.[12] Neovascularization of the cornea is a risk factor that can determine the success of corneal transplantation. Transplant recipients with vascularized corneal graft beds have significantly greater incidence of rejection.[5,13] Similarly, grafts placed in normal mouse eyes have a much higher acceptance rate than do those placed on prevascularized eyes (47% vs. 97%).[14] Studies in animal models have also demonstrated that agents that reduce neovascularization increase graft acceptance.[15,16] Thus, neovascularization plays an important role in corneal allograft success. Neovascularization of the cornea is a complicated process that involves the degradation of the basement membrane, endothelial cell migration, capillary tube formation, and endothelial cell proliferation. This process requires extracellular proteolytic activity that is crucial in endothelial cell invasion and capillary morphogenesis, leading to the formation of new capillaries from preexisting blood vessels.[17] The matrix metalloproteinases (MMPs), such as collagenase, gelatinase, and stromelysin, are intimately involved in the degradation of the extracellular matrix, which allows penetration of new vessels into the cornea.[18] Mice that were either treated with tissue inhibitor of metalloprotease (TIMP)-1 or were genetically incapable of producing MMP-9 had significantly less angiogenesis after infection with herpes simplex virus.[19] Thus, neovascularization and corneal disease are associated with increased expression of MMPs.[20–23] Because surface FasL expression is sensitive to MMP activity, it is possible that that one effect of the MMPs is to cleave FasL and reduce its ability to protect the cornea from new blood vessels and inflammatory infiltrates.

The importance of FasL to the control of inflammation and angiogenesis in the eye suggests that it may be a target for therapeutic intervention. At present, very little is known about factors that modulate FasL in the eye; however, it is known that membrane FasL is highly sensitive to the activity of MMPs,[24] which cleave FasL from the cell surface. In this study we examined the ability of inhibitors of MMPs to increase the expression of Fas and thereby prolong corneal graft acceptance.

Materials and Methods

Mice

Female C57BL/6 (H-2^b, B6) and BALB/c (H-2^d) mice were purchased from the National Cancer Institute (Frederick, MD). Female and male

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B6-*gld* and B6-*lpr* mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were housed in accordance with National Institutes of Health guidelines, and all study procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents

The metalloprotease inhibitors used in these assays were doxycycline (Sigma-Aldrich, St. Louis, MO), batimastat (British Biotech, Ltd., Oxford, UK), KB-8301 (BD Biosciences-Pharmingen, San Diego, CA), and SIMP (Peptides International, Louisville, KY).

Orthotopic Corneal Grafting, Treatment, and Evaluation

The procedure for corneal transplantation is described in detail elsewhere.[19,25] The day after removal of the sutures (day 5), all grafted eyes were examined with a vertically mounted slit-lamp biomicroscope (Marco, Jacksonville, FL), and those with surgical complications such as hyphema, cataract, wound leak, or an opacity score of 4, were considered technical failures and excluded from the study. Mice were treated with a standard antibiotic ointment that contains neomycin, polymyxin B, and bacitracin emulsified in petrolatum and mineral oil, or they were treated with the same ointment supplemented with 1% doxycycline. To determine whether such treatment inhibits MMPs in the cornea, zymography was performed as previously described, using SDS-PAGE with gels containing substrates, gelatin (for gelatinase), or casein (for stromelysin and serine proteinases).[26] Two masked observers using a previously described 0-to-5 scoring system[11,27,28] evaluated transplanted corneas for opacity by slit lamp biomicroscopy every 2 to 4 days after day 5. In agreement with previous studies, corneal grafts with an opacity score in excess of 2 at any time point after 2 weeks were considered to be undergoing a rejection reaction. Those mice that presented opacity scores greater than 2 that persisted until the end of the observation period (8 weeks) were defined as having rejected the corneal grafts.

Orthotopic Skin Grafting and Evaluation

Skin grafting was performed using tail skin-to-tail skin grafts according to previously described techniques.[29] Skin grafts are exchanged between mice and evaluated at 4 days for technical failures, with those mice whose grafts were determined to be technical failures excluded from any subsequent studies. Skin grafts are then monitored daily for signs of rejection, as previously described.[29] Briefly, grafts were scored on the basis of appearance of the skin and hair on a scale of 1+ to 4+ and were considered rejected when the scored reached 3+ for the skin.

Human and Murine Corneal Endothelial and Epithelial Cell Cultures

Cell cultures were prepared from human corneas (obtained from the Mid-America Transplantation Association, St. Louis, MO) and from C57BL/6 and BALB/c mouse corneas, as previously described.[30] Briefly, corneal buttons were removed, and Descemet's membrane was removed and placed in DMEM supplemented with 10% fetal bovine serum (FBS), fibroblast growth factor, epidermal growth factor, and insulin. Corneal endothelial cells typically migrated from the explanted tissue within 7 to 10 days after explantation. Cells were passaged after trypsin treatment at a split ratio of 1:10. These disassociated cells were then grown and maintained in the same medium as just described.

Flow Cytometry

We stained corneal endothelial cells after MMP inhibitor treatment with the FasL-specific monoclonal antibody, NOK-1 (human) Kay-10 (mouse) (BD Biosciences-Pharmingen), as previously described.[31] Cells were also stained with an major histocompatibility complex (MHC) class I antibody (culture supernatants of 34-1-2 for mouse or W2/34 for humans) as a positive control.

Apoptosis Assay

Functional assays for measuring FasL-induced apoptosis have been described.[7,11] Briefly, L-1210 cells, which express Fas (2×10^5/mL) were labeled with 5 μCi/mL ^{3}H-thymidine at 37°C in complete DMEM for 2 hours. These cells were then washed twice and incubated (2×10^4 cells/determination) with corneal endothelial cells ($2–5 \times 10^4$ cells/determination), a FasL-expressing L cell (positive control), or alone (negative control) in a 96-well plate overnight at 37°C. After incubation the L-1210 Fas target cells were harvested onto glass microfiber filters, and the radioactivity counted using a microplate scintillation counter (TopCount; PerkinElmer Life Sciences, Boston, MA). Because the fragmented DNA associated with apoptosis does not bind to the filters, the counts associated with the filters reflect nonapoptotic cell DNA only. The percentage of cells undergoing apoptosis is therefore defined as:

$$\%\ \text{DNA fragmentation} = \frac{\text{CPM L}-1210\ \text{Fas incubated alone} - \text{CPM L}-1210\ \text{Fas incubated with cornea}}{\text{CPM L}-1210\ \text{Fas incubated alone}} \times 100$$

To confirm that cell killing is due to Fas-FasL interactions, we added either the competitive inhibitor Fas-Fc (10 μg/mL) or an inhibitor of TNF-mediated apoptosis, TNFR1-Fc (10 μg/mL; both obtained from R&D Systems, Minneapolis, MN), as previously described.[7,11]

Statistical Analysis

Differences in mean fluorescent values and percent apoptosis were compared using Student's *t*-test. Survival curves for the grafting experiments were constructed using the Kaplan-Meier method. Nonparametric tests, both the Wilcoxon and the log rank test, were conducted to test whether the difference in survival curves was statistically significant.

Results

We demonstrated that FasL plays a critical role in the acceptance of corneal allografts. Grafts that do not express FasL are universally rejected, with accelerated kinetics.[11,32] Thus, procedures that would increase the expression of FasL in corneal grafts should result in increased rates of acceptance. It is known that FasL expression is highly sensitive to the activity of MMPs, which act to cleave FasL from the surface of FasL-expressing cells.[14] Therefore, we thought that MMP inhibitors might stabilize FasL expression on cells and thus enable them to stimulate apoptosis in Fas^+ cells more efficiently.

We initially tested this hypothesis by treating corneal endothelial cell lines with several MMP inhibitors to determine whether such treatment results in increased expression of surface FasL. When these cells were treated with doxycycline, there was a significant increase in FasL surface expression (Fig. 1). Similar increases were noted after treatment with batimastat and KB-8301 (data not shown). To demonstrate further that this expression was functionally relevant, we tested the ability of these cells to stimulate apoptosis in a Fas-expressing cell line, L-1210-Fas. As shown in Figure 2, treatment of corneal endothelial cells with the MMP inhibitors, doxycycline (Fig. 2A), batimastat (Fig. 2B), or KB-8301 (Fig. 2C), led to significantly greater apoptosis in the target cells. Addition of Fas-Fc, but not TNFR1-Fc, blocked killing in all cases, confirming that the cell death was mediated by Fas/FasL interactions. Because there is no evidence that MMP inhibitors act by stimulating FasL gene expression, we conclude that increased expression

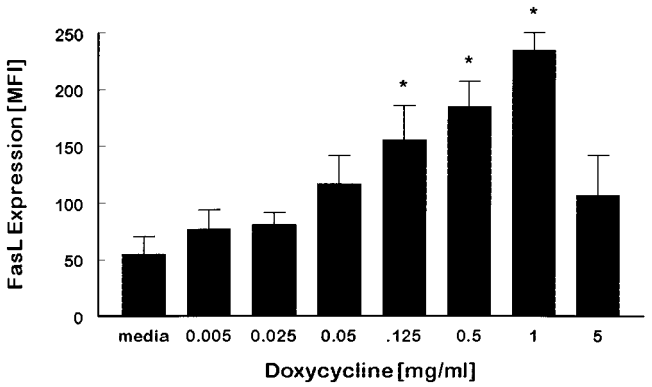

FIGURE 1. Effect of doxycycline on FasL expression on corneal endothelial cells. Cells were treated for 24 hours with the indicated amounts of doxycycline, stained for FasL expression, and analyzed by flow cytometry. Data are the mean ± SEM of results in three independent experiments. *Statistical significance: $P < 0.001$–0.05 compared with cells not treated with doxycycline.

and cell death were due to accumulation of FasL molecules on the surface of these cells.

Because MMP inhibitors can increase functional FasL on corneal cells in vitro, we tested their ability to affect corneal graft acceptance in vivo. We engrafted corneas from C57BL/6 mice into the eyes of BALB/c mice and treated the mice topically twice each day with a standard antibiotic ointment, with or without doxycycline. The mice were then observed and graded for corneal allograft rejection for 8 weeks. Results indicate that mice treated with ointment containing doxycycline had prolonged acceptance compared with those treated with control ointment (Fig. 3). Statistical analysis of these curves revealed that the two curves were different. Wilcoxon analysis yielded $\chi^2 = 4.97$ ($P = 0.02$).

We then tested whether the observed effect of the MMP inhibitor was related to FasL expression. Initially, we performed Western Blot analysis on doxycycline-treated corneas; however, none of the antibodies that we tested identified a specific band of the proper molecular weight (data not shown). Consequently, we engrafted BALB/c mice with corneas from C57BL/6-*gld* mice and then treated with either doxycycline or control ointment. Because these C57BL/6-*gld* corneas do not express functional FasL, they would not be expected to benefit from therapies designed to increase its expression. Results in Figure 4 show that doxycycline ointment had no effect on the rejection of the *gld* grafts, demonstrating that the presence of FasL was necessary for the ointment to be effective and that any other possible benefits that doxycycline treatment might have had were minor.

DISCUSSION

Previous work from this laboratory[11] and another[32] has demonstrated the critical role that FasL plays in corneal allograft acceptance. In the strain combinations used in these studies, corneal allografts that express FasL show a 40% to 60% rate of rejection, whereas those donor corneas that do not express FasL are almost universally rejected.[11,32] These reports raise the intriguing possibility that increasing FasL expression on donor corneas may also increase the acceptance of corneal allografts.

There are several potential mechanisms for increasing FasL expression. These include, increasing FasL expression by inducing protein expression, upregulating FasL gene expression by genetically altering donor corneas to overexpress FasL, or increasing FasL surface expression by blocking the normal turnover of the protein from the cell surface. We chose the final option for three reasons. First, transgenically driven FasL

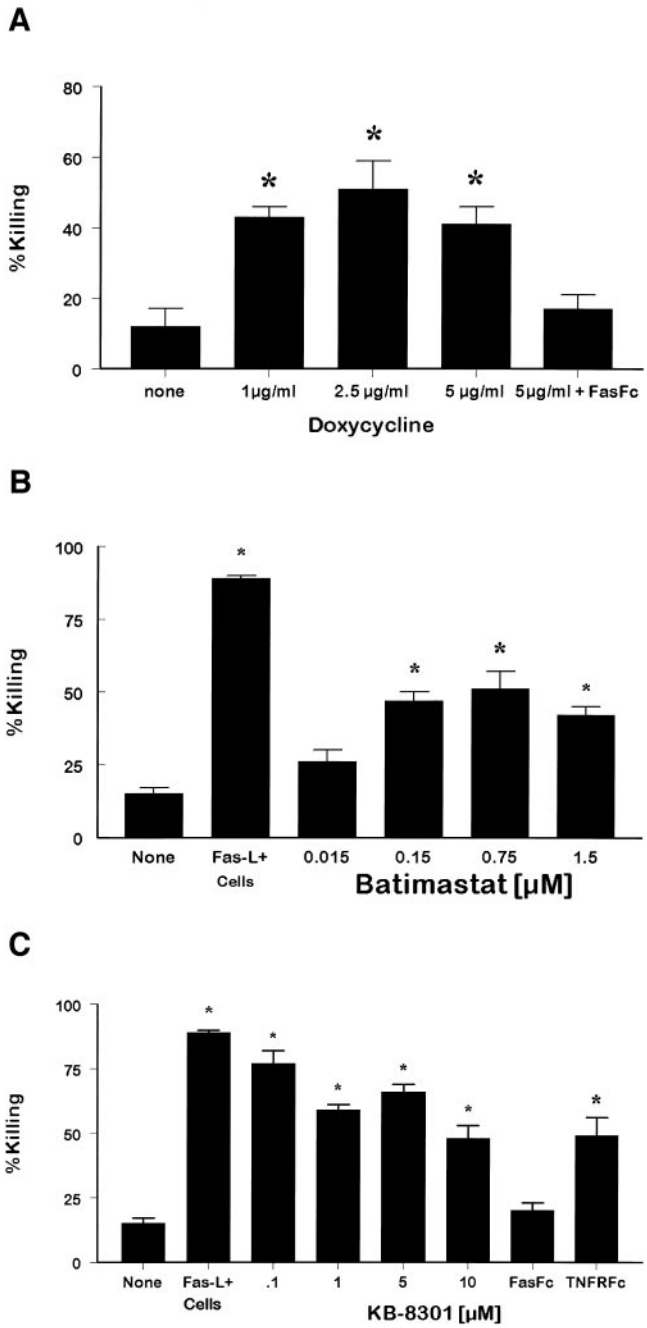

FIGURE 2. Effect of MMP inhibitors on FasL-mediated killing of a Fas-expressing cell line. Human (**A**) and mouse (**B**, **C**) corneal endothelial cells were treated with the indicated amounts of the MMP inhibitors doxycycline (**A**), batimastat (**B**), or KB-8301 (**C**) for 24 hours. Cells were incubated with [^{3}H]thymidine-labeled L-1210 Fas target cells overnight. In (**B**) and (**C**), FasL$^+$ refers to the addition of FasL-expressing L cells to separate wells containing L-1210 Fas cells, which is a positive control for FasL-mediated cell death. (**C**) Inhibitors of apoptosis, Fas-Fc (10 μg/mL) or TNFRFc (10 μg/mL), were added to duplicate wells for all concentrations of the indicated MMP inhibitor used to determine whether apoptosis was mediated by FasL or TNF-α, respectively, in cultures containing 1 μM KB-8301. Data are the mean ± SEM of results in six independent determinations. *Statistical significance at $P < 0.001$–0.02, compared with cells not treated with an MMP inhibitor.

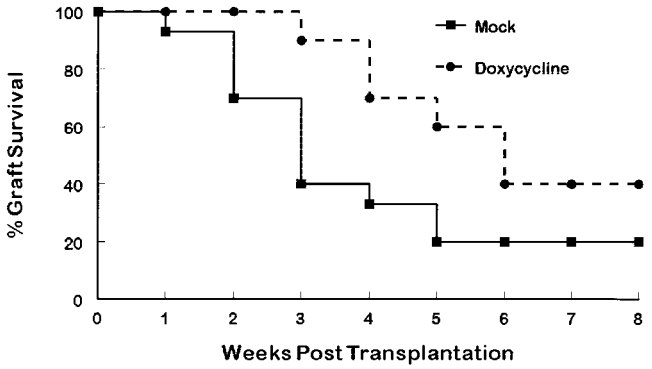

FIGURE 3. Corneal allograft survival in mice treated with doxycycline. BALB/c mice engrafted with C57BL/6 allogeneic corneas were topically treated twice daily with standard antibiotic ointment that either contained the MMP inhibitor doxycycline ($n = 10$) or ointment alone ($n = 15$). Eyes were observed for 8 weeks and evaluated for corneal graft rejection. Statistical comparison of these curves reveal that graft survival in eyes treated with antibiotic ointment containing doxycycline was significantly different from survival in eyes treated with control ointment ($P = 0.02$).

overexpression has, in several instances, resulted in increased inflammation and rejection of transplanted tissues.[33,34] Second, it is unlikely that delivery of FasL with viral vectors will be used clinically, because of the unknown effects that the virus would have on the transplanted cornea. Third, there are various drugs that are MMP inhibitors and thus could be used therapeutically in treating transplanted corneas to increase their survivability. We also chose to use doxycycline, an analogue of tetracycline, because this antibiotic, which is an MMP inhibitor, is an FDA-approved drug and thus may be a good candidate to be used in future clinical studies. Results from these studies indicate that treatment with doxycycline significantly increased the mean survival time and led to a modest increase in the rate of corneal allograft acceptance.

Previous reports have clearly demonstrated that FasL protein is found on the corneal epithelium and endothelium,[7,11] but we know from clinical corneal transplantation that maintenance of an intact corneal endothelium is critical to the clarity of the cornea.[35] Graft rejection is considered irreversible when corneal opacity becomes permanent and is usually attributed to edema or water retention by the stroma and epithelium. Because the corneal endothelium is the major cell type involved in deturgescence of the cornea, it is assumed that irreversible graft rejection involves permanent loss of the donor endothelium without being replaced by host endothelium.[36] After experimental corneal transplantation, the donor cornea typically develops some degree of opacity.[26,27,37,38] This opacity may be permanent, and thus the cornea is rejected, or it may be transient as the cornea recovers. The mechanism for this transient opacity in some corneas is not known, but recent speculation has focused on two possibilities.[36] In both cases, it involves the dysfunction of the corneal endothelium due to either an innate cell attack on the endothelium or an attack by allospecific cytotoxic T cells. We hypothesized that these responses might be limited, at least in part, by Fas-FasL–mediated mechanisms, once again emphasizing the critical nature of FasL expression on the corneal endothelium.[36]

In light of these observations and hypotheses, we believe that our data indicating that MMP inhibitor treatment, which results in prolonged corneal allograft clarity, are due to the stabilization of FasL expression on donor corneal endothelial cells. These cells would then be able to control the infiltration of Fas-expressing inflammatory cells more efficiently, whether they are mediators of innate or adaptive immune responses. In addition, treatment with MMP inhibitors would help to control new vessel growth into the cornea by both stimulating apoptosis in the vascular endothelial cells that express Fas[12,13] and inhibiting tissue remodeling that occurs when new vessels penetrate avascular tissues.[20] As a result of such treatments, we propose that the number of corneas that are susceptible to this early attack by these inflammatory cells would be reduced. Furthermore, we believe that the later rejection of these corneas was probably due, either to the development of cornea-specific T cells that were not sensitive to FasL-induced apoptosis; or, as the donor cornea healed, the efficiency of the ointment's ability to penetrate the cornea to the critical endothelial layer was reduced; or a combination of both of these potential factors. Attempts to overcome the lack of penetration of the drug by systemic (intraperitoneal) treatment of mice with doxycycline (30 mg/kg body weight) did not provide any additional benefits to the corneal allograft (data not shown). In addition, these mice did not tolerate systemic administration with doxycycline, as evidenced by weight loss, lessened activity, and changes in coat appearance.

In either case, the fact that all corneas in the doxycycline-treated eyes maintained clarity much longer than in untreated control eyes also suggests that other immunosuppressive treatments during this time may lead to further increases in corneal allograft acceptance. It was recently reported that treatment of mice with recombinant tissue inhibitors of metalloproteases (TIMPs) induces apoptosis in melanoma cell lines by a mechanism that directly involves stabilization of death receptors, including FasL.[39] It was further demonstrated that TIMP-3–mediated stabilization of death receptors also leads to a concomitant increase in the activation of caspase-3 and -8, confirming that apoptosis is indeed potentiated by stabilized expression of death receptors such as FasL.[39]

In addition to doxycycline's ability to block MMP activity, it has been reported that it can inhibit the activation of IL-1β in corneal epithelium.[40] The mechanism for this observation is probably its inhibition of MMPs, which have been shown to be involved in converting IL-1β into its active form.[41] Thus, another possible mechanism to explain these data is that doxycycline inhibits the activation of IL-1β, which is known to be critical in promoting inflammation that is a precursor to rejection of corneal allografts.[42] However, the fact that B6-*gld* corneas did not display prolonged corneal allograft survival argues that the primary effect of doxycycline treatment was to stabilize FasL expression on donor corneal cells. Studies focusing on increasing FasL function by manipulating surface expression

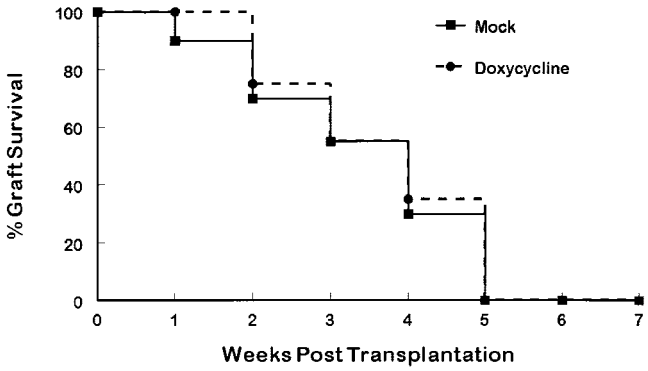

FIGURE 4. Corneal allograft survival in corneas that do not express functional FasL. BALB/c mice were engrafted with C57BL/6-*gld* allogeneic corneas and treated with doxycycline ($n = 11$) or control antibiotic ointment ($n = 10$), as described in Figure 3. Eyes were observed for 8 weeks and evaluated for corneal graft rejection. Statistical analysis did not detect significant differences.

without the need for genetic overexpression may lead to effective methods for improving corneal graft survival.

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