Corneal Neovascularization Induced by Xenografts or Chemical Cautery
Inhibition by Cyclosporin A

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Purpose. Neovascularization of the cornea occurs in numerous pathologic states causing decreased visual acuity and blindness and is a major complication of corneal allotransplantation. The purpose of this study was to investigate the effect of topical and systemic cyclosporin A (CsA) on corneal angiogenesis induced by xenotransplantation or by chemical cautereization. The subcutaneous disc angiogenesis system (DAS) also was used to study the effects of CsA on angiogenesis in a nonocular site.

Methods. Corneal angiogenesis was provoked by either xenotransplantation or chemical cautery. Rats from experiments using both of these models were subdivided into four treatment groups. Topical treatment was administered by using 4% CsA eye drops or vehicle (castor oil) four times daily for 10 days. Systemic therapy consisted of daily (5 mg/kg per day) subcutaneous injections of CsA or vehicle. In the DAS experiments, rats received CsA or vehicle systemically or intradisc. The amount of neovascularization was quantitated by digital image analysis in corneal flat preparations and sections of discs.

Results. Rats that received xenografts or cautery manifested less corneal neovascularization than did control animals after topical or subcutaneous CsA treatment. CsA also enhanced the survival of corneal xenografts. A difference between CsA and vehicle-treated animals in the DAS experiments was not detected.

Conclusions. CsA effectively retards the growth of new vessels in the cornea after xenotransplantation or chemical cautereization and prolongs xenograft survival. However, CsA does not suppress angiogenesis in all systems, because it was ineffective in blocking vessel growth in the subcutaneous DAS. Invest Ophthalmol Vis Sci. 1997; 38:274–282.

The formation of new blood vessels is associated with a wide variety of pathologic conditions, including neoplasms, psoriasis, arthritis, inflammatory reactions, and some ocular diseases.1,2 Corneas vascularized after ulceration, herpetic keratitis, thermal and chemical burns, rejected transplants, and other causes are at high risk for allograft rejection after penetrating keratoplasty. The growth of new vessels also accompanies the rejection of allografts in nonvascularized corneas.3

Animal experiments have shown lymphatic vessels within vascularized corneas.4,5 Based on a study of 160 consecutive penetrating keratoplasties in rabbits, Kevick and Shepherd8 suggested that vessels in the transplanted cornea may enhance both recognition of the nonself antigens in the graft and destruction by the immune system of the sensitized recipient. Corneal vessels presumably enhance allograft rejection because of increased contact of the graft with the afferent and efferent arms of the systemic immune system.9 For example, vascular endothelium is capable of expressing a variety of adhesion molecules (CD 36, ICAM-1, high endothelial cell antigen) that can lead to alterations in normal lymphocyte trafficking patterns.7,8 It also is possible that endothelial cells participate in the presentation of graft antigens to the immune system after induction of major histocompatibility class II or other antigens by inflammatory
cytokines. Corticosteroids, the conventional means of treating neovascularization and graft rejection of corneal tissue, are not always effective and sometimes cause serious complications such as infection, glaucoma, and cataract formation.

Cyclosporin A (CsA), a metabolite of the fungus Tolyphonadactium, is an effective immunomodulatory agent. This cyclic peptide, which is insoluble in water but is soluble in ethanol and lipids, suppresses various T-lymphocyte functions and is, hence, widely used in organ transplantation and in the treatment of autoimmune diseases. To investigate whether CsA inhibits the growth of new vessels, we evaluated the effect of topical and systemic CsA on three different models of angiogenesis in the rat. Neovascularization was induced in the cornea by xenograft transplantation or by chemical cauterization. Additionally, neovascularization was studied in subcutaneous polyvinyl alcohol foam discs (disc angiogenesis system).

METHODS

Animals

Lewis male rats (6 to 8 weeks old) were recipients of xenografts and were used in all experiments with corneal cauterization and the disc angiogenesis system. Hartley male guinea pigs (8 to 10 weeks old) were donors in the corneal transplantation experiments. All animals involved in this study were maintained and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Xenografts

Penetrating corneal grafts were performed as described previously. Guinea pigs were killed by carbon dioxide overdose and, with the aid of an operating microscope, their corneas were harvested using a 3.5-mm trephine and corneal scissors and stored temporarily in a sterile isotonic saline solution at room temperature. After this, rats were anesthetized with intramuscular ketamine (60 to 80 mg/kg) and xylazine (5 to 10 mg/kg). One drop of 0.5% proparacaine hydrochloride was applied to the eye to obtain additional local anesthesia. Recipients received a subconjunctival injection of 0.05 ml of a solution containing 1 mg/ml atropine and 1:1000 epinephrine to produce mydriasis. A 3 mm diameter trephine was used to enter the anterior chamber of the left eye of the rat recipients, and the buttons were excised with corneal scissors. Donor corneas obtained from the guinea pigs (3.5 mm diameter) were sutured into the recipient wound with 12 interrupted 11-0 nylon sutures (Alcon Laboratories, Mohnston, PA). Healon (Pharmacia, Piscataway, NJ) was injected into the anterior chamber after wound closure, and topical gentamicin ointment was administered at the end of each procedure. Half of the sutures were removed on day 5 postgrafting and the remaining six were removed on day 8. Grafted animals were divided randomly into four groups: rats that received eye drops of 4% CsA in castor oil four times per day for 10 days; rats that received eyedrops of castor oil (vehicle) four times per day for 10 days as control subjects; rats treated with subcutaneous CsA (5 mg/kg per day) in castor oil; and rats that received an equal volume of subcutaneous castor oil (vehicle).

Treatments began starting the day before surgery and continued for an additional 10 days until the experiments were terminated. Grafts were examined daily in a masked fashion by an observer, who was unaware of the experimental group of the rat. Using an operating microscope, this investigator scored the corneas on a scale that weights graft clarity and neovascularization as the two most important and consistent indicators of graft rejection, but also takes into account vessel size and degree of graft edema. The scoring system for graft clarity was as follows: 0 = completely clear; 1 = slightly hazy, iris and pupil easily seen; 2 = slightly opaque, pupil still detectable; 3 = opaque, pupil extremely difficult to detect; and 16 = completely opaque cornea, without view of anterior chamber. The scoring system for graft vascularity was as follows: 0 = no vessels in or at margin of graft; 1 = vessels at graft margin; 2 = vessels 25% of distance from margin to center; 3 = medium vessels, easily detectable; 4 = vessels 50% of distance from margin to center; and 16 = vessels reaching center of graft. The scoring system for vessel diameter was as follows: 0 = completely avascular; 1 = small vessels, detectable under operating microscope; 2 = medium vessels, easily detectable; and 3 = large vessels, detectable without operating microscope. The scoring system for graft edema was as follows: 0 = none; 1 = mild, graft slightly above recipient bed; 2 = moderate, graft up to double normal width; and 3 = severe, graft more than double normal width.

### TABLE 1. Serum Levels of CsA After Subcutaneous Administration as Determined by a Fluorescent Polarization Immunoassay

<table>
<thead>
<tr>
<th>Treatment Duration (days)</th>
<th>4 Hours</th>
<th>12 Hours</th>
<th>24 Hours</th>
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<tr>
<td>6</td>
<td>338 ± 23</td>
<td>412 ± 45</td>
<td>254 ± 38</td>
</tr>
<tr>
<td>10</td>
<td>357 ± 31</td>
<td>442 ± 47</td>
<td>280 ± 29</td>
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CsA = cyclosporin A.
Administration dose = 5 mg/kg per day.
Grafts were considered rejected if the sum of the individual grades was 12 or more. Grafted animals with completely opaque lenses, complete loss of the anterior chamber, infection, or significant adhesions between the iris and cornea (synechiae) were eliminated from the study.

Measurement of Serum Levels of Cyclosporin A

After 6 and 10 days of treatment with subcutaneous CsA (5 mg/kg per day) or topical CsA (4%, four times a day), representative animals were anesthetized and whole blood was collected from the tail vein in heparinized capillary tubes. Blood was collected 4, 12, and 24 hours after treatment from six animals at each time point, and after ultracentrifugation, the serum CsA levels were determined by a fluorescent polarization immunoassay method.14,15

Corneal Cauterization

Rats were anesthetized deeply with intraperitoneal pentobarbital sodium (40 to 50 mg/kg), and both corneas were cauterized with a 75% silver nitrate–25% potassium nitrate applicator (Graham–Field Surgical, New Hyde Park, NY) to induce the growth of new vessels. The applicator was held in place for 5 seconds, and excess silver nitrate was removed by gentle blotting with tissue paper.16,17 The animals were divided into four groups of eight rats each. The drugs used and the schedule of treatment were the same as those for the corneal transplantation experiments.

Image Analysis of the Corneas

The animals were killed 10 days after corneal transplantation or cauterization by a lethal injection of intraperitoneal pentobarbital sodium (200 mg/kg). To fill the microvasculature and quantitate corneal neovascularization,17 the upper body was perfused with 50-ml lactated Ringer’s solution until the normal pink color of the ocular fundi disappeared and then with 20 ml of a mixture of 11% gelatin (Kind and Knox, Sioux City, IA) and 10% India ink (Faber-Castell, Lewisburg, TN) in Ringer’s filtered through No. 41 Whatman filter paper (Whatman International, Maidstone, England). Immediately after perfusion, the gelatin within the corneal vessels was solidified by freezing the eyes with compressed dichlorodifluoromethane (SPI Supplies, West Chester, PA). The eyes were enucleated and submerged in 10% phosphate-buffered neutral formaldehyde for at least 24 hours. The cornea and 1-mm rim of adjacent scleral tissue then were separated from the rest of each globe, and three full-thickness peripheral radial cuts were made through the cornea to allow flattening. Corneal flat
Inhibition of Corneal Neovascularization by Cyclosporin A

FIGURE 2. Kinetics of rejection of Hartley guinea pig corneal grafts by Lewis rats. Grafts were considered rejected when the sum of the individual grades for corneal clarity, neovascularization, vessel diameter, and edema was 12 or more. Vehicle = castor oil.

preparations were masked to minimize observer bias and then analyzed by computerized image analysis (Optimas; Bioscan, Edmunds, WA) using a modification of the method of Proia et al. The total length of new vessels in each cornea was determined on flat preparations of corneas by an observer who had no knowledge of the treatment regime. Results were expressed as the ratio of length of vessels to corneal area.

Disc Angiogenesis System

Sponges made of polyvinyl alcohol foam (2 mm thick) (Kanebo PVA; Rippey, Santa Clara, CA) were dipped in 100% ethanol and air dried, then cut into discs (11 mm in diameter) using a corneal trephine. A round hole (2 mm in diameter) was punched in the center of the disc with a specially constructed trephine. Nitrocellulose filter paper (Millipore, Bedford, MA) of the same diameter of the sponge disc was cemented to one of the flat sides of the disc using a sterile glue. A laminar flow hood was used to assemble the sterile components, and all assembled discs were sterilized by a mixture of Freon and ethylene oxide (130°F) for 2 hours.

A small piece (3 mm × 3 mm × 3 mm) of sterile absorbable gelatin sponge (Gelfoam; Upjohn, Kalamazoo, MI) was impregnated with a solution of the test substance or with saline and then placed in the center hole. The open side of disc then was sealed with the gas-sterilized nitrocellulose filter paper and sterile glue.

Rats were anesthetized before the insertion of the foam discs with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Four discs were implanted subcutaneously over the back of each animal after the skin over the planned implantation sites was shaved. For each disc, the skin was incised (2 cm long) and, after a subcutaneous channel was produced lateral to the incision site by blunt dissection, the disc was slid into this space and the wound was closed with stainless steel surgical clips (Becton–Dickinson, Parsippany, NJ).

For the analysis of angiogenesis within polyvinyl alcohol foam discs, rats were divided randomly into four groups of five animals (20 discs/group). Different groups received discs containing CsA (50 mg/disc) or vehicle (castor oil) into the gelatin sponge. Other groups treated subcutaneously at a site remote from the discs with CsA (5 mg/kg per day) or vehicle (castor oil) received a gelatin sponge containing sterile saline. Systemic treatment was started the day before the implantation of discs.

Fifteen days after the implantation of discs, the rats were killed by a lethal intraperitoneal injection of sodium pentobarbital (200 mg/kg). The discs were removed and fixed in 10% neutral formaldehyde solution, embedded in paraplast, sectioned through the center of disc in a plane perpendicular to the central well or parallel to the nitrocellulose filter paper, and

FIGURE 3. Effects of topical or systemic cyclosporin A on corneal neovascularization induced by xenotransplantation. The ratios between the total length of new vessels and the area of the corneas are given. Statistical significance was calculated by comparing each experimental group of animals to the corresponding control group using the Student’s t test. *P < 0.005 versus control subjects. Each value represents the mean ± standard error of 10 corneas. Vehicle = castor oil.
stained with hematoxylin and eosin. To determine the amount of fibrovascular growth, images of the sectioned discs were captured by video camera, and the area that fibrovascular tissue extended centrally into the discs was measured by image analysis.

Data Analysis
Results are expressed as the arithmetic mean ± standard error of the mean (standard error). Each experimental group for the corneal transplantation and cauterization experiments had 10 and 16 animals, respectively. The disc angiogenesis experiments consisted of four discs in different animals per experimental group. The Student's t-test was used to measure differences between groups. P ≤ 0.05 was deemed significant.

RESULTS
Serum Levels of Cyclosporin A
The serum CsA levels as determined by a fluorescent polarization immunoassay in representative rats after subcutaneous or topical administration are summarized in Table 1. In the systemically treated animals, the serum levels of CsA reached levels sufficient to achieve immunosuppression. These levels are similar to those reported previously by Wassef et al. None of the topically treated animals achieved serum concentrations of CsA that were within the detection limits of the assay (<25 ng/ml).

Corneal Xenografts
Corneal xenografts became thickened and slightly opaque in almost all animals during the first day after corneal transplantation. This edema usually disappeared within 1 to 2 days. By 6 to 8 days post-transplantation, the grafts of the control groups (groups 2 and 4) that received topical or systemic vehicle (castor oil) had lost clarity, were markedly edematous, and intensely vascularized (Fig. 1) and were considered rejected. In the group treated with 4% topical CsA (group 1), the grafts in 6 of the 10 rats remained clear and did not swell or have relevant neovascularization by day 10. Nine of the 10 rats treated with systemic
TABLE 2. Effects of Topical or Systemic Cyclosporin A on Corneal Neovascularization Induced by Chemical Cautery

<table>
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<tr>
<th>Treatment</th>
<th>Total Vessel Length/Corneal-Area (mm) (mean ± standard error)</th>
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<tr>
<td>Topical cyclosporin A (4% in castor oil)</td>
<td>8.92 ± 0.58*</td>
</tr>
<tr>
<td>Topical vehicle (castor oil)</td>
<td>12.87 ± 0.63</td>
</tr>
<tr>
<td>Systemic cyclosporin A (5 mg/kg per day)</td>
<td>8.25 ± 0.69*</td>
</tr>
<tr>
<td>Systemic vehicle (castor oil)</td>
<td>12.37 ± 0.56</td>
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* P < 0.005 versus controls.

CsA (group 3) had clear corneas and did not reject their grafts by 10 days postsurgery. The difference between control and treated groups was particularly evident at day 7 when almost all grafts were rejected in the control animals but in none of the treated animals (Fig. 2).

In both CsA-treated groups, clinical examination results also disclosed a significant reduction of new vessel length and diameter (Fig. 1). The differences in corneal vascularization between the experimental groups were confirmed by computerized image analysis on flat preparations of perfused corneas. The ratio between the length of new vessels and the area of the corneas in the group treated with topical CsA was significantly less than in the control group (Fig. 3). Systemic CsA also inhibited the vascularization significantly compared with the control group (Figs. 3, 4). Systemic CsA reduced the corneal angiogenesis more than topical CsA (P = 0.05).

**DISCUSSION**

In our quantitative evaluation of the effect of topical and systemic CsA on angiogenesis, we found this 11 amino acid-containing cyclic peptide to suppress angiogenesis in 2 of 3 different models. CsA effectively reduced blood vessel formation in a model of rat xeno-transplantation, which induces marked corneal neovascularization. It also inhibited angiogenesis induced by cautereization of corneas with silver—potassium nitrate. CsA was, however, not effective in the noncorneal subcutaneous disc angiogenesis system.

In humans, topical corticosteroids have been the primary treatment for actively proliferating corneal vessels, and this mode of therapy still is used clinically in humans to reduce the growth of new vessels. Corticosteroids alone and in association with heparin or sulfated cyclodextrins inhibit angiogenesis significantly. Other potential modes of therapy for corneal vascularization include photocoagulation of vessels with lasers and nonsteroidal antiinflammatory agents (such as indomethacin and flurbiprofen). In addition to the two corneal models of corneal vascularization investigated in the current study, CsA is reported to suppress corneal allograft rejection in rats and in rabbits when administered systemically and topically. CsA also caused the regression corneal angiogenesis in dogs with spontaneously occurring keratoconjunctivitis sicca after topical CsA treatment. CsA also reduced corneal neovascularization that developed in mice after an intracorneal injection of interleukin-2 (IL-2). CsA given subcutaneously (4 mg/kg per day) also has been found to suppress new vessel formation induced in the mesenteric-window assay in rats. CsA also is reported to produce clinical improvement in psoriasis and certain epithelial neoplasms in which angiogenesis is prominent.

The mechanism whereby CsA reduces angiogenesis in some, but not all models, remains uncertain and


