Stromal vascularization prevents corneal ulceration

Howard Conn, Michael Berman, Kenneth Kenyon, Robert Langer, and Janet Gage

Experiments were performed with a model of focal, thermal-induced ulceration to test the clinical impression that vascularization prevents ulceration of the corneal stroma. Slow-release polymers containing a vasoproliferative agent (tumor angiogenesis factor) were placed in corneal pockets 2 mm central to the limbus of albino rabbits. These polymers elicited blood vessel ingrowth up to the implant. Control eyes received empty polymers which caused minimal to no vessel growth. Polymers were removed, and each cornea received a focal, thermal burn placed just central to the polymer site. All control corneas ulcerated: most (79%) developed deep stromal or perforating ulcers. Only 25% of prevascularized corneas developed stromal ulcers, and none was deep or perforating. After thermal burns, vessels in both groups grew at the same linear rate toward the burned area. There was a direct relationship between the distance separating the nearest blood vessel and the burned area at the time of burning and the maximum depth of stromal ulceration. Thus prevention of or less severe stromal ulceration is correlated with the earlier presence of vessels in the burned area.

Key words: cornea, vascularization, ulceration, collagenase, thermal burns, collagen degradation

The clinical impression that stromal vascularization prevents or arrests ulceration draws support from three observations. (1) Corneal ulceration often occurs central to advancing blood vessels and rarely in the vascularized areas of the cornea.1-5 (2) Ulceration seems to be arrested when vessels reach the injured area.1-4 (3) Conditions or agents which inhibit corneal neovascularization, such as damage to limbal vessels, are associated with increased tissue destruction.5-7

Although vascularization of the corneal stroma appears to be correlated clinically with the arrest of further ulceration, there have been no direct tests of the role of vascularization under controlled conditions. To assess this role, we have used a model of focal, thermal-induced injury which produces ulceration with high frequency within a discrete burned area of the avascular cornea.8 The role of vascularization was assessed by first bringing vessels into the stroma, adjacent to the region to be burned, with a vasoproliferative agent, tumor angiogenesis factor (TAF).9

From the Department of Surgery, Children's Hospital Medical Center (H. C. and R. L.) Department of Cornea Research, Eye Research Institute of Retina Foundation; (M. B., K. K., and J. G.) and the Departments of Ophthalmology, Harvard Medical School (M. B. and K. K.) and Nutrition and Food Science, Massachusetts Institute of Technology, (R. L.), Boston.
This study was supported in part by research grant 2RO1CA14095 (J. Folkman) from the National Cancer Institute; research grant EY-01246 (M. Berman); Program Project EY-00208, Institutional National Research Service Award EY-07018, Biomedical research support grant PHSS07890537 from the National Eye Institute, National Institutes of Health; Academic Investigator Award EY00156 from the National Eye Institute, National Institutes of Health (K. K.); and the Massachusetts Lions Eye Research Fund, Inc.
Reprint requests: Dr. Howard Conn, Wilmer Institute, Johns Hopkins Hospital, 601 North Broadway, Baltimore, Md. 21205.

0145-0404/80/040362+09$00.90/0 © 1980 Assoc. for Res. in Vis. and Ophthal., Inc.

Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933091/ on 11/11/2018
Vascularization prevents corneal ulceration

Materials and methods

Male New Zealand albino rabbits, weighing 3 to 4 kg, were anesthetized with intravenous sodium pentobarbital and topical proparacaine hydrochloride. Corneas to be prevascularized received a slow-release pellet containing 300 μg of TAF derived from Walker 256 rat carcinoma (Fig. 1). The pellet was placed inside an intralamellar pocket, so that the central-most edge of the pellet was 2 mm from the limbus (Fig. 2). The pocket was made at a 30° angle from the horizontal axis of the eye to avoid disturbing the area of the cornea to be burned. Control corneas received a slow-release ("empty") pellet without TAF at the same location in the stroma as the corneas to be prevascularized. Eight to ten days after implantation, when vessels had converged on the TAF-containing pellets (Fig. 2), pellets with or without TAF were removed through a 1 mm incision over the pellet. Standard thermal burns were then made at a position central and adjacent to the former pellet site, so that the peripheral border of the burned area and central border of the pellet site coincided (Fig. 1). Burns were made with a Frigitronics 4.5 mm curved-tip thermokeratophore with a probe temperature of 130°C. Each cornea received two 1 sec applications of the probe and was irrigated with saline prior to and immediately after each probe application. Previous studies had demonstrated that avascular corneas burned in this way ulcerated in 100% of cases. All eyes received erythromycin ophthalmic ointment immediately after the burns and at each subsequent examination.

Clinical examination. Corneas were examined on alternate days with a Zeiss slit-lamp stereomicroscope. Three variables were measured: accuracy ±0.1 mm and documented photographically. First, the maximum blood vessel length was determined, from which the rate of vessel growth was calculated. Second, ulcer width was measured, and ulcer depth was graded according to the following scale: 0, no stromal ulcer; 1+, trace stromal defect in slit-lamp beam; 2+, superficial stromal ulcer; 3+, deep stromal ulcer; 4+, perforating ulcer or descemetocele. Third, distance from the limbus to the peripheral edge of the ulcer was measured. All values given are sample means ± S.D. In addition, corneal opacity within the burn was estimated according to the degree of opacity and the area involved. All rabbits not used for histology were sacrificed after 10 weeks by an intravenous overdose of pentobarbital.

Histology. One animal each in the control and prevascularized groups was sacrificed by intravenous pentobarbital at the following times: immediately before burning; immediately after burning; and at 2, 4, 8, 12, and 16 days after burning. Eyes were enucleated and fixed in 10% buffered formalin. Corneas were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Organ culture of control and prevascularized corneas. Twelve ulcerating (control) corneas were arbitrarily divided into two groups of six corneas each, and the six corneas in each group were minced and distributed to organ culture plates to give two control cultures. Similarly, six prevascularized, nonulcerating corneas were minced and distributed to a single culture plate. Media were changed daily; harvests were stored at -20°C until used for assays of collagenase and hydroxyproline.

Collagenase activity. Collagenase activity was assayed in a capillary heat gel system in which 1 mm of lysis per hour is equivalent to 0.35 μg of collagen hydrolyzed per minute in the radiofibril assay. Results are given as the mean ± S.D. for the two control plates and as a single value where only a single culture plate of a given type was assayed. "Active" ("manifest") collagenase in the collagenase activity detected without prior trypsin activation of latent collagenase; "total" collagenase

EXPERIMENTAL PROTOCOL

Fig. 1. Corneas to be prevascularized received a slow-release pellet containing 300 μg of TAF. Control corneas received a slow-release, "empty" pellet without TAF. Eight to 10 days after implantation, when vessels had converged on the TAF-containing pellets, pellets with or without TAF were removed. Standard thermal burns were then made at a position central and adjacent to the former pellet site. (See text.)

Histology. One animal each in the control and prevascularized groups was sacrificed by intravenous pentobarbital at the following times: immediately before burning; immediately after burning; and at 2, 4, 8, 12, and 16 days after burning. Eyes were enucleated and fixed in 10% buffered formalin. Corneas were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Organ culture of control and prevascularized corneas. Twelve ulcerating (control) corneas were arbitrarily divided into two groups of six corneas each, and the six corneas in each group were minced and distributed to organ culture plates to give two control cultures. Similarly, six prevascularized, nonulcerating corneas were minced and distributed to a single culture plate. Media were changed daily; harvests were stored at -20°C until used for assays of collagenase and hydroxyproline.

Collagenase activity. Collagenase activity was assayed in a capillary heat gel system in which 1 mm of lysis per hour is equivalent to 0.35 μg of collagen hydrolyzed per minute in the radiofibril assay. Results are given as the mean ± S.D. for the two control plates and as a single value where only a single culture plate of a given type was assayed. "Active" ("manifest") collagenase in the collagenase activity detected without prior trypsin activation of latent collagenase; "total" collagenase

Downloaded From: https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933091/ on 11/11/2018
activity is the sum of activity that results from active collagenase plus that due to latent collagenase activated by prior trypsin treatment.\textsuperscript{15} 

\textbf{Measurement of hydroxyproline.} Hydroxyproline, as an amino acid marker of solubilized stromal collagen, was determined by the method of Rojkind and Gonzalez.\textsuperscript{16}Data were expressed as micrograms of hydroxyproline per milliliter of medium.

\section*{Results}

\textbf{Clinical course of ulceration in controls.} All corneas developed epithelial defects immediately after burning. Control corneas first showed stromal ulceration within 3 to 8 days (average 4.8 ± 1.5 days) after the thermal burns. Ulceration always occurred within the burned area and appeared with a peripheral edge 2.8 ± 0.79 mm from the limbus. Ulcers ranged in size from 1.5 to 3.5 mm in width (2.3 ± 0.55 mm). Ulceration always occurred central to the vessel tips which advanced straight and brushlike from the limbus (Fig. 2). The distance between the ulcer and the nearest vessel tip, when an ulcer was first noted, was 1.40 ± 0.79 mm. Ulceration was first detected usually as a mere defect by slit-lamp examination. Ulceration progressed to a variable extent, but the maximum ulcer depth was reached usually within 5 to 6 days after onset of ulceration and no later than 16 days after thermal injury. In many corneas, stromal tissue within the ulcer region was degraded extensively, leaving only a bulging Descemet's membrane (descemetocele) at the ulcer base. Perforation sometimes followed the appearance of a descemetocele.

Fig. 2. A, Control cornea with "empty" pellet, showing no vessels in the cornea prior to burning. B, Control cornea 6 days after thermal burn, showing a deep ulcer. C, Same control cornea 2 days later, with a descemetocele (circle). The blood vessels have advanced but have not reached the burned area. D, Prevascularized cornea containing a TAF pellet, with blood vessels growing into the cornea prior to burning. E, Prevascularized cornea 6 days after burning. The vessels have grown into the burned area, and there is no ulcer. F, Same prevascularized cornea 2 days later, showing further vessel ingrowth and no ulcer.
Vascularization prevents corneal ulceration

Fig. 3. Blood vessel length vs. time. **Right,** Average blood vessel length in the control corneas when ulceration first appeared (day 5) was 1.4 mm. **Left,** Average blood vessel length in the prevascularized corneas when controls began to ulcerate (day 5) was 3.1 mm. **Inset,** Blood vessels in the control and prevascularized corneas grew at the same linear rates (0.28 and 0.31 mm/day, respectively).

Fibrous tissue was observed to fill in the perforations in animals permitted to survive; all corneal ulcers eventually healed (22.7 ± 2.8 days after burning) in association with neovascularization of the burned area. Thermal-burned areas that did not ulcerate remained opaque for several weeks and were also invaded by blood vessels. Most blood vessels were seen to regress after several weeks.

**Effect of prevascularization on the clinical course of ulceration in thermal-burned corneas.** Table I shows the incidence and depth of ulceration in the control and prevascularized groups. All control corneas (vessel length, 0 to 0.5 mm at time of burning) ulcerated; 78.6% (11 of 14) developed deep stromal ulcers, perforations, or descemetocytes. Of the prevascularized corneas with vessels 1.5 to 3.0 mm in length at the time of burning, 25% (4 of 16) developed stromal ulcers; none was deep, perforating, or a descemetocoele (Fig. 2). Histologic examination confirmed the clinical grading of ulceration.

After thermal burns, vessels in the control and prevascularized corneas grew at the same linear rate (0.28 and 0.31 mm/day, respectively) toward the burned area (Fig. 3). After 14 days (data not shown), the vessels reached a limiting length and then gradually regressed over a period of weeks. At the time when ulcers first appeared in the control corneas (day 5), the average blood vessel length in those corneas was 1.4 mm. In contrast, the average blood vessel length in the prevascularized corneas, at the same time (5 days) when control corneas were ulcerating, was 3.1 mm. Although the blood vessel growth rate was identical in the 2 groups, the vessels in the pre-vascularized corneas, having started from a position closer to the burned area, arrived earlier in the burned area. Thus prevention of or less severe ulceration was correlated with the earlier presence of vessels in the burned area.

**Table I. Incidence and severity of ulceration**

<table>
<thead>
<tr>
<th>Ulcer depth</th>
<th>No. of corneas developing ulcers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>0 (0)*</td>
</tr>
<tr>
<td>1+</td>
<td>1 (7)</td>
</tr>
<tr>
<td>2+</td>
<td>2 (14)</td>
</tr>
<tr>
<td>3+</td>
<td>2 (14)</td>
</tr>
<tr>
<td>4+</td>
<td>9 (65)</td>
</tr>
<tr>
<td>Total</td>
<td>14 (100)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses = percent of total corneas.
Fig. 4. Relationship between ulcer depth and the distance between blood vessels and the burned area. There is a direct relationship between the maximum ulcer depth and the distance between the nearest blood vessel and the burned area ($r = 0.76, p < 0.001$).

Relationship between ulcer depth and the distance between blood vessels and the burned area. At the time of burning, prevascularized corneas differed in the maximum length of blood vessels between the limbus and the TAF pellet. Thus, considering the control corneas (vessel length, 0 to 0.5 mm; average, 0.18 mm), the prevascularized corneas with maximal vessel length of 0.6 to 1.4 mm, and those with vessels 1.5 to 3.0 mm in length, there was a spectrum of corneas with maximum blood vessel lengths ranging from 0 to 3 mm before burning. As shown in Fig. 4, there was a direct relationship between the maximum ulcer depth and the distance between the nearest blood vessel and the burned area ($r = 0.76, p < 0.001$).

Corneal opacification. Corneal opacification during the first 9 weeks after thermal burning was comparable in the control and prevascularized groups. At 10 weeks, however, there appeared to be less opacification in the prevascularized corneas due perhaps to the fact that control corneas had more severe ulceration and consequently more scarring.

Histologic observations. Control corneas exhibited severe ulceration histologically as early as day 4 after burning (Fig. 5). Ulceration was associated with infiltration of polymorphonuclear leukocytes (PMNs) which persisted through day 12. In contrast, in prevascularized, nonulcerating corneas, the PMN infiltrate was less at day 4 than in controls and had subsided by day 12.

Collagenase activity. Active and latent collagenase were present in both the control cultures and the prevascularized culture (Fig. 6). Although the variability in activities between the control cultures was wide and precluded an interpretation of definite differences in comparison with the prevascularized culture, the activities in the single prevascularized culture appeared to be lower than in the control cultures and were comparable to those observed in other cultures of nonulcerating corneas that had been burned centrally. Moreover, twice as much of the enzyme in the prevascularized culture than in the control cultures was in a latent form.

Collagen degradation. Control cultures demonstrated much higher levels of hydroxyproline in vitro than did the culture of prevascularized corneas (Fig. 7), which had a profile of hydroxyproline levels similar to that produced in culture by nonulcerating corneas that had been burned centrally.

Discussion

The results of the current study support the clinical impression that ulceration is arrested by corneal vascularization. Moreover, it would not appear that the ability to prevent ulceration is limited to blood vessels brought...
Fig. 5. A, At day 4 after burning, a prevascularized cornea shows minimal inflammatory cell infiltration of the nonulcerated stroma. A fibrocellular layer (asterisk) has developed posterior to Descemet’s membrane. (×100.) B, Control cornea at this time has undergone severe stromal ulceration with rupture of Descemet’s membrane (arrow) and intense PMN infiltration. (×50.) C, At day 8 after burning, a prevascularized cornea displays only superficial stromal ulceration and mild PMN infiltration, whereas the control (D) has undergone Descemetocle formation. (Both ×80.) E, By day 12 after burning, a prevascularized cornea has an intact epithelium, proliferating stromal fibroblasts, and numerous vessels (circled), with rare inflammatory cells (×250.) F, In contrast, the control cornea has continued to undergo stromal degradation in association with intense inflammatory cell response. (×200.)
Fig. 6. Collagenase activities in organ culture. Active collagenase (○) and total collagenase (●) in control, ulcerating corneas appear higher than in prevascularized corneas. A, Control corneas. B, Prevascularized corneas. Shown also for comparison in B, are collagenase profiles from nonburned, nonulcerating corneas (active ○; total ●) and centrally burned, nonulcerating corneas (active △; total ▼). Collagenase levels from prevascularized corneas appear higher than corresponding levels from nonburned, nonulcerating corneas and comparable to levels from centrally burned, nonulcerating corneas. Collagenase activity in control cultures is the mean activity ± S.D. of two replica cultures. Collagenase activity from nonburned, nonulcerating corneas, from prevascularized corneas, and from centrally burned, nonulcerating corneas was determined upon harvests from one culture of each type.

in by TAF. Our additional studies (unpublished data) have similarly demonstrated that vessel ingrowth to the 2 mm position, stimulated by burning with AgNO₃, also resulted in significant reduction in the incidence (6 of 14) and severity (1+ and 2+) of ulceration after the standard thermal burns. Furthermore, TAF itself would not appear to have prevented ulcers, since controls in which TAF-containing pellets were implanted immediately prior to burning and were left in place after the burn still had as many (12 of 12) and as severe (3+ and 4+) ulcers as did the standard control corneas.

Although it is not known how vascularization prevents ulceration, it is possible that vessels bring in antiproteases to inhibit destructive proteases and that vessels supply cells and nutrients for wound repair. In a previous study, the stromal ulceration caused by thermal burns was apparently mediated, in part, by collagenase, and collagenases are inhibited by serum antiproteases. Indeed, evidence has been presented that day 1 culture medium from alkali-burned, ulcerating corneas contains collagenase in complex with the rabbit α₁-macroglobulin.

The variability in collagenase levels between control plates of ulcerating corneas was significant in the present experiments, but the levels of active and latent collagenase in such cultures were comparable to those observed in individual cultures in previous work. The observed variability in the control cultures is due to the fact that the extent of
collagenase secretion, collagen degradation, and ulceration varies considerably from one cornea to another, and cultures of ulcerating corneas which are replicas with regard to collagenase secretion and collagen degradation are established only when sectors of each cornea to be cultured are distributed to all culture plates. In the present work, control ulcerating corneas were not distributed so as to produce replica plates because we sought to determine the variability between control plates with regard to the profiles observed in the single plate of prevascularized corneas.

Although only one culture of prevascularized corneas was studied, it would seem that such corneas that were not ulcerating in vivo by clinical criteria still retained the capacity to secrete some collagenase in culture and that lack of ulceration in vivo was not due to irreversible shutting off of collagenase production. Indeed, the levels of active and latent collagenase and of solubilized hydroxyproline in the prevascularized culture, although lower than in the ulcerating controls, were between those of nonburned, nonulcerating corneas and burned, ulcerating corneas and burned, ulcerating corneas.

Explanting the nonulcerating, prevascularized corneas removed them from the blood supply and, hence, possibly from a source of serum inhibitors. Whatever the mechanism(s) by which vascularization prevents ulceration in vivo, it would seem, especially on the basis of the hydroxyproline data, that prevascularized corneas have markedly suppressed ability to degrade stromal collagen during subsequent culture in vitro. It is noteworthy that neither corneas burned centrally but not ulcerating nor corneas burned peripherally but prevascularized and nonulcerating demonstrated significant numbers of PMNs upon histological examination.

In previous studies of ulceration after thermal and alkali burns, stromal ulceration was found to be associated invariably with PMNs that were active phagocytically. Thus, although the role of PMNs in ulceration is not yet understood, it was of interest in the present work that prevention of ulceration by prior vascularization was correlated with a reduced PMN infiltration, as compared to the more numerous PMNs in the ulcerating control eyes. It has been suggested that the epithelium might generate chemotactic factors, possibly via a serine protease, that are responsible for the PMN infiltration of the stroma after injury. Perhaps, then, vascularization brings in inhibitors of the hypothetical "chemotactic" protease to prevent PMN infiltration and subsequent stromal ulceration.

Evidence has been presented recently that the degradation of stromal matrix occurs simultaneously with the synthesis of repair components. Thus, as suggested by the work of Pfister and Paterson, ulceration can be prevented by supporting repair of the injured cornea. In the present study, the early vascularization of the burned region might also bring in nutrients, thus preventing ulceration, in part, by supporting repair.

In addition to supporting the clinical impression that vascularization prevents stromal ulceration, the current study presents an experimental model which should be useful in understanding the mechanisms of the prevention of ulceration by vascularization.

Fig. 7. Collagen degradation in organ culture. Collagen (solubilized hydroxyproline) was lowest in nonburned, nonulcerating corneas (●), was higher in prevascularized, nonulcerating corneas (○) and in centrally burned, nonulcerating corneas (△), and was highest in control ulcerating corneas (▲). Values from control ulcerating corneas are means ± S.D. for two cultures; for the other types, symbols represent harvests from one culture each.
We thank Dr. Judah Folkman for discussion and support. We also thank Herbert Kayne, Ph.D., for help in the statistical analysis of the data.

REFERENCES