Effect of Irradiation on Vascularization of Corneas
Grafted onto Chorioallantoic Membranes

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Several studies have shown that total body irradiation decreases the angiogenic response to corneal cauterization. This inhibition could be due to alterations in angiogenic stimuli within injured corneas and/or to a decreased ability of irradiated animals to respond to such stimuli. To determine whether total body irradiation specifically affects angiogenic stimuli within injured corneal tissue, cauterized corneas from mice exposed to 900 rads of total body irradiation and from non-irradiated controls were grafted onto the chorioallantoic membranes (CAM) of chick embryos and their abilities to stimulate the ingrowth of healthy embryonic blood vessels were compared. Cauterized corneas incorporated into CAM mesenchymal tissue were invaded by blood vessels in 34.6% of the irradiated group and in 75% of the non-irradiated controls. This difference in the two groups was statistically significant ($P < 0.03$).

Total body irradiation significantly decreased the frequency of vascular invasion of cauterized corneal tissues by healthy CAM blood vessels. This finding suggests that total body irradiation can specifically affect the stimulus for angiogenesis within cauterized corneas. Invest Ophthalmol Vis Sci 26:1533-1542, 1985

Lethal doses of total body irradiation have been shown to inhibit angiogenesis after corneal injury. Fromer and Klintworth found that 1500 rads of total body irradiation completely suppressed corneal vascularization after chemical cauterization in 40–75 gram Fischer albino rats. Corneal angiogenesis after similar chemical injuries was partially, but not completely, inhibited in 200–400 gram Sprague-Dawley rats exposed to 800 rads of total body irradiation and antineutrophil serum, and in 12–16-week-old inbred C57BL/6 × DBA/2 F1 mice that received 900 rads of total body irradiation. Vascularization of the cornea after thermal injury was partially inhibited in 2–3 kg New Zealand albino rabbits exposed to 700–1400 rads of total body irradiation.

The mechanisms by which total body radiation could inhibit corneal neovascularization include a reduced capacity of an animal to respond to angiogenic stimuli, and/or a reduction in the production of the angiogenic stimulus or stimuli. To determine whether total body radiation affects the local production of an angiogenic factor in an injured cornea, we removed chemically cauterized corneas from normal and irradiated mice and compared their abilities to elicit angiogenesis in the chorioallantoic membranes of healthy chicken embryos.

Materials and Methods

Irradiation of Mice

Male C57BL/6 × DBA/2 F1 mice between the ages of 12 and 16 wk (from the Jackson Laboratory; Bar Harbor, ME) were housed for the duration of the experiments in autoclaved microisolator systems (Laboratory Products Inc.; Maywood, NJ) containing bedding, food, and water. Hydrochloric acid was added to the water (1 ml HCl/liter H₂O) to prevent growth of Pseudomonas. Mice were not fed the night before irradiation. Gamma irradiation was delivered to the entire bodies, including heads, of the mice in a single dose of 900 rads at 120.7 rads/min in a Gammacell 40 unit (Atomic Energy of Canada Ltd.; Ottawa, Canada) that employs a double encapsulated cesium137 source. The investigation utilizing animals as described in this manuscript conforms to the ARVO Resolution on the Use of Animals in Research.

Corneal Cauterization

While deeply anesthetized with ether, the corneas of mice were cauterized by pressing applicator sticks coated with 75% silver nitrate and 25% potassium nitrate (Graham-Field Surgical Company Inc.; New Hyde
made on the sides of the cylindrical mid portion and the humidifying base so that these two segments fitted snugly. Assembled culture chambers were sterilized with ethylene oxide prior to use.

By swiftly and gently separating the two halves of the shells, the egg contents were delivered to the assembled sterile culture chambers. Four milliliters of Eagle's minimal essential medium with Earle's salt and L-glutamine (Grand Island Biological Company; Grand Island, New York), 0.4 mg/ml of gentamycin, and 200 units/ml of mycostatin were then added. The Petri dish covers were placed over the culture chambers after the split rings were raised 0.4 cm over the inner cylinder to permit ventilation.

The culture units were placed on top of humidifying bases and incubated in a well-ventilated tissue incubator (Model 51810, Hotpack Corp.; Philadelphia, PA) maintained at 37.5°C with 1–2% CO2 and a humidity of 60%. The humidifying bases contained sterile 1% cupric sulfate (CuSO4 • 5H2O) to prevent fungal growth. Before each batch of embryos was cultured, the incubator was wiped clean with 70% ethanol, and then exposed to ethanol vapor.

**Preparation of Chorioallantoic Membranes**

After washing with cool tap water and rinsing in 70% ethanol, fertilized white Leghorn eggs were incubated in a commercial egg incubator for 3 days at 37.5°C and turned twice daily to minimize adhesion of egg contents to the shell membrane. The humidity was controlled at 70–80%. After 68–72 hr-incubation, the eggs were washed with Betadine, rinsed with 70% ethanol and transferred to a laminar flow hood. With the eggs on their long side on a slightly concave wax mold, 0.5 cm cracks were produced in each shell by a C-clamp. The cracks were then extended by alternately applying pressure from the C-clamp's screw and turning the eggs about their long axes. After the crack traversed the circumference, the eggs were then kept briefly under a heating lamp on their sides to allow the embryos to return to the top.

The embryos with intact yolk and albumin were then removed from the shell and cultured in chambers modified from the plastic wrap/tripod apparatus developed by Dunn et al.7,8 This device consisted of three segments: (1) a 15 × 100 mm Petri dish cover (Corning Glass Works; Corning, NY); (2) a plastic wrap/cylindrical Plexiglas mid portion; (3) and a cylindrical Plexiglas humidifying base. The midsection was a thick-walled plexiglass cylinder (3.5 cm tall, 7.8 cm inside diameter) with four equidistant semi-circular ventilation ports (2.5 cm diameter) at the base. A Handi-Wrap plastic wrap (Dow Chemical; Indianapolis, IN) was suspended within this cylinder by a thin wall split ring (12 mm tall, 7.8 cm inside diameter) clasped around the cylinder. The inside rim of the plastic wrap was lined with a 0.4 cm white paper tape to prevent capillary action at the wrinkles and to hold the plastic wrap in a concave shape (about 3 cm deep). The cylindrical humidifying base was made by glueing a Plexiglas disc (8.3 cm diameter) to a 4.0 cm tall thick-walled cylinder of the same outside diameter. Matching shoulders were

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* Specimens from CAMS that did not survive 6 days were excluded from this study.
by a modification of the method of Form and Auerbach for each observation day by dividing the total of the assigned grades by the maximum possible scores. Separate coefficients of angiogenesis were determined for the stereoscopic observations on the living specimens and the photographic evaluations.

Histologic Evaluation

Six days after placement on the CAMs, corneal grafts and adjacent CAMs were excised en bloc, fixed in 10% buffered formalin, and processed for light microscopy according to standard procedures. Three step sections of each specimen were cut and stained with hematoxylin and eosin. The histologic specimens were evaluated in a masked fashion. This examination focused on the presence or absence of intracorneal blood vessels and the reaction of the adjacent CAM to the grafted cornea.

Statistical Analysis of Data

The correlations between the coefficients of angiogenesis determined by stereoscopic observation and photography in different experimental groups were determined by the Goodman-Kruskal index, \( \gamma = (C - D)/(C + D) \), where C is the number of concordant pairs of observations and D is the number of discordant pairs. In this context, a pair of observations \((X, Y)\) is concordant if \(X > X'\) and \(Y > Y'\) or \(X < X'\) and \(Y < Y'\), discordant if \(X > X'\) and \(Y < Y'\) or \(X < X'\) and \(Y > Y'\) and tied otherwise. This index is distributed as a random normal variable even if the observations are not, hence a Z score and probability value can be determined. Calculation of these values was done by a matched-pair partial correlation procedure (proc matpar) of SAS (SAS Institute Inc.; Cary, NC) using an IBM 3081 computer (IBM Corp.; White Plains, NY).

The correlations between the frequency of vascular invasion of corneal grafts in the different experimental groups were determined by \( \chi^2 \) analysis.

Assessment of the Effects of Irradiation

Complete blood counts were obtained on a separate group of irradiated male C57BL/6 \( \times \) DBA/2 Fl mice at daily intervals for 8 days after total body irradiation with 900 rads under identical conditions to those mice whose corneas were cauterized. In anesthetized animals a 25-gauge needle on a 1.0 ml syringe coated with disodium ethylenediaminetetracetic acid (EDTA), was used to withdraw 0.3–0.5 ml of blood from the right atrium and this was immediately placed in a glass tube coated with disodium EDTA. For each measurement, blood samples were combined from 2–3 mice. Total leukocyte and platelet counts were measured by an Ortho ELT-8/ds hematology analyzer (Ortho Diagnostics Systems, Inc.; Westwood, MA). Smears were prepared from the same samples and the differential counts were determined. Blood counts were similarly obtained from normal non-irradiated mice. To evaluate the cellular components present within corneas at the time of grafting onto the chorioallantoic membranes, eyes were obtained from a separate group of mice for light microscopic evaluation. Two days after bilateral corneal cauterization of irradiated and non-irradiated mice, mice were killed with 0.3 ml of intraperitoneal 1% sodium pentobarbital. Their eyes were immediately enucleated, fixed in 10% buffered formalin, and processed for light microscopy according to standard procedures. The corneas were evaluated in a masked fashion for the amount of leukocytic infiltration and other pathologic alterations.

Results

Stereoscopic Observations

A masked analysis of the coefficients of angiogenesis derived from in vivo stereomicroscopic observations revealed that uninjured corneas, from non-irradiated animals, induced significantly less vascular reaction than the cauterized-irradiated or cauterized-nonirradiated groups after the grafts had been on the CAM for 4 or more days (Figs. 1 and 2) \( (P < 0.001, \text{ day } 4; P = 0.003-0.0001, \text{ day } 6) \). The coefficients of angiogenesis of cauterized corneas grafted onto the CAM were significantly altered by irradiation \( (P = 0.4, \text{ day } 4; P = 0.3, \text{ day } 6) \).

Evaluation of Photographs

Similar to the results by stereomicroscopy, analysis of photographs taken of corneas that had been on the
CAM for 6 days disclosed significant differences between the uninjured non-irradiated, and cauterized irradiated mice ($P = 0.0003$), as well as between the uninjured non-irradiated, and cauterized non-irradiated experimental groups that were cauterized (Figs. 1 and 2, $P = 0.1-0.7$). The coefficients of angiogenesis of injured corneas from irradiated mice did not differ significantly from those of injured corneas from otherwise normal mice ($P = 0.7$, day 4; $P = 0.8$, day 6). Coefficients of angiogenesis derived from stereomicroscopic and photographic evaluations correlated well ($R^2 = 0.70$) (Fig. 3).

**Histologic Evaluation of Corneal Grafts on Chorioallantoic Membranes**

In all experimental groups, some corneal grafts were incorporated into the mesenchyme of the chorioallantoic membranes, while others remained external, or superficial, to the epithelium of the CAM (Fig. 4). The stroma of incorporated corneas was in direct contact with the mesenchymal tissue of the CAMs, whereas the stroma of non-incorporated corneas was separated from CAM mesenchymal tissue by hyperplastic sheets of epithelial cells. The percentage of corneas incorporated into the mesenchyme of the CAMs varied between the different experimental groups (Fig. 5). Normal, uninjured mouse corneas were incorporated into CAM mesenchymal tissue in 95% of cases. Injured corneas from otherwise normal mice were incorporated into CAM mesenchymal tissue in 66% of cases. Injured corneas from irradiated mice were incorporated into CAM mesenchymal tissue in 93% of cases. The frequency of incorporation into CAM mesenchymal tissue of injured corneas from normal mice was lower than that from irradiated mice ($P = 0.02$). All corneas that failed to become incorporated into the CAM mesenchymal tissue had a flattened, desiccated appearance and were never invaded by blood vessels, regardless of their experimental category. In contrast, some corneas incorporated into the mesenchyme of the CAMs stimulated on ingrowth of blood vessels, an ability that showed marked variation between the different experimental groups (Figs. 6 and 7).

Uninjured corneas from normal mice that were incorporated into CAM mesenchymal tissue were invaded by blood vessels in 5% of cases. The stroma of the uninjured corneas typically had normal-appearing
keratocytes and collagen fibers, and did not contain leukocytes after being on the CAM for 6 days (Fig. 8). The surrounding CAM displayed minimal leukocytic infiltration and minimal epithelial hyperplasia.

Injured corneas from otherwise normal mice incorporated within CAM mesenchyme were invaded by blood vessels in 75% of cases, a frequency of vascularization that was significantly greater than in uninjured corneas ($P < 0.005$). Grafts of injured corneas from otherwise normal mice typically contained fibroblasts and leukocytes (Fig. 9).

Cauterized corneas from irradiated mice incorporated in CAM mesenchymal tissue were invaded by blood vessels in 34.6% of cases. This frequency of vascularization was greater than in uninjured corneas ($P = 0.02$) and less than in cauterized corneas from otherwise normal mice ($P = 0.002$). Vascularized corneal grafts from irradiated mice also contained leukocytes and fibroblasts whereas non-vascularized grafts were acellular (Fig. 10). These corneas induced the same degree of leukocytic infiltration and epithelial hyperplasia in the surrounding CAMs as injured corneas from otherwise normal mice.
Fig. 7. Percentage of vascularized corneas in different experimental groups. Only specimens that became incorporated into the CAMs are included. (In Figures 1, 2, 4-7, the experimental groups were non-cauterized normal corneas (1), corneas from normal mice 2 days post cauterization (2), and corneas from irradiated mice 2 days post cauterization (3)).

The coefficients of angiogenesis, as determined from stereomicroscopic and photographic evaluations, were higher in corneas that became vascularized than in those that did not manifest intracorneal blood vessels histologically (Fig. 11).

Assessment of the Effects of Total Body Irradiation

Mice receiving total body irradiation were markedly leukopenic and thrombocytopenic from the time of corneal injury (6 days after irradiation) to the time of killing and removal of the cornea for grafting onto the CAMs (8 days after irradiation). Light microscopic examination of cauterized eyes from otherwise normal mice revealed an absence of all corneal cellular layers in the region of the cautery site, while injured corneas from otherwise normal mice contained numerous leukocytes. Neutrophils were the predominant cell type, but mononuclear cells were also present. Cauterized corneas from lethally irradiated mice displayed an absence of keratocytes and corneal endothelial cells in the region of the burn. The corneal epithelium was intact in approximately half the mice and absent in the other half. Injured eyes from lethally irradiated mice exhibited minimal leukocytic infiltration in the cornea or the anterior chamber.

Discussion

Stereoscopic evaluation of mouse corneas grafted onto chick chorioallantoic membranes revealed that...
uninjured corneas produced significantly less vascular reaction than injured corneas from either normal mice or irradiated mice. Moreover, histologic examination established that blood vessels seldom invaded uninjured mouse corneas after being on the CAM for 6 days.

While the vascular response of the CAM to cauterized corneas from mice that had received total body irradiation did not differ from those obtained from non-irradiated mice according to stereomicroscopic and photographic evaluations, histologic examination of the grafted corneas differed in these two experimental groups as pointed out under Results. This observation reinforces the suggestion by Vu et al.\textsuperscript{13} that histological examination of grafted corneas should be done as a complement to stereomicroscopy. Injured corneas from irradiated mice were incorporated into CAM mesenchymal tissue more frequently than injured corneas from otherwise normal mice ($P = 0.02$), but total body irradiation markedly decreased the ability of these incorporated cauterized corneas to induce vascular invasion of the corneal tissues on the CAM ($P = 0.002$). Since the blood vessels in the CAM should have been fully capable of responding to angiogenic stimuli within the cauterized corneas, it is clear that total body irradiation suppressed the stimulus for angiogenesis within the injured corneas either by decreasing the amount of angiogenic substances within the corneas or by causing an accumulation of inhibitors of angiogenesis within the donor tissue. The experiments of course do not exclude the possibility not tested in these experiments that in vivo irradiation also has an effect in the pericorneal vessels compromising their ability to proliferate and vascularize the cornea.
While the present experiments do not address the nature of specific stimuli in injured corneas altered by total body irradiation, several possibilities warrant consideration. The association between events in the inflammatory response, such as the leukocytic infiltration, and angiogenesis is well established and a causal relationship seems possible. Profound leukopenia was present in the irradiated mice during the 2 days between the corneal cauterization and the time of grafting onto the CAMs. Moreover, histologic examination of representative corneas from similarly treated mice at the time that others were grafted to the CAM revealed only a minimal leukocytic infiltration, and this was considerably less than that in cauterized corneas of comparable non-irradiated mice at this period of time. Hence, the degree of leukocytic infiltration in the injured incorporated corneas correlated with the frequency of vascular invasion found on histologic examination.

In addition to an effect mediated by leukopenia, total body irradiation theoretically could have influenced vascularization of corneal grafts on the CAM by other mechanisms as well. Because a number of studies have linked platelets with vascular endothelial cell proliferation and new vessel growth, the profound thrombocypenia in the irradiated mice during the period between corneal cauterization and grafting onto the CAMs is of potential significance in the impaired

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**Fig. 10.** Cornea injured during life from lethally irradiated mouse. After 6 days on the CAM, the cornea is avascular and the central stroma is acellular (hematoxylin and eosin, upper, X40; lower, X100).

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**Fig. 11.** Coefficients of angiogenesis of corneas after different periods of time on the CAM based on stereomicroscopy and photographic evaluations. The specimens with histologically confirmed vascularization tended to have higher coefficients of angiogenesis than those that did not vascularize.
angiogenesis of irradiated animals. Although platelets accumulate in the pericorneal vessels after corneal cautery, they would not be expected within the avascular corneal tissue grafted onto the chorioallantoic membranes in these experiments, as they do not leave the intravascular space under normal circumstances. However, platelet-derived growth factor, which can conceivably diffuse into the central cornea, may have been diminished in the corneal tissue of irradiated mice grafted onto the CAMs. In addition to platelet-derived growth factor, a number of other humoral blood components have been implicated in angiogenesis including epidermal growth factor, fibroblast growth factor, prostaglandins, fibrin, and plasminogen activator. Although the effects of irradiation on these substances were not assessed in this study, irradiation could conceivably have affected their synthesis or function.

In addition to altering components of the blood, total body irradiation also could affect the ability of endogenous corneal cells to undergo mitosis. Corneal cauterization, as carried out in this study, causes necrosis of central corneal cells and increased mitotic activity of the surviving epithelium, fibroblasts and endothelium of the cornea. Since irradiation is known to inhibit mitosis, it is likely that the corneal cells of the irradiated mice were unable to proliferate following cautery, and this inability may have been associated with other functional changes, including an ability of corneal cells, such as those of the epithelium, to induce neovascularization.

Lastly, tissues damaged during total body irradiation could conceivably release angiogenic substances which might accumulate within the cornea to inhibit the growth of blood vessels into the injured corneal tissue.

While we found that uninjured mouse corneas rarely became vascularized on the CAM, other investigators have shown that normal corneal tissue from other species (canine puppy and rat) can eventually become invaded by blood vessels when grafted to the CAM. However, it is difficult to compare such studies to ours, since the grafts varied not only in size but also in the duration of time that they remained on the CAM. The normal puppy corneas that Eisenstein et al. found to vascularize were left on the chorioallantoic membranes for 7 days, one day more than in our study. In addition, they did not specify the percentage of grafts that were invaded by blood vessels. Vu et al. found that only 20% of normal rat corneas grafted onto chorioallantoic membranes for up to 9 days were incorporated into CAM mesenchymal tissue in contrast to this study's finding that normal mouse corneas were incorporated in 95% of cases. In both studies, rat and mouse corneas that remained external to the CAM epithelium did not vascularize. Blood vessels invaded 2 of the 4 normal rat corneas that were incorporated into CAM mesenchymal tissue. As with cauterized rat corneas that were grafted onto the CAM, injured corneas from otherwise normal mice frequently induced a vascular response as assessed by both stereomicroscopy and histologic examination. All of the above underscore the importance in CAM studies of considering multiple variables, including the subjective impression of the CAM response, the presence or absence of incorporation of the graft into the CAM, and the histologically documented presence or absence of new vessels within the grafted cornea.

Key words: angiogenesis, cornea, chorioallantoic membrane, irradiation

References

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