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M. W. Scroggs

A. D. Proia

C. F. Smith

E. C. Halperin

G. K. Klintworth

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The Effect of Total-Body Irradiation On Corneal Neovascularization in the Fischer 344 Rat After Chemical Cauterization

Mark W. Scroggs,* Alan D. Proia,*† Clayton F. Smith,* Edward C. Halperin,‡ and Gordon K. Klintworth*†

Previous investigations of corneal neovascularization after irradiation yielded discordant results. Most studies indicated that new blood vessel formation in the cornea is inhibited by irradiation. However, others reported that angiogenesis after corneal cauterization is similar in both irradiated and nonirradiated animals. To assess the effect of total-body irradiation on neovascularization further, the amount of angiogenesis was determined in irradiated rats after chemically induced corneal injury. Corneal tissue was evaluated quantitatively with computerized image analysis 2, 3, or 4 days postcautery in rats perfused with India ink and gelatin immediately after death. The rats were exposed to a single dose (9 Gy) of total-body irradiation 6 days before corneal cauterization. In both the nonirradiated and irradiated rats, neovascularization increased with the duration of the postcautery interval. The amount of corneal neovascularization was not significantly different in the irradiated and nonirradiated rats at any of the postcautery intervals studied. This investigation suggests that endothelial cell migration plays a more important role than cell replication in the pathogenesis of corneal angiogenesis in the Fischer 344 rat. Moreover, the suppression of corneal angiogenesis by irradiation may be dependent on the experimental conditions and species examined. Invest Ophthalmol Vis Sci 32:2105–2111, 1991

Neovascularization of the cornea is a significant cause of decreased visual acuity and blindness. It occurs in many inflammatory, infectious, and traumatic settings. Corneal angiogenesis involves three phases: a prevascular latent period, a stage of active neovascularization, and a stage of vascular maturation and regression.[1] Despite knowledge of the morphologic progression of new vessel formation in the cornea, the mechanism of this process is not understood completely. Factors which may be responsible for angiogenesis include cellular and humoral components of the inflammatory response, such as leukocytes,[2–6] platelets,[7–11] cytokines,[12] and eicosanoids,[13–16] and plasma constituents that enter the cornea during the increased permeability of the pericorneal blood vessels.[6] Another site of putative angiogenic factors is the corneal epithelium.[17–19] To clarify the role of some of these elements in corneal neovascularization, previous investigators studied the effect of irradiation on the process.[4,17,18,20–24]

Several prior reports using different experimental conditions, different animals, and different methods to measure new blood vessel growth in the cornea indicated that the amount of corneal neovascularization decreases after irradiation.[4,17,18,20–24] This presumably results from altered angiogenic stimuli in the injured cornea,[23] from the prevention of cell division in the pericorneal microvasculature, and from a modification in the ability of the host to respond to angiogenic stimuli in other ways. One study of total-body irradiation (TBI) with 8 Gy, however, did not diminish corneal angiogenesis in Sprague-Dawley rats 4 days postcorneal cauterization with silver nitrate.[20] In view of this discrepancy, we assessed the effects of TBI on corneal neovascularization in an inbred strain of rats with a recently developed technique for quantifying corneal angiogenesis by computerized image analysis.[25]

Materials and Methods

Animals

Male rats of the inbred Fischer 344 stain (Charles River, Research Triangle Park, NC) were used when they weighed between 200–225 g. The animals were

From the Departments of *Pathology, †Ophthalmology, and ‡Radiology, Duke University Medical Center, Durham, North Carolina.

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Reprint requests: Gordon K. Klintworth, MD, PhD, Box 3802, Duke University Medical Center, Durham, NC 27710.

housed for the duration of the experiments in autoclaved microisolator systems (Laboratory Products, Maywood, NJ) containing bedding, food, and water. Sterilized rodent laboratory chow (#5001; Ralston Purina, St. Louis, MO) and tap water were given ad libitum to all animals throughout the experiment. Experimental procedures conformed to the ARVO Resolution on the Use of Animals in Research and were approved by the Duke University animal care committee. Animal care was supervised by a veterinarian.

Irradiation

For animals receiving TBI, photon irradiation from a 4-MeV linear accelerator (Clinac 4; Varian, Palo Alto, CA) was delivered at an 80-cm source-to-axis distance. The animals were irradiated while awake and breathing air in a custom-designed box which provided adequate build-up and back-scatter material. The radiation field was 25 × 25 cm or 30 × 30 cm at the treatment distance. A dose of 9 Gy in a single fraction at 2 Gy/min was given to the midplane of the animals.

Corneal Cauterization

The eyes were cauterized by applying the tip of a new silver–potassium nitrate applicator (75% silver nitrate:25% potassium nitrate; Graham-Field, Hauppauge, NY) to the surface of the cornea at a point approximately 2.5 mm from the corneoscleral limbus. An initial group of animals underwent corneal cauterization for 5 sec. A subsequent group was cauterized for 1 sec to determine if the duration of corneal injury affected the amount of vascularization. Rats were anesthetized before cauterization with intraperitoneal (IP) injections of ketamine (60 mg/kg) and xylazine (10 mg/kg). In irradiated rats, chemical cauterization was done 6 days after TBI.

India Ink Perfusion

The animals were killed with an IP injection of pentobarbital 2, 3, or 4 days after corneal cautery. A 22-gauge butterfly syringe was then placed into the left ventricle of each rat, and the head, neck, and upper extremities were perfused with 50 ml of lactated Ringer's solution delivered by an automated infusion pump (Harvard Apparatus, Dover, MA). This was followed immediately by 20 ml of a mixture containing 10% waterproof drawing ink (Faber-Castell, Louisburg, TN) and 11% gelatin in lactated Ringer's solution at 37°C. After perfusion, these eyes were cooled immediately with compressed dichlorodifluoromethane to solidify the gelatin. Then the eyes were enucleated and fixed in 3.7% neutral buffered formaldehyde for 24–48 hr. The cornea and scleral rim were dissected from each globe, and three radial incisions were made through the cornea at equidistances to allow flattening of the tissue. Each excised cornea was dehydrated through a series of graded alcohols and xylene and mounted on a glass slide.

Quantitation of Corneal Neovascularization

To minimize observer bias, each corneal flat preparation was assigned a random number so that corneal neovascularization could be quantified in a masked fashion using computerized image analysis according to the method of Proia et al.[25] Briefly, corneal flat preparations were examined with a Lemont OASYS video input image analyzer (State College, PA). The images were magnified and then digitized for gray-scale analysis. In each specimen the areas of the blood vessels, cauterized site, and cornea were determined independently. In all corneas, the distance from the corneoscleral limbus to both the center and the edge of the cauterized site was measured as was the distance of the longest vessel from the corneoscleral limbus to the cauterized site. In one group (irradiated rats, 1 sec, 4 days) extravasated blood in the corneal stroma interfered with the quantitation of blood vessel area. All animals in this group and in the corresponding control group were analyzed by placing a red filter (K610) between the light source and the flat mounted cornea at the time the image was digitized. This process removed the effect of extravasated blood from the area of analysis. The data was stored in a database using KnowledgeMan, an integrated software package (Micro DataBase Systems, Lafayette, IN) and then analyzed with an IBM 3081 mainframe computer (Kingston, NY).

Statistical Analysis

The software used for statistical analysis was SAS (Cary, NC). The vessel areas were analyzed by analysis of variance, using a model which included postcautery interval and treatment group (control or TBI) and individual animal identification numbers (animal ID). The animal ID term was included because left and right eyes from the same animal are more alike than eyes selected at random. Other variables (corneal area, burn area, distance of center of burn from limbus, and distance of edge of burn from limbus) were analyzed using a student t-test.

Blood Analysis

To assess the influence of TBI on the bone marrow, the total leukocyte, differential cell count, platelet, hemoglobin, and hematocrit values were determined 3, 4, 6, 8, 9, and 10 days after 9 Gy of TBI using an

Ortho ELT-815 hematology analyzer (Braintree, MA) in representative TBI rats that were not subjected to India ink perfusion. These times were selected for the determination of these values since they represented periods before corneal cauterization (days 3 and 4 postirradiation), the day of corneal cauterization (day 6 postirradiation), and the days of India ink perfusion (days 8, 9, and 10 postirradiation). Samples from nonirradiated rats served as controls. Blood from five rats was analyzed at each time after killing the animals with a lethal IP injection of pentobarbital. Blood was collected in a 3-ml syringe with a 22-gauge needle from the right ventricle and immediately transferred into a plastic container (Microvette CB 1000; Sarstedt, West Germany) coated with dipotassium disodium ethylenediaminetetraacetic acid.

Results

Corneal Neovascularization

In both the nonirradiated and irradiated rats, the amount of corneal neovascularization increased with time over the duration of the postcautery intervals studied. The difference between the experimental and control groups did not differ significantly at any time (Fig. 1). The length of the longest vessel extending from the corneoscleral limbus to the cauterized site, the area of the cauterized site, and the distance from the corneoscleral limbus to the burn edge and to the center of the cauterized site were also similar in the nonirradiated and irradiated rats.

In control animals cauterized for 1 or 5 sec, the amount of corneal angiogenesis was similar at 2 days postcautery, but the degree of new vessel formation was significantly greater in rats cauterized for 5 sec compared with rats cauterized for 1 sec on days 3 ($P = 0.018$) and 4 postcautery ($P = 0.0001$). Increasing the duration of corneal cauterization in irradiated rats from 1 to 5 sec increased the amount of corneal angiogenesis at 4 days postcautery, but this effect was not detected at the 2- and 3-day intervals (Fig. 1).

The density and length of the vessels arising from the limbal arcade was similar in the nonirradiated and irradiated groups. By contrast with the nonirradiated animals, erythrocytes frequently became extravasated by day 3 postcautery from the corneal blood vessels in the TBI rats, resulting in a blood-stained cornea. In the irradiated, but not the control rats, the tips of small vessels closest to the cauterized site often leaked minute amounts of India ink during perfusion, causing staining of mainly the avascular region of the cornea. This leakage did not affect significantly the quantitation of neovascularization.

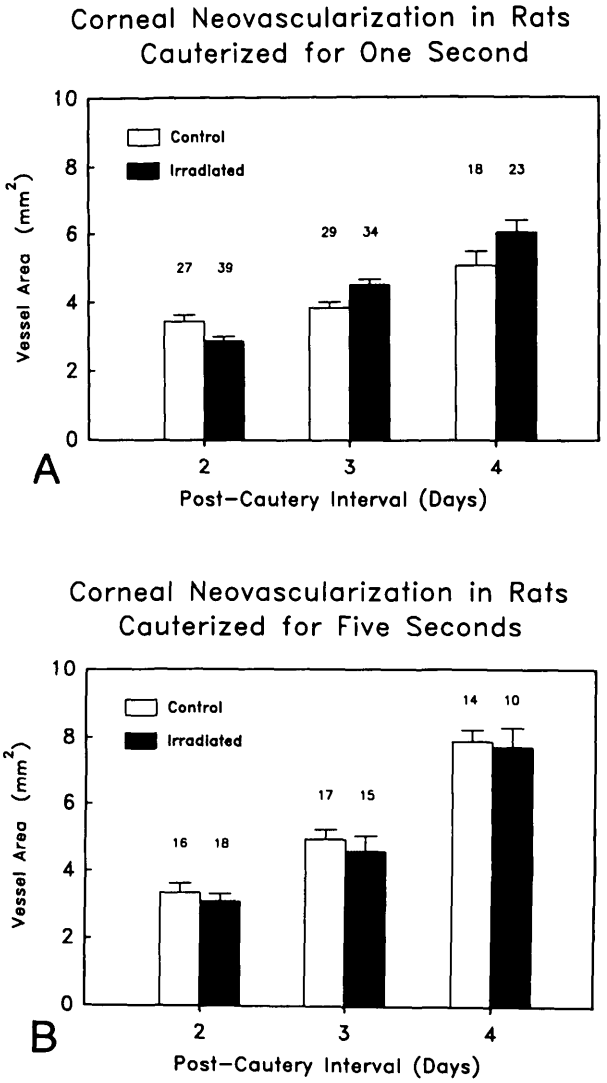

Fig. 1. Histograms comparing corneal neovascularization in TBI and control rats cauterized for 1 sec (**A**) or 5 sec (**B**). Vertical bars represent standard error of the mean, and the numbers above the blocks represent sample size. Neovascularization increased in both groups as the post-cautery interval increased. Corneal neovascularization in TBI rats was not suppressed relative to the control rats.

Effect of TBI on Blood Components

Total leukocytes and platelet counts were lower in TBI rats than in nonirradiated control rats when measured 3, 4, 6, 8, 9, and 10 days after radiation. Hematocrit, hemoglobin, and erythrocyte volume indices were similar in TBI and nonirradiated animals (Table 1).

Discussion

In an early evaluation of the role of leukocytes in corneal angiogenesis, Fromer and Klintworth[4] observed that 15 Gy of TBI produced a profound leukopenia and an absence of a leukocytic and vascular

Table 1. Blood values in irradiated rats

	Units		Day following irradiation						
			0	3	4	6	8	9	10
WBC	$\times 10^3/mm^3$	Mean	7.53	0.70	0.50	0.30	0.30	0.33	0.30
		STD	0.34	0.13	0.12	0.10	0.07	0.10	0.08
Platelets	$\times 10^3/mm^3$	Mean	759.33	493.67	372.00	25.33	10.33	15.33	13.75
		STD	39.78	17.49	10.42	2.23	1.20	1.43	1.94
Erythrocytes	$\times 10^6/mm^3$	Mean	8.71	10.38	10.33	9.81	8.66	8.96	6.86
		STD	0.26	0.08	0.07	0.19	0.10	0.20	0.21
Hemoglobin	mg/dl	Mean	15.07	15.30	15.47	16.30	14.70	15.40	11.55
		STD	0.27	0.13	0.27	0.15	0.15	0.37	0.40
Hematocrit	%	Mean	47.70	54.33	56.03	53.57	52.60	51.83	47.60
		STD	1.50	0.45	0.76	0.46	0.80	1.30	1.49
MCV	u^3	Mean	69.33	69.00	69.00	68.50	68.17	68.17	69.25
		STD	0.42	0.00	0.00	0.22	0.17	0.40	0.16
MCH	uugm	Mean	17.50	16.98	16.58	17.13	16.98	17.37	16.84
		STD	0.12	0.08	0.18	0.13	0.06	0.10	0.11
MCHC	%	Mean	25.28	24.55	24.07	25.03	24.93	25.47	24.25
		STD	0.19	0.10	0.26	0.19	0.10	0.28	0.14

Abbreviations: WBC = white blood cell count; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobulin concentration.

invasion of the cornea after corneal cauterization in weanling Fischer albino rats (two rats died 2 days after corneal injury, and two survived 4 days after corneal cauterization). Rats of a similar strain and size that received 11 Gy (four rats) or 13 Gy (four rats) of TBI, a dose that did not eliminate circulating leukocytes totally, had both a vascular and leukocytic invasion of the cornea in a manner comparable to controls. When corneas (four rats) were cauterized immediately after 15 Gy of TBI before the onset of leukopenia, angiogenesis ensued, suggesting that the extent of the new vessel formation was related to the timing of TBI with respect to corneal cauterization. Moreover, when 15 Gy of irradiation was administered only to the heads of four rats, corneal cauterization still induced neovascularization. These authors did not attempt to quantify the amount of corneal neovascularization in this study and only determined whether angiogenesis was present or absent by examining the corneas with the aid of a dissecting microscope before the animals were killed. These observations supported the notion that leukocytes play an important pathogenetic role in corneal neovascularization, and this theory has been bolstered by the vast body of information that has accumulated since then.[4,6,17,20,21,26–32]

In an investigation of corneal neovascularization and the associated leukocytic infiltration, others[20] found corneas of four male Sprague-Dawley rats (weight, 200–400 g) to contain scant leukocytes after silver nitrate corneal cauterization; however vascularization after 8 Gy TBI occurred similarly to control rats. However, when antineutrophil serum was administered to the irradiated animals, neovascularization was diminished relative to control rats, and corneal leukocytes were absent. In this study, new blood vessels were quantified as the average length of new blood vessels in the cornea after perfusion with colloidal carbon.

Subsequently impairment of corneal angiogenesis was reported in New Zealand albino rabbits that survived 3 days (two animals), 4 days (three animals), and 5 days (four animals) after corneal cauterization after 7 Gy TBI (with the eye and part of the head shielded).[17] In that study corneal angiogenesis was quantified in mounted preparations of the cornea by measuring the distance between the most centrally located vessel tip extending from the limbus and the cauterized site. In a later study homogenates of corneal epithelial cells induced less neovascularization, as determined from photographs taken before the animals were killed, in corneas from four rabbits after an intracorneal inoculation after 15 Gy TBI (but with the eyes shielded) relative to nonirradiated rabbits.[18]

Also, Ryu and Albert[20] found 9.5 Gy of TBI to suppress corneal angiogenesis in 12 New Zealand albino rabbits relative to non-irradiated controls if administered approximately 12 hours prior to the intracorneal injection of viable melanoma or retinoblastoma tumor cells. Ryu and Albert[21] quantified the corneal angiogenic response with a slit-lamp biomicroscopic camera and routine histologic sections.

Others demonstrated that 20 and 80 Gy of irradiation administered to the eye alone suppressed corneal angiogenesis after corneal cauterization in the Sprague-Dawley rat.[22] They found that these massive doses of irradiation totally suppressed endothelial cell division in irradiated corneas, as measured by ^{3}H-thymidine labeling.

To investigate the association between TBI and the production of angiogenic substances in injured corneas, Glatt et al[23] cauterized mouse corneas with silver–potassium nitrate and then grafted them onto chick chorioallantoic membranes. In this experimental model, 9 Gy of TBI was administered to mice 6 days before cauterization. Histologic examination of the cauterized corneas 6 days after being grafted to the chorioallantoic membrane indicated that corneas from irradiated mice incited less inflammation and less angiogenesis than those from nonirradiated mice. Therefore TBI either decreases the production of angiogenic factors in an injured cornea or allows for the accumulation of factors which inhibit angiogenesis in the injured corneas.

To ascertain the role of leukocyte subsets in corneal angiogenesis, the effects of fractionated doses of total lymphoid irradiation (TLI) were evaluated in the Sprague-Dawley rat.[24] This study, which quantified corneal angiogenesis by the same technique we used, disclosed significantly less neovascularization in corneas of rats after chemical cautery, if the injury was preceded by TLI. This procedure decreased circulating lymphocytes to a greater degree than other bone marrow-derived elements.

Surprisingly, in contrast with these studies, we found that 9 Gy TBI did not inhibit corneal angiogenesis in inbred Fischer F344 rats when administered before corneal cauterization with silver–potassium nitrate. Our study, and that of others,[20] raises the question of whether irradiation suppresses corneal angiogenesis. A critical evaluation of the published reports of the putative suppressive effect of irradiation on corneal angiogenesis discloses notable weaknesses in the methods used for quantifying corneal angiogenesis and evaluating differences between experimental and control groups. With the exception of the study by Suvarnamani et al,[24] all of these studies used small sample sizes and less precise methods for quantifying of corneal angiogenesis compared with our computerized image analysis; they did not analyze data with detailed biostatistical methods. Nevertheless, the experiments of others[23,24] strongly suggest that irradiation suppresses corneal angiogenesis and that another explanation is necessary. Several potential explanations may account for our failure to detect an inhibitory effort of irradiation on corneal angiogenesis. The variety of radiotherapy techniques used, including differences in field homogeneity, build-up, back-scatter, photon energy, and other technical factors may account for the variable effect on corneal angiogenesis. Moreover, in contrast with our study, previous investigations used genetically heterogeneous populations of animals, and the biologic spectrum of responses in their experiments would be expected to be greater than in our study. This may have allowed differences to be detected. Alternatively, the induction of corneal angiogenesis in the inbred Fischer F344 rats may be resistant to an inhibitory effect of irradiation. Because some strains of the rat appear to be more susceptible to corneal neovascularization than others,[33] it is conceivable that irradiation may have a variable effect on different species or strains of animals.

New vessel growth is a result of endothelial cell migration and replication.[34,35] We found that corneal neovascularization occurred in Fischer F344 rats despite 9 Gy of TBI which should impair endothelial cell division. The radiosensitivity of endothelial cells was measured by several investigators.[36] Early experimental systems measured radiosensitivity with animal models that relied on quantitative assessment of the formation of capillary loops, capillary sprouts, or use of fluorescent dye to measure revascularization of a subcutaneous air pouch.[37–41] A common measurement of radiosensitivity is Do, the dose required (on the exponential part of the radiation cell survival curve) to reduce the probability of survival to e^{-1} (ie, to 0.37). The Do for endothelial cells using the early experimental systems ranged from 170–260 cGy. DeGowin et al[42] found that a single fraction of 870 cGy completely inhibited the growth of cultured human umbilical vein endothelial cells for 1 week. Subsequent studies by the same authors showed that 100 cGy suppressed endothelial cell replication by 37%.[43] As early as a few hours after high-dose irradiation, endothelial cells showed changes in ultrastructure. These included dilation of the endoplasmic reticulum and formation of cytoplasmic lipid droplets and osmiophilic precipitations.[44]

In more recent years, techniques were developed to harvest endothelial cells from the vessels of humans or animals and to culture them as a homogeneous population in vitro for prolonged periods for the purpose of clonogenic radiosensitivity assays. Recently reported clonogenic assays of rabbit, bovine, and human endothelial cells demonstrate Do values in the range of 101–200 cGy.[45–49] It is interesting to note that the Do obtained from systems which involved the counting of capillary sprouts is higher than from systems which use endothelial cells grown in culture. The capillary sprout system may be assessing, in part, a subclonogenic endothelial cell population.[48]

There appears to be considerable species and tissue variation in the Do of endothelial cells. Our single dose of 9 Gy TBI was likely, based on the previously cited experimental data, to have significantly impaired endothelial cell division. As a result of variable turnover rates in the stem cell population, however, radiation damage may not be expressed for some

time. In a detailed study of the vasculature of the mouse mesentery, a significant reduction in the number of endothelial cells was not seen until 6 weeks after irradiation of 20–30 Gy.[48] Even though the dividing capacity of the endothelial cells had been abolished by the 9 Gy of TBI we administered, the death of the cells would not be apparent for several weeks because of a slow cell turnover time. The integrity of the tissue was preserved for the period of the experiment, without cell division.

Our data suggest that corneal angiogenesis in the Fischer F334 rat occurs predominately by endothelial cell migration rather than cell division—the latter presumably was suppressed by the 9 Gy of TBI. This finding is consistent with the observation that endothelial cell migration was not inhibited by 15 Gy even though ^{3}H-thymidine labeling of the cells was abolished.[34] Further studies will be needed to clarify the mechanism whereby different forms of irradiation, at different doses, and in different species, have a variable effect on the pathophysiology of corneal neovascularization.

Key words: angiogenesis, Fischer 344 rat, cornea, chemical cautery, total body irradiation

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