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Authors: Dynlacht, Joseph R., Tyree, Craig, Valluri, Shailaja, DesRosiers, Colleen, Caperell-Grant, Andrea, et al.

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Effect of Estrogen on Radiation-Induced Cataractogenesis

Joseph R. Dynlacht,[a,1] Craig Tyree,[a] Shailaja Valluri,[b] Colleen DesRosiers,[a] Andrea Caperell-Grant,[c] Marc S. Mendonca,[a] Robert Timmerman[a,2] and Robert M. Bigsby[c]

[a] *Department of Radiation Oncology,* [b] *Department of Ophthalmology, and* [c] *Department of Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, Indiana 46202*

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Cataractogenesis is a widely reported late effect that is observed in patients receiving total-body irradiation (TBI) prior to bone marrow transplantation or radiotherapy for ocular or head and neck cancers. Recent studies indicate that estrogens may protect against age-related and drug-induced cataracts. Moreover, other reports suggest that estrogen possesses antioxidant properties. Since the effect of estrogen on radiation cataractogenesis is unknown, we wished to determine whether estrogen modulates radiation-induced opacification of the lens. Intact or ovariectomized Sprague-Dawley rats were treated with either 17-β-estradiol or an empty silastic capsule. The right orbit was then irradiated with either 10 or 15 Gy of ^{60}Co γ rays using a Leksell Gamma Knife, and lenses were examined at various times postirradiation with a slit lamp or evaluated for light transmission. We found that for ovariectomized rats irradiated with 15 Gy, the lens opacity and the incidence of cataract formation in the estradiol-treated group were significantly increased compared to the control group at the end of the 25-week period of observation. Cataract incidence was also high in irradiated eyes of ovary-intact animals at 25 weeks postirradiation but was greatly reduced in the ovariectomized control group, with less than half of irradiated eyes showing evidence of cataractogenesis. Thus, after irradiation with 15 Gy of γ rays, estrogen increased the incidence of cataract formation. We also observed that although the incidence of cataract formation in rats irradiated with 10 Gy and receiving continuous estrogen treatment was not altered compared to rats in the control group that did not receive estrogen, the latent period for posterior subcapsular cataract formation decreased and the severity of the anterior cataract increased. Taken together, our data suggest that estrogen accelerates progression of radiation-induced opacification.

INTRODUCTION

Postmenopausal women are at a higher risk for development of age-related opacities than age-matched men, and this gender-based difference may be attributable in part to hormonal differences. Epidemiological data suggest that estrogens may protect against some forms of cataract. Retrospective studies have shown that postmenopausal estrogen replacement therapy reduces the incidence of age-related nuclear and anterior capsular cataracts, or cortical opacities in women (*1–4*). However, the effect of estrogen on the incidence of age-related posterior subcapsular cataracts is controversial, with either no change or an increased risk in two studies involving women receiving hormone replacement therapy (*3, 5*) and an apparent protective effect in another study (*6*). The protective effect was also evident in women who had been subjected to elevated estrogen exposure by virtue of past pregnancy or in women who had never been pregnant but who had received past hormone replacement therapy (*4*). A fifth study showed no association between hormone replacement therapy and any type of cataract, but a reduced risk of cataract was found in ovariectomized women (*7*).

Data from animal studies suggest that a lack of estrogen is associated with cataractogenesis and that slightly elevated or physiological levels of estrogen may even be protective against cataract induction. Prolonged exposure to the antiestrogen tamoxifen increases the incidence of cataracts in rats (*8*). Bigsby *et al.* (*9*) found that estrogen protected ovariectomized rats from methylnitrosourea (MNU)-induced cataractogenesis. Furthermore, estrogen inhibits anterior opacities in cultured lenses from ovariectomized female rats induced by Tgfb2 (*10*).

Radiation cataractogenesis is often an unavoidable complication if the orbit is included in the treated volume during conventional radiotherapy or as a result of total-body irradiation (TBI) prior to bone marrow transplantation (*11–15*). It has long been assumed that a minimum dose of ~2 Gy of X rays in a single exposure will cause opacification in the human lens (*16*). While radiation therapy generally involves high doses of radiation administered in single or fractionated regimens (6–14 Gy delivered in one to four fractions in the case of TBI, for example), more recent data [(*17–19*) and references therein] now argue against such a high threshold dose for radiation-induced cataractogenesis. In addition, the lower doses incurred by astronauts during manned space missions have been shown to result in an

[1] Address for correspondence: Indiana University, Department of Radiation Oncology, 535 Barnhill Drive, Indianapolis, IN 46202; e-mail: jdynlach@iupui.edu.

[2] Current address: Department of Radiation Oncology, University of Texas Southwestern, 5801 Forest Park Road, NF3.302B, Dallas, TX 75390.

increased risk for cataractogenesis in the years after active flight (*20, 21*). Furthermore, doses to the lens in the range of those used for diagnostic radiology procedures such as computed tomography of the head (<10 cGy) may be sufficient to cause an increased incidence of posterior subcapsular cataracts (*17*). Currently, the cure for cataracts is surgery. However, surgical procedures are not available to everyone, do not produce identical outcomes, and are not without risk (*22*). Therefore, the development of agents that increase the latent period before cataract formation or decrease the severity of radiation-induced cataracts would be of great clinical relevance. Cataract induction is also a potentially serious outcome that could occur in astronauts involved in planetary missions during which they would be exposed to densely ionizing space radiation (*20*). With lengthy planetary missions and extended sojourns on space stations planned within the next two decades, the identification of agents which modulate cataractogenesis due to exposure of astronauts to ionizing radiation in space would also be of interest to NASA.

Radiation-induced cataracts can be distinguished from age-related cataracts in that they begin to form in the posterior subcapsular region of the lens (*23, 24*). As the radiation-induced cataract progresses to later stages, it becomes non-specific and indistinguishable from other types of cataracts and gradually extends to the cortex and the nucleus. The time between exposure and the detection of opacities, the latent period, varies inversely with dose and reflects the time during which damaged lens epithelial cells become altered and migrate from the equatorial region to the posterior pole, where they accumulate as abnormal nucleated lens fibers. Worgul and coworkers have hypothesized that postirradiation proliferative activity of surviving but damaged cells is a prerequisite for cataractogenesis (*24, 25*).

Radiation damage to cells involves the production of free radicals, notably the hydroxyl radical. The free radicals can subsequently interact with DNA, resulting in base damage and the formation of single-strand breaks (SSBs) and double-strand breaks (DSBs). It is generally agreed that DSBs are the critical lesions leading to radiation-induced cell death. Unrestituted or misrepaired DSBs can result in the formation of chromosomal aberrations, the presence of which correlates well with cell killing (*26*). An accumulation of or failure to repair radiation-induced DNA damage in lens epithelial cells may be a precursor to cataractogenesis (*27–29*). Since lens fiber cells are derived from epithelial cells, incomplete, inhibited or otherwise inefficient DSB repair in epithelial cells may lead to the development of radiation-induced lens fiber opacity (*28–30*).

Estradiol has been shown to significantly decrease the formation of SSBs and DSBs induced in naked plasmid DNA by hydrogen peroxide as well as the number of mutations resulting from low-dose irradiation of immortalized lymphocytes (*31, 32*). These and other studies suggest that the estradiol molecule has antioxidant properties (*33, 34*); however, the direct antioxidant effect requires very high, non-physiological concentrations of the steroid. To our knowledge, no studies have been conducted to determine the effects of hormonal modulation on radiation-induced cataractogenesis.

Since estrogens may behave as antioxidants and have been shown to inhibit formation of age-related and TGFB- or alkylating agent-induced cataracts, we hypothesized that estrogen could be useful in the protection against radiation-induced cataracts. However, we now present data that indicate that estradiol reduces the latent period and increases the incidence of radiation-induced cataracts in rats.

MATERIALS AND METHODS

Treatment of Animals prior to Irradiation

All experimental procedures performed on animals were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Prior to each experiment, 35–40-day-old Sprague-Dawley female rats were received from Harlan (Indianapolis, IN). One week after acclimation, the 42–47-day-old animals either were ovariectomized and randomly assigned to either an empty implant or an estradiol implant group or were left intact (designated ''ovary intact'') (15–16 rats per group). Ovariectomies, implants and irradiations were performed while animals were under general anesthesia. To induce general anesthesia, each ~150-g rat was injected with 12.45 mg ketamine, 0.27 mg acepromazine, and 0.06 mg atropine. In the estradiol implant group, a Silastic capsule containing 20 mg of crystalline 17 β-estradiol (E2) (Sigma-Aldrich, St. Louis, MO) (*35*) was implanted subcutaneously on the back of each rat. Each capsule provided a continuous course of estrogen therapy throughout the period of observation (*9*). As controls, ovariectomized rats received an empty Silastic capsule (control group). Silastic capsules (1 cm long) were fashioned from Veterinary Silicone Silastic Tubing (0.062″ inside diameter; 0.125″ outside diameter) (Dow Corning, Konigsber Instruments, Inc., Pasadena, CA) and sealed with Dow Corning Medical Adhesive (Silicone Type A).

Dosimetry and Irradiation of Rats

The right eyes of anesthetized 49–56-day-old rats were irradiated with either 10 or 15 Gy of ^{60}Co γ rays using a Leksell Gamma Knife. Dosimetry and irradiation of rat eyes have been described in detail previously (*36*). Briefly, an anatomically correct silicone model of a 6-week-old Sprague-Dawley rat was constructed. Lithium fluoride thermoluminescence dosimeters (Bicron Harshaw, Newbury, OH), were placed in slits located in the position of each eye for determination of dose to the target and contralateral eyes.

To enable reproducible positioning, the model or live rats were placed on a 6″ × 8″ wood resting plate, which in turn was placed on an 8″ × 10″ wood platform designed to fit securely into the Gamma Knife helmet and Gamma Knife trunnions during irradiation. The platform was fixed to an 8-mm collimator helmet. The right eye of each rat was centered in the radiation field by visual observation through two 4-mm collimator conduits at right angles to each other. All conduits whose beam trajectories passed through the left eye were plugged to prevent irradiation of the left lens. A 1-cm bolus (Bolx I, Med-Tec, Orange City, IA) was placed over the head of each rat to provide adequate buildup for dose to the right eye.

Either intact, ovariectomized empty capsule-implanted (control), or E2-implanted Sprague-Dawley rats (15 animals per group) were anesthetized and immobilized ~10 min prior to irradiation. Each rat received a single fraction of 10 or 15 Gy of ^{60}Co γ rays to the right eye. The dose rates for the 10- and 15-Gy irradiations ranged from 1.95–2.11 Gy/min and were determined on the day of each irradiation. The total exposure time for the largest dose (15 Gy) did not exceed 8 min. The contralateral eye

TABLE 1
Grading Scale for Slit Lamp Evaluation

Estimated surface area (%)	Score (based on interval midpoint)
0	0
5–10	7.5
11–20	15
21–40	30
41–60	50
61–80	70
81–100	90

Note. Grading scale for assessment of radiation-induced cataracts by slit-lamp analysis.

received less than 2% of the total dose to the target eye (*36*). No cataracts developed in contralateral lenses during the period of observation [see ref. (*36*) and Results]; thus, for purposes of comparison, contralateral eyes are hereafter referred to as unirradiated eyes.

Measurement of Lens Opacification and Transparency

Animals whose right eyes had been irradiated with 10 or 15 Gy were observed for cataracts every 2 to 4 weeks and killed humanely at various times postirradiation. Lenses were examined macroscopically by gross examination for complete opacity (15-Gy-irradiated lenses) or with a slit lamp (10-Gy-irradiated lenses). For animals irradiated with 10 Gy, anterior and posterior subcapsular lens opacities were graded based on the estimated percentage surface area of the opacity as described in Table 1. Because some of the animals irradiated with 10 Gy had developed complete anterior opacification after 41 weeks, it was not possible to measure posterior subcapsular opacities accurately after this time. Therefore, to eliminate any uncertainties in assessment, scores for posterior cataracts are presented only through 41 weeks.

All animals irradiated with 15 Gy were killed after 25 weeks, and lenses were examined microscopically to determine the degree of opacity by evaluating the effective light transmission (*9*). Lenses were removed and placed in a petri dish containing PBS. Using a stereoscopic dissecting microscope with an attached CCD video camera (Model DXC-960MD, Sony), a standardized intensity of light was passed through the lens from underneath the petri dish. The image of the lens was captured on a computer and the intensity of the light was analyzed by an imaging program (IPLAB SPECTRUM, Signal Analytics, Vienna, VA) run on a computer. By comparing the intensity of the light passing through the lens against an image of the light outside of the lens, determinations of the percentage light transmission were made (*9*). A 2-mm-thick piece (1 cm^2) of opaque, white Teflon was included in the microscopic field for measurement of zero transmitted light. The intensity of the light (in arbitrary units) transmitted at the center of the lens was measured with the IPLAB SPECTRUM program. Likewise, the intensity of light transmitted through the culture dish to a position just outside the lens was measured and used to define 100% transmission. The units of light intensity measured from the Teflon piece were considered background and used to correct the light transmission measurements made at the lens and outside the lens. The light passing through the lens was calculated as the percentage transmission. Both gross examination of cataract formation and light transmission were used to compare the estrogen-supplemented and non-supplemented groups. After evaluation of light transmission, lenses were fixed in formalin and analyzed histologically to determine the type of cataract (anterior, posterior) present.

Lens Histology

Entire eyes were removed and fixed as described earlier (*9*). Paraffin-embedded eyes were sectioned at 6 μm, and sections were stained with hematoxylin and eosin.

Statistical Analyses

Differences in cataract (complete opacification as determined by gross examination) incidence at 25 weeks after 15 Gy irradiation were determined by Fisher's exact test. Treatment effects on lens opacity/transparency after 15 Gy irradiation, as measured *ex vivo*, were analyzed by a *t* test. After testing for normal distribution (D'Agostino and Pearson omnibus normality test), slit-lamp scores were analyzed across times after irradiation (10 Gy) by two-way ANOVA; differences between treatments at individual times were determined by Bonferroni post tests. The slit-lamp scores were subjected to linear regression analyses. Cataract incidence was plotted using the fraction of animals with lens opacification >15, calculated by the Kaplan-Meier method; these incidence curves were compared using the logrank test. Significance levels were set at $P < 0.05$.

RESULTS

The Leksell Gamma Knife was used to selectively irradiate only one eye of each test animal with 10 or 15 Gy of ^{60}Co γ rays. The contralateral eye received a dose less than 2% of the total dose to the target eye (<25 cGy in the case of 15 Gy irradiation to the target eye). No cataracts developed in contralateral eyes during the course of observation after irradiation of the target eye with 10 or 15 Gy when rats were followed for up to 53 or 25 weeks, respectively. Therefore, for purposes of this study, the contralateral eye was regarded as an internal unirradiated control eye in both E2-treated and untreated animals.

An initial experiment was performed in which intact or ovariectomized female Sprague-Dawley rats were randomly assigned to either an empty implant or 17-β-estradiol (E2) implant group (15–16 rats per group). In the E2 implant group, a Silastic capsule containing 20 mg of E2 was implanted subcutaneously in each rat. The slow-release capsule provides physiological serum levels of hormone throughout the course of the experiment (*9*). The right orbit was then irradiated with 15 Gy. At various times postirradiation, irradiated lenses and contralateral (unirradiated) lenses were examined grossly for opacities by simple visual inspection. The technique of lens inspection in this initial experiment did not allow a determination of the position of cataracts within the lens. All rats were killed at 25 weeks postirradiation, and the lenses were evaluated microscopically to determine the degree of opacity by evaluating the effective light transmission. The incidence of visually apparent cataracts was increased when estrogen was present, either from an endogenous source (ovary intact) or administered exogenously (ovariectomized plus estradiol) (Table 2). Almost all irradiated eyes from both ovary-intact and ovariectomized, estrogen-treated animals became cataractous during the 25-week period of observation, while less than half of the irradiated eyes in the ovariectomized (control) group showed evidence of cataractogenesis. Light transmission of lenses from 15-Gy-irradiated ovariectomized animals that received continuous estradiol was significantly reduced (that is, opacity was significantly increased) compared to lenses from irradiated animals that received an empty implant (Fig. 1). Histological examina-

TABLE 2
Cataract Incidence after Irradiation with 15 Gy

Treatment	Irradiated eye	Nonirradiated eye
Ovary intact	15/16	0/16
Ovariectomized	5/13*	0/13
Ovariectomized + estradiol	12/14	0/14

Notes. Rats were left intact (ovary intact) or were ovariectomized and received an empty implant or an implant containing E2. The fraction of cataractous lenses with complete opacity was determined by gross examination of eyes at 25 weeks after irradiation. * $P < 0.05$ compared to intact or ovariectomized + estradiol (Fisher's exact test).

tion of cataractous lenses revealed that both anterior and posterior subcapsular cataracts developed within 25 weeks postirradiation with 15 Gy (data not shown). Based on these results, it would appear that either the absence of E2 decreases the incidence of cataract formation, or its presence increases the incidence. However, these data could also indicate that E2 reduced the latent period of cataractogenesis. A slit-lamp analysis of opacities throughout the period of observation would be necessary to obtain data to establish the true time course for cataractogenesis.

A slit lamp was used to measure the level of opacities in subsequent experiments in which ovariectomized rats received empty or E2 implants and were irradiated with 10 Gy. Criteria were developed for grading the severity of cataracts based on estimates of the percentage surface area of opacity (see the Materials and Methods).

The data shown in Figs. 2 and 3 depict the time course for the development and incidence, respectively, of anterior subcapsular and posterior subcapsular cataract formation in irradiated lenses of control animals or animals that received estradiol. In untreated (control) animals, the development of posterior subcapsular cataracts occurred nearly concomitantly with the development of anterior subcapsular cataracts (Fig. 2). Linear regression showed that the rate of increase in anterior cataract formation (slope) was higher in the estradiol-treated animals compared to controls ($P < 0.01$). ANOVA showed that the scores differed between treatments from 41 weeks onward for anterior cataracts and from 38 weeks onward for posterior cataracts ($P < 0.05$). The slope of the lines for posterior cataracts did not differ between the two treatments, but the X intercepts were different ($P < 0.001$). We observed that the latent period for posterior cataract formation decreased in 10-Gy-irradiated rats that had received continuous estrogen treatment, while in the anterior region of the lens, the severity of the cataract increased. The cataract incidence curves tended to differ between treatments for both the anterior ($P = 0.058$) and posterior regions ($P = 0.095$), showing an earlier onset of cataractogenesis in the estradiol-treated group (Fig. 3). Taken together, these data suggest that while estradiol did not significantly alter the overall incidence of cataract formation in animals irradiated with 10 Gy, it did accelerate the onset and progression of opacification.

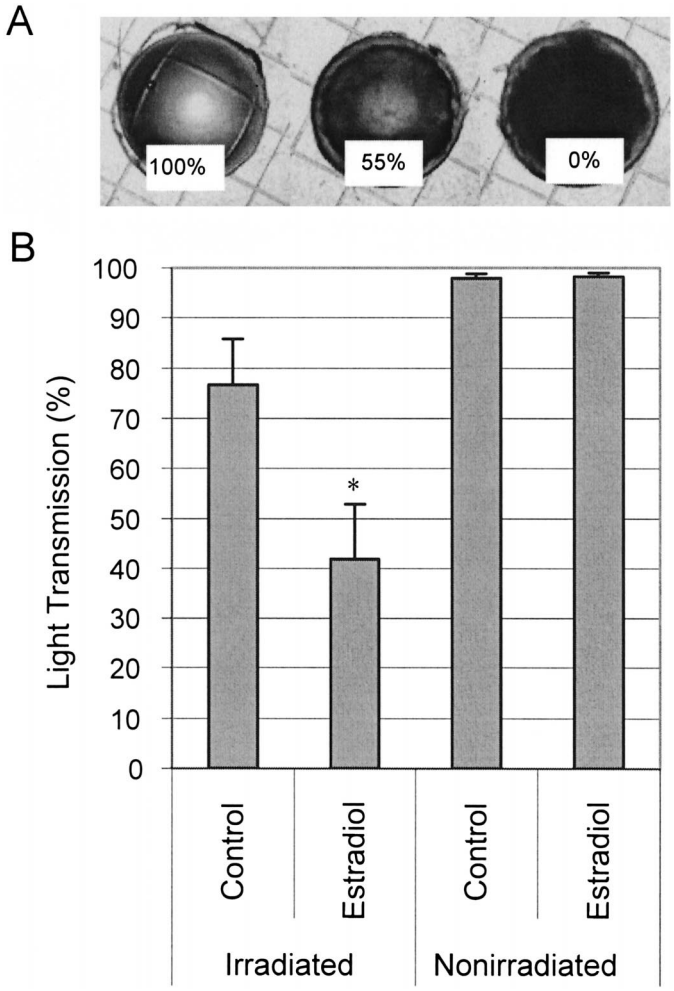

FIG. 1. Percentage light transmission in the lens after 15 Gy irradiation. Panel A: The lens was viewed with a dissecting microscope, and the intensity of the light was measured in the center of the lens and expressed as percentage transmission compared to light transmitted outside the lens. The percentage transmission is shown beneath each of these three lenses. Panel B: At 25 weeks postirradiation, the average light transmission (±SEM) was determined for both irradiated and nonirradiated (contralateral) lenses from 13 animals implanted with an empty silastic capsule (Control) or 14 animals treated with estrogen (Estradiol). *$P < 0.05$ compared to control, irradiated lens.

DISCUSSION

Several reports indicate that estrogens protect women against some forms of age-related cataracts (*1–4*). Induction of cataracts in rats by alkylating agents mimics age-related cataractogenesis, and estrogen is protective in this model (*9*). Since alkylating agents and radiation are both effective at inducing DNA damage in lens epithelial cells, which may lead to the development of lens fiber opacity (*28–30*), it might be predicted that estrogen would protect against radiation-induced cataracts as well. Contrary to the predicted effects, our data indicate that estrogen reduces the latent period for radiation-induced cataractogenesis (Figs. 2 and 3). Anterior subcapsular opacification was first detected in some E2-treated animals within 28 weeks after irradia-

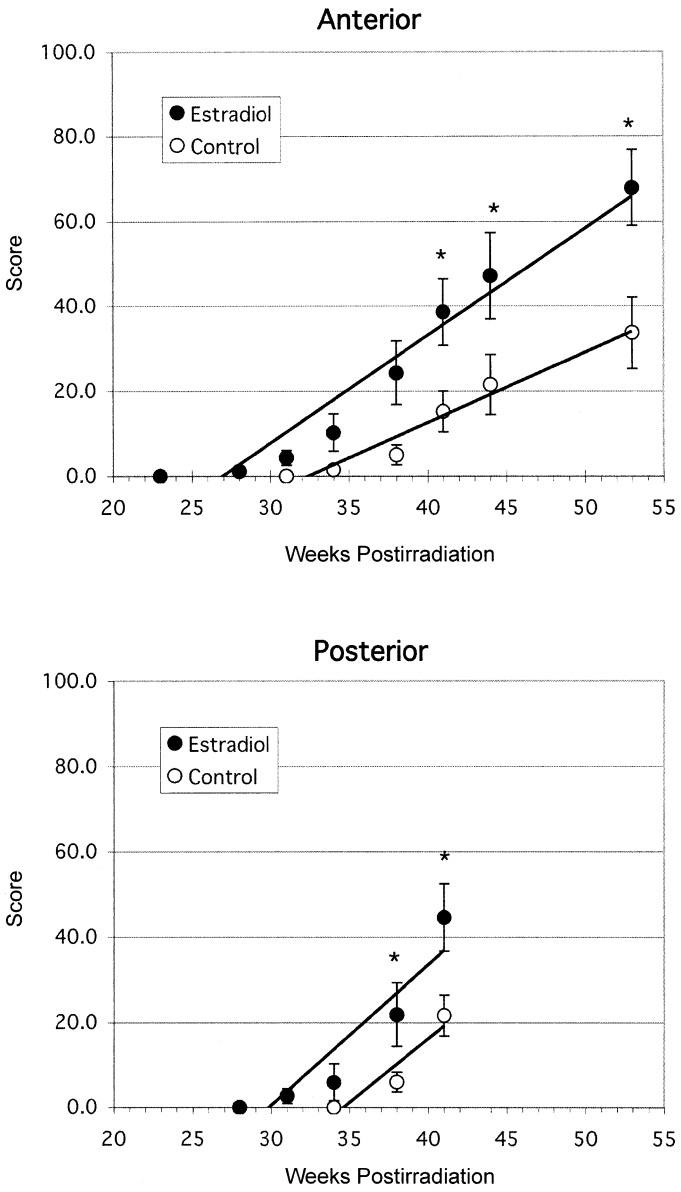

FIG. 2. Progression of anterior and posterior subcapsular cataracts after 10 Gy irradiation. Ovariectomized rats either were treated with continuous estradiol commencing 1 week prior to irradiation (Estradiol) or received an implant consisting of an empty silastic capsule (Control). Data represent scores based on estimated percentage surface area opacification (see Table 1) for anterior subcapsular and posterior subcapsular cataracts of all irradiated eyes within each treatment group (±SEM); the control group consisted of 15 animals and the estradiol group consisted of 14 animals.

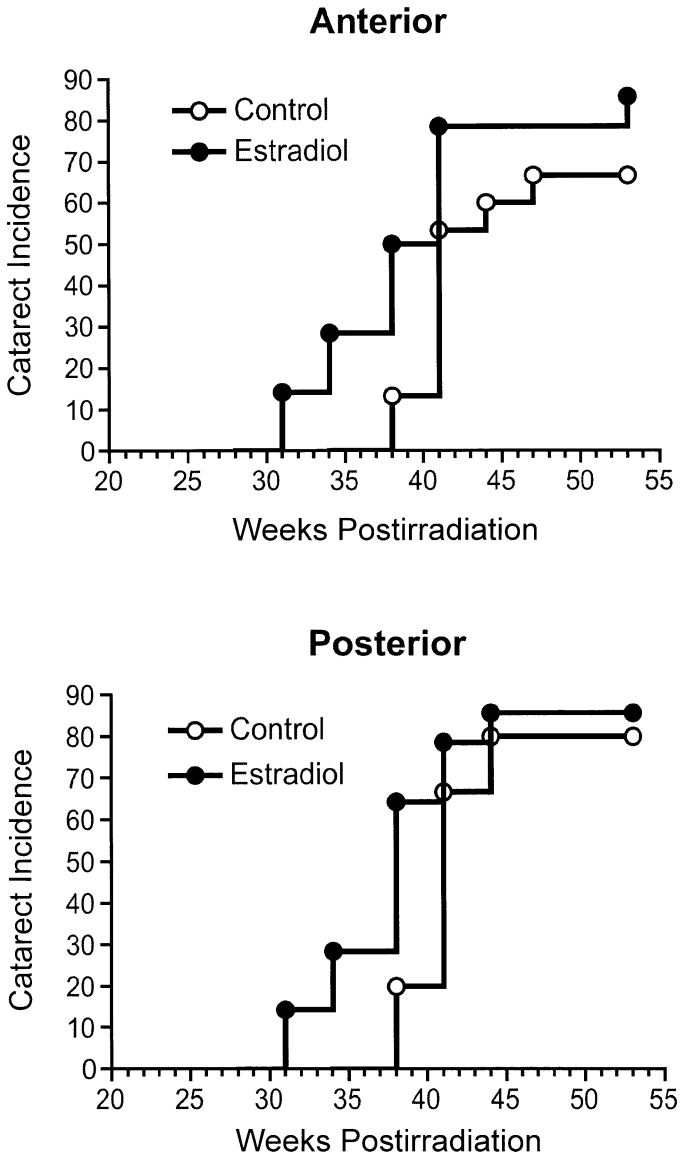

FIG. 3. Incidence of anterior and posterior subcapsular cataracts as a function of time after 10 Gy irradiation. Data represent the percentage of animals (as described in Fig. 2) with cataract scores ≥15; the control group consisted of 15 animals and the estradiol group consisted of 14 animals.

tion, compared to 34 weeks in untreated control animals. We observed that in both control and E2-treated animals, anterior subcapsular opacification occurred nearly concomitant with posterior subcapsular opacification. While this finding appears to conflict with other data that suggest that cataracts begin to form in the posterior subcapsular region (*23–25*), several investigators have also reported that initial changes may occur in the anterior subcapsular region after treatment with high-LET radiation or high doses of low-LET radiation (*37, 38*).

The difference between the effect of the hormone on age-related and radiation-induced cataractogenesis may be linked to the different mechanisms of cataract production in the anterior and posterior subcapsular regions of the lens. Opacification of the anterior portion of the lens occurs when the architecture of the subcapsular fiber cells become distorted and vacuolated. Radiation-induced cataracts occur when proliferating equatorial epithelial cells migrate aberrantly into the posterior region of the lens. In both cases, oxidative damage to DNA may be the triggering event. Significant increases in the levels of hydrogen peroxide have been reported in the lens and aqueous humor of cataract patients, indicating a role for oxidative damage in cataract formation (*39*). An accumulation of or failure to repair DNA damage from reactive oxygen species in lens epithe-

lial cells may be a precursor to cataractogenesis (*27–29*). Radiation damage to cells involves the production of free radicals, notably the hydroxyl radical. The free radicals can subsequently interact with DNA to form base damage, SSBs and DSBs, the latter of which are believed to be the lethal lesion involved in radiation-induced cell killing. It is also worth noting that Fu *et al.* (*40*) found that hydroxyl radical attack on lens proteins was associated with nuclear cataractogenesis. Though the estrogen molecule is believed to possess antioxidant properties (*34*) and has been demonstrated to protect against oxidative stress in human lens epithelial cells (*41*), it did not confer protection against radiation-induced cataractogenesis in our experiments (Table 2, Figs. 2 and 3).

If one accepts the notion that cataractogenesis in the posterior subcapsular region requires postirradiation proliferation of damaged cells (*25*), one may speculate that the reduction in latent period may be related to a dysregulation of cell cycle checkpoint control in irradiated cells treated with estrogen. Normally, several molecular checkpoints act to delay cell cycle progression in irradiated cells to avoid replication and segregation of damaged DNA (*42*). It appears that the role of these DNA damage checkpoints is to provide time for repair to occur. While ionizing radiation inhibits cell cycle progression, physiological and low pharmacological doses of estrogen may stimulate cell proliferation by reducing cell cycle transit times through a reduction in the durations of G_1 and S phase (*43*). Thus it is possible that the time for full repair of DNA damage may be reduced prior to replication of the genome of irradiated lens epithelial cells (that is, estrogen may act to reduce radiation-induced inhibition of cell cycle progression), leading to the development of aberrant secondary lens fibers. In addition, estrogen might lead to retention of damaged cells that would normally be deleted through apoptosis. In hormone-dependent normal and transformed breast cell lines, E2 exerts anti-apoptosis effects by blocking JNK activation, preventing the JNK-induced, inactivating phosphorylation of BCL2 and BCL-xl, the subsequent stimulation of the caspase cascade, and cell death (*44–46*).

Women receiving therapeutic estrogen treatment showed significant suppression of the repair of UV-radiation-induced DNA damage in lymphocytes (*47*). Moreover, metabolites of estrogens, particularly catechol estrogens, induce multiple forms of genetic lesions in cultured cells, laboratory animals and human tissues, including structural chromosomal aberrations such as deletions, translocations and gene amplifications [(*48*) and references therein]. Catechol estrogens are capable of continuous redox cycling, yielding quinone intermediates; this process results in the generation of reactive oxygen species (free radicals) that can damage DNA by inducing strand breaks and increased levels of 8-hydroxy-2′-deoxyguanosine. As with ionizing radiation, among the potential free radicals generated is the hydroxyl radical, the most potent oxidizing species. Thus it is plausible that estrogens may add to radiation-induced DNA damage either directly by the covalent binding of estrogen metabolites to DNA or indirectly by free radical generation. It is therefore attractive to speculate that, in estrogen-treated irradiated lens cells, the radiation damage to DNA can interact additively or synergistically with free radicals generated through estrogen metabolism. Increased DNA damage resulting from estrogen treatment in irradiated lens cells might be expected to enhance radiation-induced cataractogenesis.

Clearly, additional experiments are required to determine whether the enhancement of radiation-induced cataractogenesis is mediated through genomic mechanisms or nongenomic mechanisms. The timing of the estrogen effect also requires further investigation.

Finally, it has been demonstrated that the administration of steroids for graft versus host disease after high-dose-rate TBI decreased the latency and increased the severity of radiation-induced cataracts (*15*). Based on these data and data from our current study, a retrospective study comparing cataractogenesis in postmenopausal women treated with radiotherapy for head and neck or ocular cancers who received or did not receive concurrent hormone replacement therapy is warranted. Data from such a study could be useful in determining whether radiation oncologists should recommend cessation of hormone replacement therapy during radiotherapy.

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