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Ultraviolet Radiation-Induced Corneal Degeneration in 129 Mice

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ABSTRACT

Ultraviolet radiation (UVR) is a risk factor for the development of ocular disease in humans, including acute photokeratitis, chronic corneal spheroidal degeneration, and cataract formation. This report describes the ocular lesions seen in 21 mice chronically exposed to UVR as part of a skin carcinogenicity study. All globes were affected to varying degrees. The primary lesion, not previously reported in UVR-exposed mice, was marked loss of keratocytes relative to age-matched controls. Secondary lesions included corneal stromal thinning, keratoconus, corneal vascularization and fibrosis, keratitis, globe rupture, and phthisis bulbi. In addition, more than 90% of UVR-exposed and unexposed lenses had evidence of cataract formation; this is the first report of the occurrence of spontaneous cataracts in 129 mice. In a subsequent study, apoptotic cells were identified histologically and by cleaved caspase 3 immunoreactivity in the corneal epithelium and, less commonly, in the corneal stroma after acute UVR exposure. Based on this finding, we propose that the loss of keratocytes observed in the chronic study was due to UVR-induced apoptosis.

Keywords. Cataract; cornea; eye; mouse; ultraviolet rays.

INTRODUCTION

Exposure to ultraviolet radiation (UVR) is a significant risk factor for ocular disease. Acute UVR exposure in man can cause photokeratitis characterized by apoptosis and exfoliation of the corneal epithelium, formation of punctate ulcers, inflammation, edema, and pain (Cullen, 2002; Young, 2006). Chronic UVR exposure, on the other hand, is associated with spheroidal degeneration of the cornea, which has also been called Bietti corneal degeneration, Labrador keratopathy, Eskimo corneal degeneration, and elastotic degeneration. Clinically, spheroidal corneal degeneration is characterized by homogeneous, translucent, yellowish, spherical lesions (0.1–0.6 mm) in the superficial corneal stroma. Histologically, the superficial cornea contains extracellular spherules thought to represent accumulations of plasma proteins that have diffused into the cornea from the limbal circulation and have been modified by UVR (Magovern et al., 2004). Lifetime exposure to chronic UVR has been directly associated with the development of cortical cataracts in man, and the World Health Organization has reported that as much as 20% of cataract-associated blindness may be due to UVR exposure. Genetic factors, diabetes, steroid use, and smoking are also strongly associated with cataract formation in humans (Robman and Taylor, 2005).

There are few reports of adverse UVR effects on the mouse eye. In one study, all male albino mice chronically exposed to high doses of UVR (>40,000 J/m^2 daily) for 1–4 months had anterior cortical lens opacities (Jose and Pitts, 1985). With chronic exposure to much lower doses of UVR (350–3600 J/m^2 daily), mice developed clinically evident posterior lens opacities in the absence of anterior lens opacities or corneal disease after 6–9 months (Jose, 1986). It was recently reported that 5000 J/m^2 UVR for periods of 1 to 8 days induced grossly apparent subcapsular, cortical, or nuclear cataracts in all exposed C57BL/6 mice (Meyer et al., 2005). UVR-induced corneal lesions in mice vary considerably in severity, depending on UVR dose and number of exposures. Mice exposed to >40,000 J/m^2 daily develop corneal scarring and vascularization (Jose and Pitts, 1985), while those exposed to 350 or 3600 J/m^2 daily are reported to have no corneal lesions (Jose, 1986) and those receiving 800 J/m^2 daily develop only corneal clouding (Downes et al., 1994, 1997). Comparative studies of corneal clouding in different strains of mice and in recombinant inbred SWXJ mice suggest that aldehyde dehydrogenase activity protects the cornea against UVR-induced clouding (Downes et al., 1994, 1997).

In this study we report previously undescribed clinical and histologic lesions in the corneas of 129 mice chronically exposed to UVR, as well as a high incidence of posterior cortical opacities in both unexposed and UVR-exposed mice.

MATERIALS AND METHODS

Study Design

Studies were conducted in compliance with all applicable state and national guidelines and were approved by The Ohio State University (OSU) Institutional Laboratory Animal Care and Use Committee. Twenty-one 129S1/SvImJ mice (14 females, 7 males) constituted the control group in a long-term skin carcinogenesis study. Mice were obtained from a

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Abbreviations: UVR, ultraviolet radiation; UVA, ultraviolet radiation, type A; UVB, ultraviolet radiation, type B; UVC, ultraviolet radiation, type C; MED, minimal erythemal dose; HA, hyaluronan; ROI, reactive oxygen intermediate.

breeding colony of 129 mice maintained at OSU from mice originally acquired from Jackson Laboratory (Bar Harbor, ME, USA). Mice were exposed to 3200 J/m^2 (2 minimal erythemal doses, MED) of UVR 3 times a week for up to 50 weeks. One MED is the dose required to cause detectable skin thickening. UVR was provided by Kodacel-filtered Westinghouse FS-40 sunlamps that emitted wavelengths between 280 and 400 nm, with a peak at 313 nm. The emitted light contained approximately 60% UVB and 40% UVA; UVC wavelengths were removed by Kodacel filters. A single mouse was sacrificed after 9 weeks of UVR exposure. Five mice were removed early from the study (38–48 weeks of exposure) due to large skin tumor size. One mouse was found dead; the eyes were not examined histologically. A total of 14 mice were alive at the end of the study (50 weeks).

For comparison, eyes from 13 untreated 129 mice maintained in our breeding colony were examined. Six of these control mice were 10–12 weeks old; and 7 of the mice were 13–17 months of age, to match the ages of the chronically exposed mice at the end of the study. In the acute UVR exposure study, 10–12-week-old 129 mice were exposed to a single dose of 4800 J/m^2 (3 MED) and sacrificed at 12 (7 females, 1 male), 24 (7 females, 1 male), 48 (1 female, 1 male), 72 (1 female, 1 male), and 96 hours (1 female, 1 male) and 1 week (2 females, 1 male) after UVR exposure. All mice were housed under the same conditions. Mice were sacrificed by carbon dioxide asphyxiation. Following euthanasia, the globes were removed and fixed in 10% neutral-buffered formalin for histologic examination.

Ophthalmic Examination

Eyes were examined using a Zeiss HSO-10 slit lamp (Dublin, CA, USA) and a Keeler All Pupil indirect ophthalmoscope with a 40 diopter lens (Broomall, PA, USA). In the chronic UVR exposure study, the first ophthalmic examination was performed when mice had been on study for 36–49 weeks. For some mice, repeated examinations were performed to document lesion progression. Six mice that were removed early or died during the study did not receive ophthalmic examinations. In the acute UVR study, selected mice were examined before UVR exposure and immediately prior to euthanasia.

Histology and Immunohistochemistry

Eyes were embedded in paraffin, sectioned at 4–5 μm, and stained with hematoxylin and eosin. For stromal cell counts, all stromal cells within a 200X field of axial cornea were counted from hematoxylin and eosin-stained sections. Axial cornea was defined as that portion of the cornea overlying the pupil. If the pupil was not present in the section, corneal stromal cells were not counted. Additionally, corneal stromal cells were not counted if there was evidence of corneal inflammation or fibrosis.

Apoptosis was evaluated by immunohistochemical detection of cleaved caspase-3. Slides were deparaffinized and re-hydrated. Antigen retrieval was performed with DakoCytomation Target Retrieval Solution (Carpinteria, CA, USA) and the Biocare Digital Decloaking Chamber (Concord, CA, USA) by heating under pressure to 125°C for 30 seconds followed by cooling in the chamber to 90°C and on the bench top for 10 minutes. Immunohistochemical staining was performed using the Dako Autostainer. Slides were rinsed with water, and then treated for 5 minutes with 3% hydrogen peroxide and with protein block (DakoCytomation Serum-free Protein Block) for 10 minutes. Slides were incubated for 30 minutes with primary antibody (Cell Signaling, Danvers, MA, USA) diluted 1:200 in DakoCytomation Antibody Diluent with Background Reducing Components. Slides were incubated for 30 minutes with secondary antibody (Vector biotinylated goat-anti-rabbit antibody; Burlingame, CA, USA) diluted 1:200 in antibody diluent and for 30 minutes with ABC reagent (Vector R.T.U. Vectastain Elite ABC). Slides were then incubated for 5 minutes in DakoCytomation Liquid DAB Substrate, counter-stained with hematoxylin, dehydrated, and coverslipped. Rinses were performed using DakoCytomation Wash Buffer (Tris-buffered saline/Tween 20).

Statistical Analysis

Statistical analysis was performed and graphs generated with SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). Since the Kolmogorov–Smirnov test showed that data violated assumptions of normality, data were analyzed using Kruskal–Wallis nonparametric analysis of variance and Mann–Whitney U-tests for pairwise comparisons if the Kruskal–Wallis test was significant. A p value of less than 0.05 was deemed statistically significant. Box-plots display medians, interquartile range (the box), and 1.5 times the interquartile range (the 'whiskers').

RESULTS

Chronic UVR Exposure

None of the control mice had evidence of corneal disease by slit lamp examination. All UVR-exposed mice developed clinical evidence of unilateral or bilateral lesions. Corneal abnormalities were noted clinically as early as 31 weeks. Repeated slit lamp examination demonstrated progression from corneal stromal cleft formation and stromal thinning to keratoconus with variable degrees of corneal vascularization to corneal perforation, iris prolapse, and phthisis bulbi (Figure 1, Figure 2A–E). Eleven of the 38 globes examined (29%) ruptured; eye rupture occurred in 9 of 21 mice (43%).

Posterior and, less commonly, anterior cortical cataracts were noted both in older unexposed mice and in chronically UVR-exposed mice. In some mice, repeated slit lamp examination demonstrated progression from no detectable cataract to a detectable posterior cataract (Figure 2F).

Histologic examination of the eyes of unexposed control mice revealed a significant age-dependent loss of keratocytes. Mice 10–12-weeks old had a median of 96 (range = 76–118) stromal cells per 200X field of axial cornea, while mice 13–15-months old had a median of 58 (range = 34–79) stromal cells per 200X field of axial cornea (Figure 4). The difference in the number of stromal cells between the 10–12-week-old and 13–15 month-old unexposed mice was significant ($p < 0.001$).

Microscopically, corneal lesions from UVR-exposed mice were characterized primarily by mild to marked hypocellularity of the corneal stroma, accompanied by collagenolysis of stromal collagen. Stromal thinning and, rarely, stromal cleft

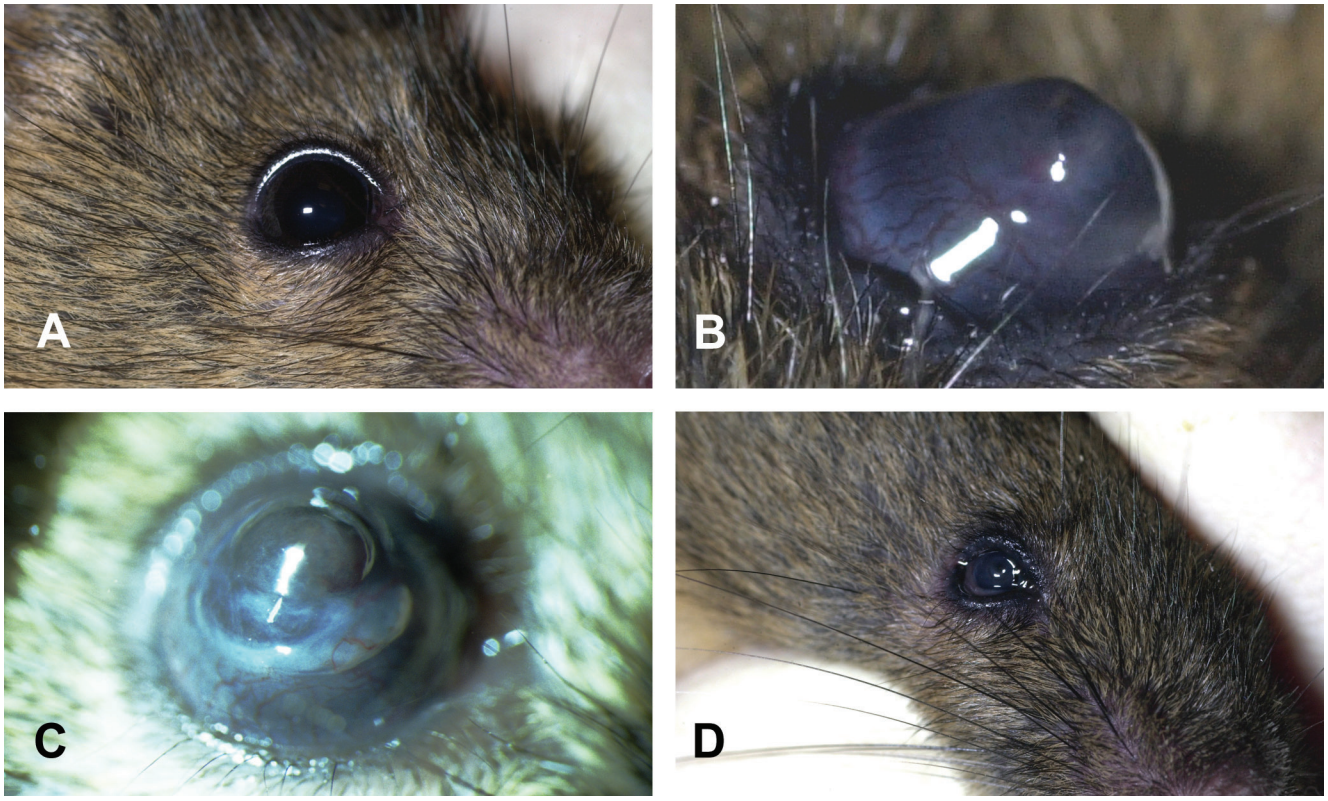


FIGURE 1.—Gross images of 129 mouse eyes. (A) Normal eye. (B) Keratoconus. (C) Corneal perforation and iris prolapse. (D) Phthisis bulbi.

formation were also observed (Figure 3B,C). Hyperplasia or dysplasia of the corneal epithelium was occasionally seen. As observed by slit lamp examination, corneal lesions often progressed to rupture of the globe. It is postulated that the severe thinning of the corneal stroma with resulting corneal fragility resulted in globe rupture following mild ocular trauma. As a result of the globe rupture, iris prolapse and phthisis bulbi occurred. Five of 21 mice (24%) had corneal fibrosis, vascularization, and inflammation (Figure 3D). Mice whose corneal lesions were characterized only by loss of keratocytes often had normal slit lamp examinations.

Chronically UVR-exposed mice averaged 18 stromal cells per 200X field of axial cornea; this was significantly fewer than in age-matched unexposed mice ($p < 0.001$) or young unexposed mice ($p < 0.001$) (Figure 4). The mouse that was exposed to 9 weeks of UVR had 44.5 stromal cells per 200X field of axial cornea. This suggested that as few as 9 weeks of UVR was sufficient to markedly decrease the number of stromal cells.

Cataracts identified by slit lamp examination in both UVR-exposed and unexposed mice were confirmed histologically in all cases. In some cases, cataracts were seen histologically that were not identified clinically. This discrepancy was due, in part, to the difficulties involved in evaluating the eyes of non-anesthetized mice and the severity of corneal disease. Of the lenses examined microscopically, 92% of UVR-exposed lenses and 93% of unexposed lenses had evidence of cataract formation. Cataracts were characterized by minimal to moderate swelling and degeneration of lens fibers at the posterior pole and, in some cases, by vacuolation of nucleated lens fibers at the equator (Figure 5C, D). There was evidence of posterior migration of lens epithelial cells in only 3 globes, from 3 different UVR-exposed mice. In these eyes, there was global lens fiber degeneration, as well as hyperplasia, metaplasia, and posterior migration of the lens epithelial cells (Figure 5E, F). This type of cataract was associated with either a ruptured cornea or severe corneal fibrosis and inflammation. Such cataracts likely developed in response to inflammatory mediators in the aqueous humor, rather than in response to UVR exposure alone. Eyes from the mouse that was exposed to UVR for only 9 weeks were not examined with the slit lamp, but microscopically there was minimal posterior cataract formation in one eye. The presence of minimal lens fiber degeneration after 9 weeks of UVR exposure suggested that mice that did not have clinical evidence of cataract prior to 47 weeks may have had histologically apparent cataractous changes too mild to be visualized by slit lamp.

Acute UVR Exposure

All eyes were grossly normal, without evidence of corneal edema, ulceration, or keratitis at any time point following a single UVR dose of 3 MED. There were no significant differences from unexposed eyes in the number of stromal cells at any time point following acute UVR exposure (data not shown).

Occasional apoptotic corneal epithelial cells and rare apoptotic corneal stromal cells were apparent histologically, but there was no histologic evidence of corneal ulceration or keratitis at any time point. Cleaved caspase 3 immunostaining was positive in the corneal epithelium at 12 (range = 0–10),

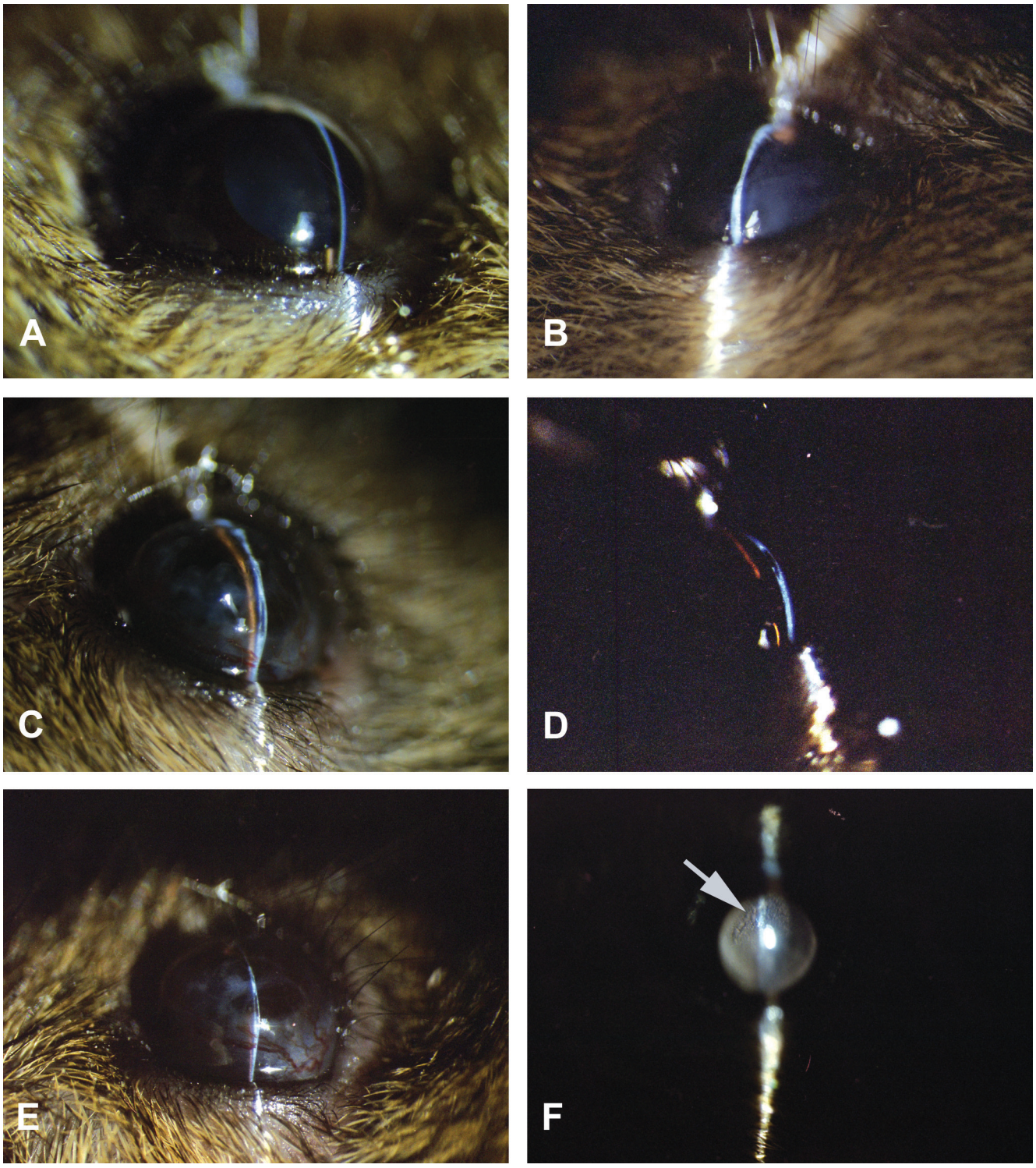


FIGURE 2.—Slit lamp images of 129 mouse eyes. (A) Normal eye. (B) Axial corneal stromal thinning. (C, D) Corneal stromal cleft. (E) Corneal stromal thinning, cleft formation and vascularization. (F) Retroillumination demonstrating posterior cortical vacuoles (arrow) and cataract formation.

24 (range = 0–14), and 48 hours (range = 0–1) post-UVR exposure (Figure 6A). Rarely, cleaved caspase 3 staining was noted in stromal cells (Figure 6B). The number of cleaved caspase 3-positive apoptotic epithelial cells in UVR-exposed eyes peaked at 24 hours and was significantly higher than the number in unirradiated eyes ($p < 0.001$) or in UVR-exposed eyes at 72 hours after exposure ($p < 0.01$) (Figure 7). Vacuolation of equatorial lens fibers was apparent in a few lenses, but no cataracts were apparent at 1 week after a single dose of 3 MED UVR.

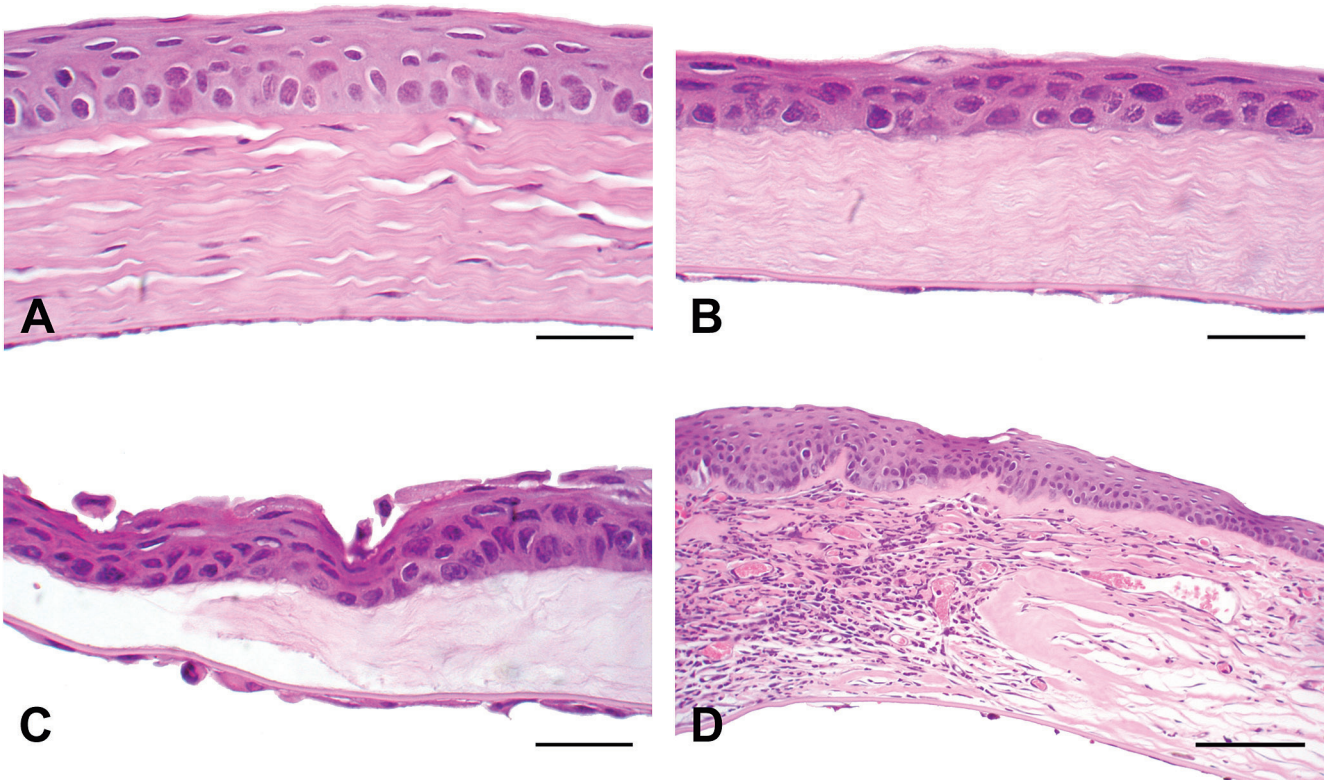


FIGURE 3.—Histopathology of the corneas of 129 mice (hematoxylin & eosin stain). (A) Normal cornea from an unexposed 10–12-week-old mouse (scale bar: 30 μm). (B) Diffuse loss of corneal stromal cells (scale bar: 30 μm). (C) Loss of corneal stromal cells, collagenolysis and cleft formation (scale bar: 30 μm). (D) Keratoconus with marked thickening of the cornea by fibrosis, neovascularization, and a mixed inflammatory infiltrate. Note that this image is taken at a lower magnification: scale bar: 100 μm).

DISCUSSION

Keratocytes function in maintaining corneal transparency, as well as structural stromal stability, by regulating collagen fibril size and spacing within the proteoglycan matrix (Kallinikos and Efron, 2004). In this study, we report both age-related and UVR-induced loss of corneal stromal cells. Age-related loss of keratocytes has been reported in humans (Moller-Pedersen, 1997), but has not previously been reported in other species.

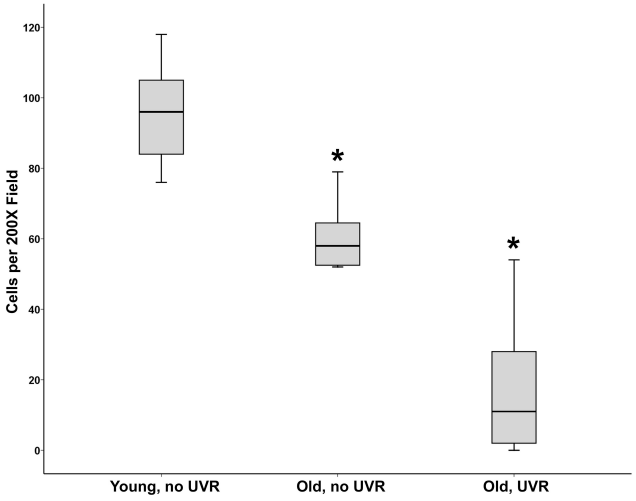


FIGURE 4.—Keratocyte numbers in the corneas of 129 mice. Asterisks indicate significant differences ($p < 0.001$) from young (12-week-old) unexposed mice. Old (1-year-old) UVR-exposed mice also had significantly fewer keratocytes than old (1-year-old) unexposed mice ($p < 0.001$).

Corneal de-epithelialization alone is sufficient to cause loss of keratocytes in the anterior stroma (Campos et al., 1994; Szerenyo et al., 1994; Ivarsen et al., 2004). Moreover, exposing the de-epithelialized rabbit cornea to UVR results in full thickness loss of keratocytes (Podskochy, 2004). The mechanism of keratocyte loss in de-epithelialized cornea is controversial. Some authors suggest that exposure of the de-epithelialized cornea to tears is sufficient to induce keratocyte apoptosis in mice (Zhao et al., 2001). Additional suggested etiologies include altered glucose metabolism and osmotic factors (Zhao et al., 2001). In the present study, however, there was no evidence of corneal ulceration or de-epithelialization at any time following UVR that could explain the observed loss of keratocytes.

Keratocyte loss has also been observed following UVR exposure of unwounded corneas. Podskochy et al. (2000) have shown that UVB focused on the center of the un-wounded cornea of an anesthetized rabbit results in full thickness corneal damage with apoptosis of corneal epithelium, keratocytes, and corneal endothelial cells. Apoptosis of corneal epithelial cells, visualized by TUNEL staining and confirmed by electron microscopy, peaked at 24 hours post-UVR, as was seen in the current study; by 72 hours postexposure, keratocytes were completely absent from the exposed region of the cornea (Podskochy et al., 2000). By 7 days there was

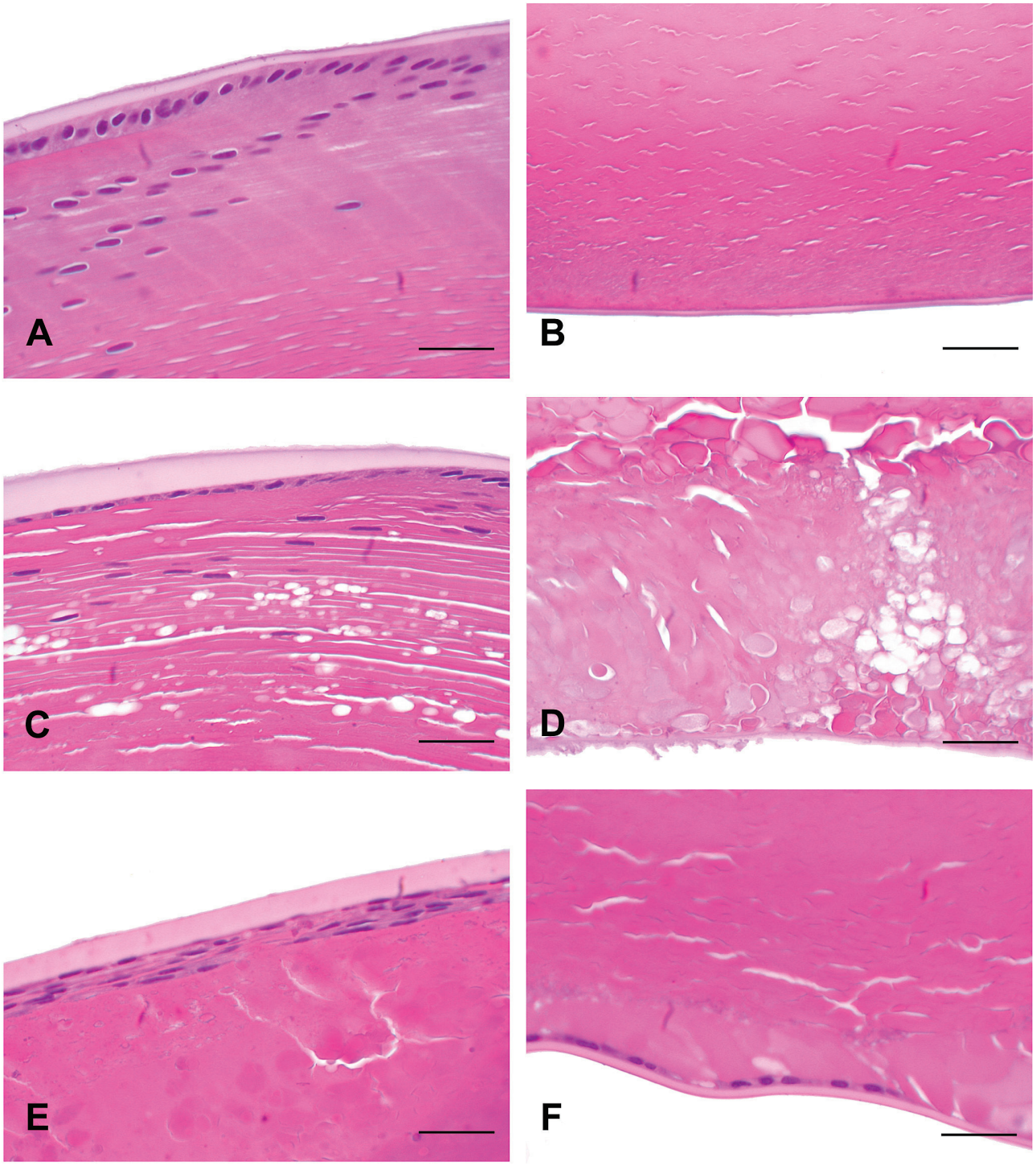


FIGURE 5.—Histopathology of the lens of 129 mice (hematoxylin & eosin stain). (A) Normal lens equator (scale bar: 30 μm). (B) Normal posterior cortex of the lens (scale bar: 30 μm). (C) Vacuolization of the equator of the lens, as seen in some old and UVR-exposed mice (scale bar: 30 μm). (D) Lens fiber swelling and degeneration in the posterior cortex of the lens of old and UVR-exposed mice (scale bar: 30 μm). (E) Proliferation and metaplasia of lens epithelial cells at the equator, as seen in some mice with keratoconus or globe rupture (scale bar: 30 μm). (F) Posterior migration of lens epithelial cells to the posterior cortex of the lens, with associated lens fiber degeneration and swelling, as seen in some mice with keratoconus or globe rupture (scale bar: 30 μm).

almost complete repopulation of the injured stroma by new keratocytes (Podskochy and Fagerholm, 1998). Also consistent with the present study, Podskochy et al., (2000) reported that there was no inflammation in the cornea at any time during following UVR exposure.

The mechanism of keratocyte apoptosis in UVR-exposed intact corneas is controversial. Keratocyte apoptosis has been reported to correlate with de novo FasL expression by keratocytes (Podskochy and Fagerholm, 2002). IL-1 from injured corneal epithelium may induce FasL expression by

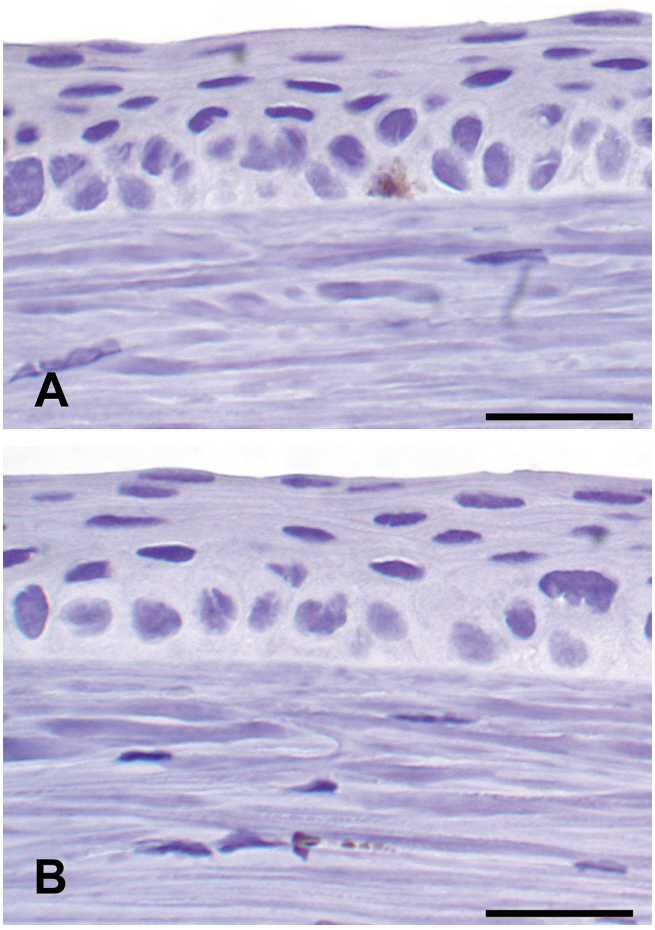


FIGURE 6.—Immunohistochemistry for cleaved caspase 3 to demonstrate apoptotic cells in 129 mice 24 hours after exposure to UVR. (A) Positive cells in the corneal epithelium (scale bar: 20 μm). (B) Rare positive cell in the corneal stroma (scale bar: 20 μm).

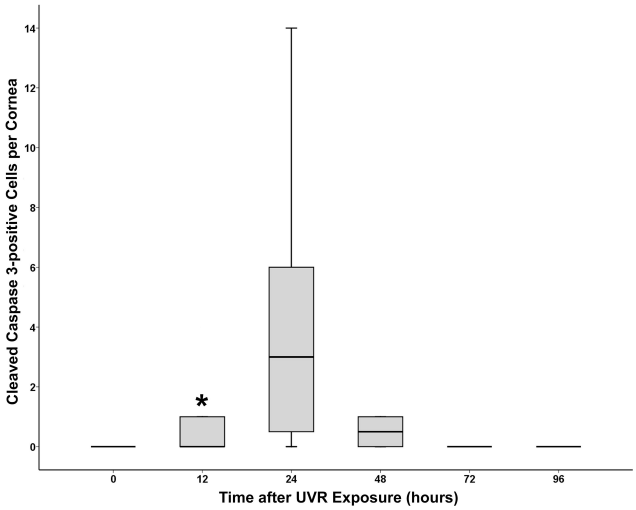


FIGURE 7.—Cleaved caspase 4-positive epithelial cells in the corneas of mice exposed to 3 MED UVR. The asterisk indicates a significant difference in mean compared to non-UVR-exposed eyes and UVR-exposed eyes at 72 hours after exposure ($p < 0.01$).

keratocytes (Podskochy and Fagerholm, 2002). Alternatively, FasL expression in keratocytes may be directly induced by UVR (Podskochy and Fagerholm, 2002). Loss of keratocytes has also been reported in humans following contact lens usage and is hypothesized to be due to apoptosis mediated by cytokines, growth factors, and other inflammatory mediators released by contact lens–induced trauma to the corneal epithelium (Kallinikos and Ephron, 2004). In support of this theory, rubbing of the eye exacerbates the loss of keratocytes.

Free radical-mediated injury may also contribute to keratocyte apoptosis. Since the cornea absorbs the majority most of the UVR entering the eye, it is very susceptible to reactive oxygen intermediate (ROI)-mediated injury and is rich in antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase) (Buddi et al., 2002). Furthermore, UVR-induction of hyaluronan (HA) may be a protective mechanism designed to scavenge ROIs generated by UVR exposure. New keratocytes that repopulate UVR-exposed rabbit corneas are positive for HA (Podskochy and Fagerholm, 1998, 2001). There is less keratocyte apoptosis 24 hours after 3 UVR doses than after a single UVR dose, and decreased apoptosis is associated with increased HA staining. This suggests HA-mediated 'resistance' to apoptosis following repeated UVR doses (Podskochy and Fagerholm, 2001). Insufficient antioxidant or HA protection may result in ROI-mediated lipid peroxidation of the cornea with the production of reactive aldehydes like malondialdehyde (Buddi et al., 2002), which may damage the corneal epithelium and contribute to the loss of keratocytes and degeneration of stromal collagen.

As another defense mechanism, 20–40% of the soluble protein in the cornea is aldehyde dehydrogenase (ALDH) (Buddi et al., 2002). ALDH is thought to have a protective effect on the cornea by directly absorbing UVR, removing cytotoxic aldehydes produced by UVR-induced lipid peroxidation, and generation of NADPH (Buddi et al., 2002; Manzer et al., 2003). Low levels of ALDH have been associated with an increased corneal sensitivity to UVR (Downes et al., 1994, 1997). Manzer demonstrated that UVR decreased the enzymatic activity of ALDH from 24 to 96 hours post-UVR in C57BL/6J/Ibg mice by altering its transcription, modulating its posttranscriptional levels, and introducing posttranslational modifications which affect its catalytic activity through structural changes and increased oligomerization. These conformational changes are suggestive of chaperone-like functions and may be beneficial to the cornea by preventing the aggregation of other proteins which would otherwise lead to precipitation of these proteins and corneal clouding (Manzer et al., 2003). Others have also demonstrated decreased levels of ALDH following UVR exposure (Downes et al., 1993, 1997). UVR-induced loss of ALDH may predispose corneal stromal proteins to aggregation and degeneration; the subsequent phagocytosis and removal of such proteins may contribute to the stromal depletion that we report.

UVR-induced apoptosis of corneal epithelial cells demonstrated in our study and by others could have resulted in the prolonged release of mediators that were responsible for the continued apoptosis and loss of keratocytes seen in this study. Continued UVR exposure may have prevented replacement of stromal keratocytes. Because one of the key functions of keratocytes is to synthesize and maintain stromal collagen,

loss of keratocytes would result in decreased collagen production of collagen in the face of normal collagen turnover, resulting in a net loss of stromal collagen and subsequent corneal thinning. UVR has also been shown to induce the production of matrix metalloproteinases (MMP) by the corneal epithelium in dogs and humans (Kozak et al., 2003).

UVR-induced MMPs are thought to contribute to the pathogenesis of photokeratitis in both species. UVR induction of MMPs in the current study may have contributed to the degradation and loss of the corneal stroma, and further investigation of the role of MMPs in the development of these lesions is warranted. Although there was no histologic evidence of inflammatory cell infiltrates in the eyes of mice described in the present study, other researchers have reported that phagocytes are occasionally apparent by transmission electron microscopy. These inapparent phagocytes may remove degenerate collagen and contribute to the stromal thinning.

Since cataracts occurred in both the unexposed and UVR-exposed mice, they were likely age-related and not UVR-induced; however, it is likely that UVR exacerbated and accelerated cataract formation. Since the corneal stroma is known to absorb UVB (Podskochy, 2004), the loss of corneal stroma might allow greater amounts of UVB to reach the lens. Cataracts in the present study were characterized by variable degrees of posterior cortical lens fiber degeneration and swelling without changes in the lens epithelial cells. In globes with severe inflammation, cataracts were characterized by global lens fiber degeneration and swelling with proliferation, metaplasia and posterior migration of the lens epithelial cells. Interestingly, in a report by Jose and Pitts, cataracts in male albino mice exposed to very high doses of UVR were characterized primarily by proliferation, metaplasia, and posterior migration of the lens epithelial cells with disruption of adjacent cortical fibers (Jose and Pitts, 1985). Thus, the UVR dose and degree of direct ocular damage may determine the character of the cataracts that develop.

This is the first report of age-related loss of keratocytes in a nonhuman species, and the first report of cataracts in 129 mice. Recognition of these changes is essential to interpretation of ocular lesions in aged mice. This is also the first report describing corneal degeneration induced by chronic UVR exposure in mice. This unique lesion was characterized primarily by loss of keratocytes and subsequent degeneration and loss of the corneal stroma and was often complicated by secondary inflammation or globe rupture. Our studies did not investigate the mechanism of UVR-induced keratocyte apoptosis. Although the exact pathogenesis of corneal degeneration remains unclear, the lesion is clearly UVR-induced.

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