A Novel Murine Model of Radiation Keratopathy

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Cancers of the head and neck, which include cancers of the oral cavity, larynx, pharynx, salivary glands, and nose/nasal passages, are a diverse group of cancers ranging from squamous cell carcinomas (91%), adenocarcinomas, melanomas and other nonspecified tumors (7%), and sarcomas (2%).1 Head and neck cancers account for approximately 3% of all malignancies in the United States2 and 4% of those in Europe.1 Worldwide, 550,000 new cases are diagnosed and 380,000 deaths occur every year3 with men having a four times higher incidence of head and neck cancers than women.1–3 Risk factors for head and neck cancers are primarily tobacco and alcohol use, followed by human papillomavirus (HPV) infection (for oropharyngeal cancer) and Epstein-Barr virus (EBV) infection (for nasopharyngeal cancer).4 Standard treatments for head and neck cancers, which depend on the tumor location, tumor stage, and patient’s age and overall health, include radiation therapy, surgery, chemotherapy, targeted therapy, or a combination of treatments.5 Advances in techniques attempt to minimize damage to surrounding healthy tissues when patients undergo radiation therapy. This includes selectively shielding key ocular structures, such as the lacrimal gland, fine focusing of the angle of the radiation field and using beam attenuators to allow the dose to be delivered uniformly.6 Nevertheless, complications from radiation therapy still occur and ocular tissues may be affected, but may not manifest for weeks, months, or even years later.7 Some of these adverse effects include chronic dry eye disease (DED) due to the apparent damaging effects of radiation on the lacrimal or meibomian glands,8 radiation retinopathy,9 or radiation keratopathy.10 Interestingly, even if the lacrimal gland is shielded during the irradiation procedure, radiation keratopathy still can develop, which means that the tear film alone is not a factor in the damage to the cornea.11 Radiation keratopathy can manifest clinically with several presentations. Acute radiation keratopathy can be transient and negligible and present with superficial punctate keratitis, stromal keratitis, stromal scarring, and corneal edema, and
typically is treated with topical anti-inflammatory and lubricating eye drops. Early signs are loss of corneal sensation that can result in neurotrophic corneal ulcers, corneal melting, and in severe cases, corneal perforation. Clinically, chronic corneal inflammation and concurrent decreased corneal sensation have been described with the development of limbal stem cell deficiency in patients with radiation keratopathy. Unfortunately, most patients are not referred to ophthalmologists for monitoring after receiving radiation therapy for cancers of the head and neck. Therefore, if damage to the cornea does occur, treatment may be started after the disease has advanced and the effects of radiation become irreversible and, thus, treatment options become more limited. This is partially due to the neurotrophic state of the cornea from corneal nerve loss and lack of symptoms in patients with early disease.

The specific mechanisms involved in the development of radiation keratopathy remain elusive. Corneal inflammation has been demonstrated during the early stages of radiation keratopathy. Further, loss of corneal sensation indicates that corneal nerves are being affected by radiation and/or inflammation, potentially due to apoptosis or necrosis. Given the current evidence, we hypothesized that there is a potential interplay between nerve damage and inflammation, resulting in the development and progression of this disease. To test our hypothesis, we developed a novel mouse model for radiation keratopathy without any direct insult to the cornea, mimicking human disease, allowing us to study the pathogenesis and specific mechanisms of this disease.

**Materials and Methods**

**Animals**

Six- to 8-week-old male BALB/c mice were obtained through Charles River Laboratories (Wilmington, MA, USA). Experiments were performed in concordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Harvard Medical Area Standing Committee on Animals and Schepens Institutional Animal Care and Use Committee. For each time point, at least three, but up to six mice were used.

**Irradiation Procedure**

The initial rationale was to mimic the human radiation treatment dose for head and neck cancers (66–74 Gy given as 2.0 Gy/fraction daily Monday through Friday for 7 weeks; Rad = 0.01 Gy) in two ways—70 Gy split 3 hours apart or 70 Gy split between 5 days once per day. In both instances even with shielding, all animals died. We next assessed if the standard radiation dose to generate bone marrow chimeras, a much lower dose than radiation to the head and neck, resulted in corneal changes. Thus, we used a similar protocol that allows sublethal irradiation, which affects the corneas without resulting in death of animals. This resulted in adaptation of the following lower radiation model, which allowed us to study the effect of radiation on the cornea and ocular surface.

Six- to 8-week-old adult male BALB/c mice were anesthetized with a mixture of ketamine (112.5 mg/kg) and xylazine (22.5 mg/kg) and placed upright in a 50 mL conical tube wrapped in 6.6 mm lead shielding (Electron Microscopy Sciences, Hatfield, PA, USA) with the heads exposed. The mice underwent sublethal irradiation from a Cesium-137 irradiator (Mark 1 Irradiator; JL Shepherd & Associates, San Fernando, CA, USA), 2 × 550 rad, 3 hours apart. Each radiation exposure lasted 2 minutes 39 seconds. Immediately afterwards, Gentecal gel (Alcon, Fort Worth, TX, USA) was added to their corneas, and they recovered in their cages. Animals were kept under viral antibody-free/specific pathogen–free (VAF/SPF) barrier conditions on standard laboratory chow and sterile water with antibiotic-containing water (sulfatrim) ad libitum (Fig. 1).

**Clinical Evaluation and Corneal Fluorescein Staining**

Irradiated mice were evaluated clinically by corneal fluorescein staining (CFS) at 1, 2, and 3 months, after which normal and irradiated corneas were excised respectively. Briefly, a FULL GLO Fluorescein Sodium Strip (Akorn, Lake Forest, IL, USA) was placed in 1 mL 1 × PBS (Life Technologies, Carlsbad, CA, USA) in a 5 mL syringe. The diluted fluorescein solution was placed in 1 mL 1 × PBS. Pictures were taken under cobalt blue light to document corneal fluorescein staining. Uveitis was assessed by eye redness or pain. Evidence of pain would be if the animals had a ruffled coat, extreme lethargy, or inability to eat/drink.

**Immunofluorescence Histochemistry**

Upon euthanizing the mice, whole corneas with surrounding bulbar conjunctiva were excised, and fixed in ice cold 100% acetone (Sigma-Aldrich Corp., St. Louis, MO, USA) for 15 minutes. Following three washings with 1 × PBS, tissues were blocked in 3% BSA (Sigma-Aldrich Corp.) containing 1% anti-CD16/CD32 Fc receptor (FcR) mAb (clone 2.4G2; Bio X Cell, West Lebanon, NH, USA) for 1 hour at room temperature (RT). Next, samples were incubated overnight at 4°C with FITC-conjugated CD45 (BioLegend, San Diego, CA, USA) or FITC anti-I-A/I-E (MHC class II; BD Biosciences, San Jose, CA, USA) to determine immune cell alterations and with Northern Lights.
557 (NL557)-conjugated anti-neuron-specific βIII tubulin antibody (R&D Systems, Minneapolis, MN, USA) to evaluate corneal nerve changes in central and peripheral corneas. Corneas then were washed with 1× PBS three times for 10 minutes each, and then were mounted on slides with Vectashield mounting media with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and underwent confocal microscopy with a Leica TCS SP5 confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA). Analysis was performed with Imaris (Bitplane, South Windsor, CT, USA) to calculate cell density of immune cells and with NeuronJ, a plugin for ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) to calculate nerve density.17

**RESULTS**

**Irradiation Results in Significant Corneal Fluorescein Staining**

The survival rate of irradiated mice was 100% with the body lead shielding at 3 months. Compared to previous studies,18,19 this method did not require generation of bone marrow chimeras to maintain survival. Mice demonstrated significant corneal fluorescein staining as well as hair loss around the eyes at 3 months after irradiation (Fig. 2). No mice showed symptoms of uveitis, such as redness or pain.

**Irradiation Results in Significant Corneal Nerve Loss Within Months**

Corneal nerve density (Figs. 3A–I) decreased in the central cornea from 135.29 ± 11.67 mm/mm² in naïve mice (Fig. 3A) to 92.84 ± 6.42 (P = 0.009) and 78.83 ± 8.17 (P = 0.005) mm/mm² at 2 (Fig. 3E) and 3 (Fig. 3G) months after irradiation, respectively. Peripheral corneal nerve density decreased from 122.01 ± 13.96 mm/mm² at 2 (Fig. 5E) and 3 (Fig. 5G) months after irradiation, respectively. In the peripheral cornea, MHC-II-positive immune cell densities increased from 103.79 ± 17.34 mm/mm² at 2 (Fig. 4B) to 159.42 ± 23.94 (P = 0.002; Fig. 4F) and 208.13 ± 10.15 (P < 0.001; Fig. 4H) mm/mm², respectively.

Further, MHC-II, a sign of antigen presenting cell activation and maturation, significantly increased in the central cornea from 11.07 ± 2.11 cells/mm² in naïve mice (Fig. 5A) to 70.58 ± 13.96 (P < 0.05) and 104.07 ± 20.69 (P < 0.05) cells/mm² at 2 (Fig. 5E) and 3 (Fig. 5G) months after irradiation, respectively. In the peripheral cornea, MHC-II immune cell densities increased from 33.90 ± 5.47 (Fig. 5B) to 174.78 ± 18.92 (P < 0.001; Fig. 5F) and 166.20 ± 26.98 (P < 0.01; Fig. 5H) cells/mm². Neither CD45⁺ nor MHC-II⁺ cell densities were significantly different between naïve mice and those at 1 month after irradiation (Figs. 4C, 4D, 5C, 5D).

**Irradiation Results in a Strong Inverse Correlation Between Corneal Nerves Loss and Increased Density of Corneal CD45⁺ Cells**

A strong inverse correlation was noted between decreased corneal nerves and increased CD45⁺ cells in the central cornea (r = −0.83; P < 0.001) using the Pearson correlation coefficient (Fig. 6).

**DISCUSSION**

Radiation doses to head and neck tumors are typically given between 50 and 74 Gray (2.0 Gy/fraction; daily Monday–Friday for 7 weeks),20 but to our knowledge no data exist on the dose received by surrounding normal tissue. Parsons et al.21 described a subset of 20 of 33 evaluated patients with extracranial head and neck tumors who received irradiation of the entire orbit and who had a higher risk of severe dry eye complications due to the location of tumor and exposure of the lacrimal gland. Specifically, the probability of complications, including edema, ulceration, bacterial infection, vascularization, opacification, and perforation, appeared to increase steeply at doses >30 Gray. Bhandare et al.22 emphasized the chronic problem of severe radiation-induced DED that can significantly affect quality of life due to compromised vision and severe pain caused by radiation injury to one or several of the ocular structures, including major and accessory lacrimal glands, conjunctival goblet cells, and meibomian glands. They suggested to reduce the total dose and fraction size to all components of the lacrimal system to reduce the incidence of delayed severe DED. Further, Fujishima et al.15 described a severe case of radiation keratopathy with temporary corneal stem cell dysfunction that developed after radiation therapy and resulted in ocular pain and loss of vision followed by partial conjunctivalization of the cornea.15 Thus, radiation keratopathy can result
in significant ocular surface disease, stem cell deficiency, vision loss, discomfort, and subsequent poor quality of life.

Kwok et al. analyzed the incidence and risk factors of severe radiation keratopathy. The incidence increased from 0% after doses <59 Gy to 100% after doses >70 Gy. They proposed that the threshold radiation dose of 45 to 60 Gy to the cornea is the most important factor in determining the outcome of radiation keratopathy and, therefore, an important prognostic factor. After this dose is reached, visual outcome is in serious jeopardy. They also refuted prior literature indicating that protecting only the lacrimal gland would prevent severe radiation keratopathy especially when the corneal dose was >70 Gy. In addition, they emphasized the impact of irradiation on the corneal nerves, resulting in epithelial defects and delaying corneal healing would healing. They noted the necessity of clinical trials on newer strategies, such as for limbal and conjunctival autographs, to treat ocular surfaces exposed to heavy irradiation and the hope that the treatment to the cornea be started as early as possible before damage occurs to the cornea.

Clinically, the management of radiation keratopathy is extremely difficult, due to lack of effective therapies and poor understanding of the disease. The goal of treating a patient with a head or neck cancer is to obtain the best possible outcome of eliminating the growth with as few side effects as possible. However, studies have demonstrated that while patients do not suffer from immediate side effects from the treatment, late stage ocular complications affect their quality of life and vision. Thus, additional mechanistic studies to understand the pathogenesis of this debilitating disease are needed. However, the lack of animal models of radiation keratopathy to date has hampered progress in this field.

We developed a murine model of irradiation keratopathy by utilizing a cesium irradiator. While the human-equivalent dose from the human treatment for head and neck tumors was lethal to mice, even our sublethal lower dose (11 vs. 70 Gy) demonstrated clinical signs of radiation keratopathy, including increased corneal fluorescein staining, indicating that the corneal epithelium is affected, although not as severely as those undergoing high radiation doses for head and neck.

**FIGURE 3.** Representative histologic images of central (A, C, E, G) and peripheral (B, D, F, H) corneas stained with neuron-specific anti-β III tubulin NL557-conjugated antibody in normal (A, B) mice, and at 1 (C, D), 2 (E, F), and 3 (G, H) months after irradiation. Graph results shown in (I). Scale bar: 100 μm. Mean ± SEM. **P < 0.01; ***P < 0.001.
Further, we demonstrate periorbital hair loss or alopecia due to the sensitivity of the hair follicle to radiation. Moreover, these mice did not suffer uveitis or symptoms of pain. Although we did not specifically look for any effects on the posterior segment in our model, studies have shown posterior injury following irradiation, including iris neovascularization, neovascular glaucoma, optic neuropathy, radiation maculopathy and radiation retinopathy.

Furthermore, Mütter et al. using a similar dose of full-body irradiation as in our model, demonstrated leukocyte infiltration in the retina. Neuropathy and progressive cognitive impairment are a well-recognized late complications after radiation therapy. Radiation neuropathy may develop and persist long after treatment. Yet, the mechanisms of radiation-induced neuronal injury are poorly understood. One hypotheses includes the role of glial cells, which are responsible for myelination in the central nervous system (CNS), which might be a prime target of radiation-induced demyelination and necrosis of the white matter of CNS. Histology has shown a significant decrease in nerve fiber density, especially affecting large nerve fibers after doses higher than 20 Gy. Another group performed electron microscopic analysis and demonstrated an increase in microtubule density and neurofilament accumulation in axons of irradiated nerves. These findings suggest radiation-induced hypoxia, resulting in axonal damage and subsequent nerve fiber loss as a mechanism of late radiation injury to the peripheral nerve. Other theories of vascular occlusion, free radical injury, direct damage to cellular DNA, and damage to the blood–brain barrier have been proposed to explain the pathophysiolo. Regardless, irradiation triggers a complex and multifactorial response involving a persistent increase of reactive oxygen species (ROS) and pro-inflammatory cytokines that actively participate in remodeling of the irradiated microenvironment. However, such changes have been suggested not to be irreversible. Acharya et al. reported that transplantation of human embryonic stem cells may rescue the radiation-induced cognitive impairment.
Neurogenic inflammation results from damage to the nociceptor sensory neurons, most widely studied with the application of the chemical capsaicin.44 This, then, results in sensation of pain and release of potent neuropeptides, notably calcitonin gene-related peptide (CGRP), substance P (SP), and neurokinin A from activated nerve terminals, which subsequently bind to their respective receptors neurokinin-1 receptor (NK-1R) located on neurons,45 leukocytes,46 and epithelial cells.47 In response to SP stimulation, macrophages, for example, can release inflammatory mediators, such as interleukins,48 chemokines,49 and growth factors.50 The resulting inflammation is due to vasodilation, microvasculature permeability, leukocyte infiltration, and mast cell degranulation.51 This process has been implicated in various human diseases of the nervous system,52 respiratory system,53 gastrointestinal tract,54 skin,55 and more relevant to this study, the ocular surface.55,56,57

Our current animal model showed that the protracted nature of radiation responses contributes to the inhibition of neuronal regeneration or persistent neuronal injury as shown

**FIGURE 5.** Representative histologic images of central (A, C, E, G) and peripheral (B, D, F, H) cornea stained with anti-MHC class II FITC-conjugated antibody in normal (A, B) mice, and at 1 (C, D), 2 (E, F), and 3 (G, H) months after irradiation. Graph results shown in (I). Scale bar: 100 µm. Mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

**FIGURE 6.** There was a severe significant reverse correlation (r = -0.83; p < 0.001) between CD45+ cell density and nerve density in the central cornea of irradiated mice (P < 0.001).
by decreased total nerve density in the central and peripheral corneas seen as early as 2 months after irradiation. Further studies are required to reveal the mechanisms of nerve loss and assess the validity of the existing hypothesis. Our study did not demonstrate changes in total nerve density or an increase in CD45+ or MHC-II+ cells at 1 month after irradiation. Nevertheless, it is possible that changes to individual trunks and branches of the nerves at that point and even earlier may have been present. Alternatively functional alterations may have preceded morphological nerve changes.

Our findings are novel in that the breakdown of immune homeostasis due to corneal nerve damage may be a key pathologic mechanism of radiation keratopathy. On a microscopic level, we showed that irradiation results in an infiltration of CD45+ bone marrow-derived cells and increased level of MHC-II+ cells, indicating infiltration and activation of leukocytes. In addition, there is a strong inverse correlation between nerve loss and increased leukocyte density in the central cornea at 2 and 3 months. MHC-II+ cells also had an inverse correlation with total nerve loss, but it was not significant (P = 0.09). Similarly, our mechanical denervation model of the cornea involving transecting the ciliary nerves using a lateral conjunctival approach results in complete loss of sensory nerves and increased expression of vascular adhesion molecules, leading to migration of bone marrow-derived immune cells to infiltrate the cornea as early as 24 hours postoperatively. Increase in proinflammatory cytokines and heme- and lymphangiogenesis follows, disrupting the homeostasis of this immune privileged site solely from loss of nerves to the cornea. Clinically, we also recently reported the homeostasis of this immune privileged site solely from loss of nerves to the cornea. 

In conclusion, we presented a novel and clinically relevant murine model of radiation keratopathy and demonstrated significant nerve loss and increase in leukocyte influx and their activation within months. This model will enable future investigations to understand the mechanisms and effects of radiation therapy on the eye, and as well as the studies of the effects of chronic nerve loss on the corneal immune homeostasis. Given the current results, future studies are necessary to assess the use of corneal shielding for patients undergoing radiation for head and neck tumors, and early monitoring by ophthalmologists for these patients may be warranted.

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