Optic nerve injury has been found to be dramatically reduced in a genetic mouse glaucoma model following exposure to sublethal, head-only irradiation. In this study, the same radiation treatment was used prior to experimental induction of elevated intraocular pressure (IOP) to determine if radiation is neuroprotective in another glaucoma model.

Methods. Episcleral vein injection of hypertonic saline was used to elevate IOP unilaterally in two groups of rats: (1) otherwise untreated and (2) radiation pretreated, n > 25/group. Intraocular pressure histories were collected for 5 weeks, when optic nerves were prepared and graded for injury. Statistical analyses were used to compare IOP history and nerve injury. The density of microglia and macrophages in two nerve head regions was determined by Iba1 immunolabeling.

Results. Mean and peak IOP elevations were not different between the two glaucoma model groups. Mean optic nerve injury grades were not different in glaucoma model optic nerves and were equivalent to approximately 35% of axons degenerating. Nerves selected for lower mean or peak IOP elevations did not differ in optic nerve injury. Similarly, nerves selected for lower injury grade did not differ in IOP exposure. By multiple regression modeling, nerve injury grade was most significantly associated with mean IOP (P < 0.002). There was no significant effect of radiation treatment. Iba1+ cell density was not altered by radiation treatment.

Conclusions. In contrast to previous observations in a mouse genetic glaucoma model, head-only irradiation offers the adult rat optic nerve no protection from optic nerve degeneration due to chronic, experimentally induced IOP elevation.

Keywords: glaucoma, radiation, optic nerve, animal models, intraocular pressure, axon degeneration
adapter molecule 1 (Iba1) positive (microglia + macrophage) cells in glaucoma model ONHs sections.

METHODS

Animals

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Oregon Health and Sciences University (OHSU) Institutional Animal Care and Use Committee. Animals were obtained in three cohorts from Charles River (Wilmington, MA, USA) and, within each cohort, randomly assigned to either the radiation treated group or the untreated group.

Glaucoma Model Induction

Unilateral IOP elevation was produced in 8-month-old, male Brown Norway rats \((n = 55)\) by a single episcleral vein injection of hypertonic saline. \(^{18}\) Rats were housed in low level, constant light to minimize circadian IOP fluctuations. \(^{19,20}\) Tissues were collected at 5 weeks following injection. Of these animals, 27 rats were otherwise untreated and eyes from these rats formed the untreated control and untreated glaucoma comparison eye groups for the radiation treatment group described below.

Radiation Treatment

The radiation group rats \((n = 28)\) received head-only radiation treatment 6 to 8 weeks prior to the unilateral induction of the glaucoma model. This protocol was designed to mimic the paradigm used in DBA/2j mice for “head-only” radiation exposure that was demonstrated to provide robust protection (see Fig. 2A in the publication). \(^{5}\) A Rad Source Rs 2000 X-ray Irradiator (Rad Source Technologies, Suwanee, GA, USA) was used to irradiate the animals. Rats were anesthetized by intraperitoneal injection of anesthetic (50 mg/mL ketamine, 5 mg/mL xylazine and 1 mg/mL acepromazine, 1 mL/kg). To expose the head while shielding the body, a box constructed from 3-mm thick lead sheeting was designed (Fig. 1). The dimensions of the box allowed it to fit inside a standard mouse cage for placement into the cage holder provided by the irradiator manufacturer. The heads were exposed to a total dose of 10 Gy, consisting of two 5 Gy treatments (dose rate 1.43 Gy/min) approximately 3 hours apart. The shielding reduced the body dose to 2% of the head dose and protected the rats from the lethal effect of whole body exposure to this dosage. \(^{21}\) The dosages to the eye and body were originally determined using a rat cadaver by the OHSU Research Radiation Safety Officer with a Radcal Accu-Dose model 2086 control unit coupled to a model 9660A Ion Chamber converter connected to a model 10X6-0.63 ionization chamber. Eyes from these animals formed the radiation control and radiation glaucoma eye groups.

IOP Measurement

Intraocular pressure was measured in unanesthetized rats prior to glaucoma model induction and then three times weekly throughout the experimental duration using a Tonolab (Colonial Medical Supply, Franconia, NH, USA) as described. \(^{22}\) Each IOP measurement was determined as the average of 10 individual instrument readings. Mean IOP was determined as the cumulative area under the curve of IOP to days over the 5-week experimental period divided by the number of days between episcleral vein injection and the last IOP measure-
Glaucoma Model Radiation Neuroprotection Failure

Table 1. Tonolab IOP History Comparison

<table>
<thead>
<tr>
<th></th>
<th>Mean IOP ± SD</th>
<th>Peak IOP ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glaucoma model</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glaucoma alone</td>
<td>27.3 ± 6.2</td>
<td>47.5 ± 11.4</td>
</tr>
<tr>
<td>Radiation + glaucoma</td>
<td>28.2 ± 6.9</td>
<td>43.9 ± 10.6</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>21.6 ± 1.5</td>
<td>28.7 ± 2.8</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>21.2 ± 2.3</td>
<td>27.1 ± 3.2</td>
</tr>
</tbody>
</table>

* For both mean and peak IOP values, glaucoma model eye IOPs were significantly elevated compared to their respective control group eyes (P < 0.01). However, there were no significant IOP differences within glaucoma model or within control eye groups (two-way ANOVA), demonstrating that radiation treatment does not have a significant effect on IOP in either control or glaucoma model eyes.

Table 2. Mean Injury Grade by Experimental Group

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Injury Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glaucoma model</td>
<td></td>
</tr>
<tr>
<td>Glaucoma alone</td>
<td>3.55 ± 0.32</td>
</tr>
<tr>
<td>Radiation + glaucoma</td>
<td>3.65 ± 0.30</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

Iba1 Immunohistochemistry and Cell Density Determination

Eyes were perfusion fixed with paraformaldehyde, myelinated optic nerves removed at 1 mm behind the globe and sectioned as previously described. Following deparaffinization and rehydration in PBS, sections were blocked in 5% goat serum in PBS and incubated overnight at 4°C with Iba1 antibodies (0.1 μg/mL in PBS + 1% BSA; Wako Chemicals USA, Richmond, VA, USA), washed in PBS, then incubated for 30 minutes at room temperature in 0.2% Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Grand Island, NY, USA) in blocking solution, washed in PBS, and the nuclei stained with DAPI Prolong Gold antifade mounting media (Life Technologies). Optic nerve heads were photographed using an Olympus Fluorescence microscope equipped with a DP71 Digital Camera and associated software (Olympus America, Center Valley, PA, USA). Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA) tools were used to delineate the anterior (Bruch’s membrane to 150 μm posterior) and transition (200 to 350 μm) regions of the ONH. Iba1 positive cells (Iba1 labeled areas surrounding a DAPI labeled nucleus) were counted within these regions and region areas determined to calculate cell densities. N ≥ 5/glaucoma model group.

Optic Nerve Injury Grading

Retrobulbar optic nerves were postfixed, embedded in Spurr’s resin, and cross-sectioned for light microscopic evaluation. Nerve cross sections, collected in the initial myelinated portion of the nerve, were stained with toluidine blue and graded for injury on a scale from 1 (normal nerve) to 5 (extensive degeneration affecting the entire nerve area). Each nerve was graded by three masked independent observers. The mean of these values was defined as the injury grade for that nerve. Previous studies using transmission electron microscopy have demonstrated that each unit increase in the injury grade is equivalent to degeneration affecting approximately 12% to 15% of the optic nerve axons.

Statistical Analysis

Statistical analyses included multiple regression modeling of the contribution of glaucoma model induction, radiation treatment, and IOP parameters: mean, peak, and SD IOP to nerve injury grade, http://www.R-project.org, plus two-way ANOVA and linear regression using GraphPad Prism version 6.0.

RESULTS

IOP Elevation Responses in Glaucoma Model Eyes Are Unaffected by Radiation Treatment

The pattern of IOP histories (Supplementary Fig. S1), the distribution of mean and peak IOP (Supplementary Figs. S2, S3) and average of mean and peak IOPs (Table 1) were statistically compared between groups. Compared to their respective control eyes, mean and peak IOP values in both glaucoma model eye groups were significantly elevated (P < 0.01). Although within group IOP values were quite variable for glaucoma model eyes, average mean and peak IOP elevations were approximately 7 mm Hg and 18 mm Hg above control (fellow) eye mean, respectively. Radiation treatment did not have a significant effect on either control or glaucoma model IOP values for any of these comparisons (two-way ANOVA).

Optic Nerve Injury in Glaucoma Model Eyes Treated With Radiation

Mean nerve injury grades are shown in Table 2. There was no significant effect of radiation exposure on the mean injury grade. For untreated glaucoma model eyes, the mean injury grade was 3.55, while it was 3.65 for the radiation treated group, both significantly greater than respective control eye group (P < 0.001). A grade of 3.5 in our grading scale is approximately equivalent to 35% of optic nerve axons in the process of degeneration. Figure 2 illustrates the distribution of nerve injury grades within glaucoma model groups, showing that in both groups there was a wide range of injury grades, including many nerves with near maximal injury as well as several with no injury. Figure 3 illustrates the appearance of nerves of approximately average injury grade for both groups. As the inserts show, the lesions have a similar appearance, with a mixture of degenerating and normal appearing axons scattered throughout the lesion areas. Table 2 and Supplementary Figure S4 demonstrate that radiation exposure did not have a discernable effect on the appearance of control optic nerves.

Relationship Between IOP Exposure History and Optic Nerve Injury

Intraocular pressure parameters mean, peak, and SD IOP were all linearly correlated with injury grade. For the radiation and untreated groups, respectively, the r² values for mean IOP to
injury grade were 0.68 and 0.62. Values for SD IOP were 0.79 and 0.68, and those for peak IOP were 0.84 and 0.72. There were no significant differences by treatment group between these correlation lines. Next, using multiple regression modeling, we examined the variables of injection status (glaucoma model or fellow eye), treatment (radiation or untreated), and the IOP variables mean, SD, and peak as predictors of optic nerve injury grade. Mean IOP was found to be the most significant predictor of optic nerve injury grade ($P < 0.002$), while SD IOP and injection status were also significantly associated (both, $P < 0.02$). In all of these analyses, radiation treatment failed to have a significant effect on optic nerve injury due to elevated IOP exposure.

This study was designed with the expectation of striking optic nerve protection following radiation exposure. However, in order to determine if there was a subtle protective effect of treatment at lower IOP exposure levels, we also examined nerve injury by comparable mean or peak IOP range.

For the mean IOP analysis (Fig. 4), glaucoma model eye nerves are divided into lower ($<25 \text{ mm Hg}$) and higher ($>25 \text{ mm Hg}$) mean IOP ranges ($n \geq 10$). All glaucoma model IOP groups had significantly more injury than the comparable control nerve group ($P < 0.05$), and the differences in injury in glaucoma model nerves between the lower and higher IOP ranges were also significant ($P < 0.05$). For both radiation-treated and untreated eyes, the effect of mean IOP level was highly significant ($P < 0.0001$). However, no significant effect of radiation treatment occurred at either the lower or the higher mean IOP range (two-way ANOVA).

Similarly for the peak IOP analysis (Fig. 5), glaucoma model eye nerves were divided into lower ($<45 \text{ mm Hg}$) and higher ($>45 \text{ mm Hg}$) peak IOP ranges ($n \geq 11$). Again, all glaucoma model range had significantly more injury than the respective controls, and there were significant differences in injury between the lower and higher peak IOP ranges ($P < 0.05$, two-way ANOVA). As in the previous analysis, the effect of peak IOP level was highly significant ($P < 0.0001$), but there was no significant effect of radiation treatment at either peak IOP range.

Finally, we compared the IOP history of nerves with injury grades less than 4.0 (see Fig. 2 for injury distribution). In this comparison, there was no significant difference between mean and peak IOPs for these nerves, although for radiation exposure.
treatment nerves, the average peak IOP was slightly lower ($P = 0.11$ ANOVA).

To summarize, in these last three analyses, we could find no significant effect of radiation treatment on optic nerve injury at the lower IOP exposure levels.

**ONH Microglia and Macrophages Cells**

Microglia, as well as macrophages recruited from circulating monocytes, can be labeled in neural tissues utilizing the antibody Iba1. In DBA/2J mice, there is growing evidence that radiation exposure hinders monocyte entry into the ONH and that this results in axonal protection. We used IBA1 immunohistochemistry to determine if radiation affected the density of these phagocytic cells in fellow or glaucoma model ONH. Figure 6 demonstrates that both in the anterior ONH (just under Bruch’s membrane) and the transition region (where myelination begins) there was no significant difference between treatment groups in the density of IBA1 labeled cells in fellow ONH, ONH with mean IOP $< 25$ mm Hg, or ONH with IOP $> 25$ mm Hg. However, for both treatments and in both regions, mean IOP $> 25$ resulted in a significant increase in IBA1+ cell density compared to fellow and to IOP $< 25$ groups ($P < 0.005$). Additionally, comparison of densities in the two ONH regions (Figs. 6A, 6B) demonstrates that these phagocytic cells are approximately twice as dense in the transition region as in the anterior ONH. This difference is observed in fellow ONH and persists as the ONH responds to radiation and untreated groups (two-way ANOVA).

**Other Treatment-Related Effects**

After radiation treatment, rats experienced an initial weight loss of approximately 5% over a period of several days, followed by a steady recovery over the next weeks. No ocular or other treatment-related effect was observed in the animals.

**DISCUSSION**

This experiment indicates that prior exposure to high dose, head-only irradiation has no detectable effect on either the subsequent development of elevated IOP or optic nerve injury in a commonly used, experimentally induced glaucoma model in rats. This is in sharp contrast to observations in a popular genetic glaucoma model in DBA/2J mice using a comparable exposure paradigm. In these mice, head-only irradiation treatment resulted in preservation of over 80% of optic nerves with little or no injury, at an age when over 60% of nerves in eyes from untreated mice were severely injured. At the same time, radiation treatment was found to have no effect on the age-related IOP elevation that spontaneously occurs in this mutant mouse.

There is no obvious explanation for the difference in response to radiation between these two glaucoma models. In both, elevated IOP is produced by obstruction of the outflow pathways. In our study, experimentally induced outflow obstruction was produced in rats by vascular sclerosis with hypertonic saline. In the DBA/2J mice, spontaneous mutations in both the Gpmb and Tyrp1 genes result in pigment dispersion and iris atrophy, producing a condition similar to pigmentary glaucoma in humans.

In both models, IOP elevation patterns appear unaffected by irradiation. It is important to consider, however, that accurate IOP histories are difficult to obtain in any glaucoma model and anesthesia can have a dramatic lowering effect on measured IOP in rodents adding to this difficulty. In our study, rats were unanesthetized and IOP measurements were made three times per week over the 5-week period of IOP elevation. In the DBA/2J study, IOP was measured once by cannulation in anesthetized animals in all groups, although not in every mouse. Mice with elevated IOP (above 21 mm Hg) were found in both groups at every age between 9 and 12.5 months, and there was no obvious difference in IOP profile between the DBA/2J groups. While neither method provides a complete IOP history on each animal, in our study, awake IOP in every eye was measured repeatedly over the experimental time course.

The age of the animals at the time of radiation treatment differed in the two experiments. In this study, adult rats aged 6 to 7 months old were used. In the DBA/2J study, radiation exposure occurred at 2 months of age, 4 months prior to the onset of detectible IOP elevation or nerve injury in this strain. So there is the potential that the observed radiation neuroprotection is dependent on exposure early in the lifespan of the animal.

Obviously, there is a species difference between the two models. In addition, the DBA/2J strain of inbred mice develops other pathologies besides glaucoma. Young DBA/2J mice are susceptible to audiogenic seizures. Older mice develop progressive hearing loss and abnormal calcification of the pericardium and other tissues. In addition, they are deficient in complement component 5 (C5) and lack CD94 (Kird1), a receptor that is primarily involved in modulating natural killer cell cytotoxicity (Jackson labs http://jaxmice.jax.org/strain/000671.html). In DBA/2J mice, C5 sufficiency ameliorates, while CD94 sufficiency does not affect, the development of glaucoma.

There are also strain characteristics of Brown Norway rats (http://www.harlan.com/products_and_services/research_models_and_services/research_models/brown_norway_inbred_rat.html), but in general, these animals are resistant to spontaneous disease during the first years of their lifespan.

Spontaneous optic nerve axon loss with aging appears to proceed differently between the two species. In DBA/2J mice, optic nerve damage to up to 5% of axons is morphologically indistinguishable from early glaucoma in this strain and from age-matched nerves from mice of various strains that do not develop glaucoma. In contrast, for Brown Norway rats, we have demonstrated that there is no detectible axon loss between 5
and 24 months of age. At 32 months of age, when axon loss is significant in these rats, 87% of axons still remain intact.38

In microarray analysis studies of ONHs with early glaucomatous injury, immune responses appear more dominant in the DBA/2J mouse strain compared to the Brown Norway rats.37,39 In the molecularly defined stage 2 DBA/2J nerve heads (early glaucoma, <5% of axons degenerating), the DAVID (http://david.abcc.ncifcrf.gov/) gene ontology category of immune response, with closely related categories of leukocyte activation and chemotaxis appear predominant compared to cell proliferation. In contrast, in our Brown Norway analysis of early injury (<15% degenerating axons), the categories of cell proliferation and cytoskeleton were more significantly regulated than immune response. Therefore, it is possible that there are critical differences in immunological susceptibility between the two models.

Howell et al.5 have identified the inhibition of monocyte entry into the ONH via transendothelial migration as a key

Figure 6. IBA1 immunostaining was used to determine the density of microglia and macrophages in two regions of the ONH: (A) the anterior region (within 150 µm of Bruch’s membrane) and (B) the transition region. Mean cell densities were determined for each treatment and ONH group (Fellows, and the two glaucoma model mean IOP groups illustrated in Fig. 4). There were no significant differences due to radiation treatment. Densities in both regions for mean IOP > 25 groups were significantly higher than respective fellow or mean IOP < 25 groups (P < 0.005). Note that the higher density of IBA1+ cells in the transition region, compared to the anterior ONH, is maintained as glaucomatous injury progresses. Representative images of IBA1 (red) and DAPI (blue) labeled ONH sections are shown in (C) (fellow) and (D) (glaucoma model). In the images, the nerves are oriented with the retina up, transition region toward the bottom and superior to the right. The vertical bar in C (150 µm) illustrates the extent of the anterior ONH.
component of radiation neuroprotection in DBA/2J mice. While still under investigation in our experimental glaucoma model in rats, we have yet to find convincing evidence of monocyte entry into the ONH. However, we have found in this study that radiation treatment did not affect the density of Ihba1-ONH cells (microglia plus monocyte-derived macrophages).

This difference in response to radiation has important implications for all glaucoma models. At least from a population standpoint, genetic models have the advantage of a relatively gradual onset of glaucomatous pathology, and in this respect, may mimic the time course of human glaucoma. However, linking the specific genetic mutations in these models to the actual mechanisms and affected genes in human glaucoma has been challenging. Elevated IOP is probably the best recognized risk factor for the development of glaucoma, and lowering IOP is an effective therapy to limit glaucoma progression. Most experimentally induced glaucoma models, including ours, rely on the production of elevated IOP to induce glaucoma-like pathology over a period of days to months. The assumption is that the biological processes that occur in these models will be similar to those that occur in human glaucoma. However, in using any model it is important to avoid excessively high IOPs that may induce pathological mechanisms that are not principal components of human glaucoma. To maximize the potential that promising glaucoma therapies will lead to effective human treatment, a combination of both approaches is necessary, utilizing multiple well-characterized and executed animal models.

In the case of radiation neuroprotection, we suggest that further investigation in other experimental and genetic glaucoma models is warranted, based on the dramatic difference in the effectiveness of radiation in protecting against glaucomatous injury observed in these two well-established models. While safer and effective options to the clinical use of radiation exist to reduce glaucomatous progression, defining the biological basis of the difference in response in these two animal models is likely to reveal important information about the pathogenic mechanisms of glaucomatous nerve injury and, potentially, offer relevant insights to improve therapeutic treatment of human glaucoma.

Acknowledgments
Supported by National Institutes of Health Grants EY016866, EY010145, and 5P30EYO10572 (Bethesda, MA, USA); Medical Research Foundation of Oregon; and an unrestricted grant from Research to Prevent Blindness, Inc., New York, New York, United States.

Disclosure: E.C. Johnson, None; W.O. Cepurna, None; D. Choi, None; T.E. Choe, None; J.C. Morrison, None

References


