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Mrad9 and Atm Haploinsufficiency Enhance Spontaneous and X-Ray-Induced Cataractogenesis in Mice

Norman J. Kleiman,[a,b] Janice David,[b] Carl D. Elliston,[c] Kevin M. Hopkins,[c] Lubomir B. Smilenov,[c] David J. Brenner,[a,c] Basil V. Worgul,[b,c,1] Eric J. Hall[c] and Howard B. Lieberman[a,c,2]

[a] *Department of Environmental Health Sciences, Columbia University, Mailman School of Public Health, New York, New York 10032;* [b] *Eye Radiation and Environmental Research Laboratory, Columbia University, College of Physicians and Surgeons, New York, New York 10032; and* [c] *Center for Radiological Research, Columbia University, College of Physicians and Surgeons, New York, New York 10032*

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***Rad9* and *Atm* regulate multiple cellular responses to DNA damage, including cell cycle checkpoints, DNA repair and apoptosis. However, the impact of dual heterozygosity for *Atm* and *Rad9* is unknown. Using 50 cGy of X rays as an environmental insult and cataractogenesis as an end point, this study examined the effect of heterozygosity for one or both genes in mice. Posterior subcapsular cataracts, characteristic of radiation exposure, developed earlier in X-irradiated double heterozygotes than in single heterozygotes, which were more prone to cataractogenesis than wild-type controls. Cataract onset time and progression in single or double heterozygotes were accelerated even in unirradiated eyes. These findings indicate that the cataractogenic effect of combined heterozygosity is greater than for each gene alone and are the first to demonstrate the impact of multiple haploinsufficiency on radiation effects in an intact mammal. These observations may help explain observed interindividual differential radiosensitivity in human populations and have important implications for those undergoing radiotherapy or exposed to elevated levels of cosmic radiation, such as the astronaut corps. These findings demonstrate that *Mrad9* and *Atm* are important determinants of lens opacification and, given the roles of Atm and Rad9 in maintaining genomic stability, are consistent with a genotoxic basis for radiation cataractogenesis.**

INTRODUCTION

The cellular response to DNA damage caused by normal metabolic processes or exposure to exogenous insults is critical for determining whether deleterious effects will occur. It is well established that complete loss of gene function (homozygous defects) for cellular components involved in DNA damage recognition, DNA repair or cell cycle checkpoint control might lead to genomic instability, mutation, carcinogenesis or lethality (*1–3*). There is also compelling evidence that heterozygous gene defects that lead to haploinsufficiency can have negative biological effects [for reviews, see refs. (*4, 5*)]. For example, haploinsufficiency for *pTEN, Blm, Rb, Atm* or *Plk4* enhances carcinogen-induced tumor formation (*6–11*). In mice, heterozygosity for *Atm* causes early onset of cataracts induced by X rays or heavy ions (*12, 13*). In humans, *ATM* heterozygosity might predispose individuals to deleterious late effects of radiotherapy (*14*). Women heterozygous for *BRCA1* or *BRCA2* might have increased risk for breast cancer after diagnostic chest X rays (*15*), and individuals with heterozygous defects in the melancortin 4 receptor might be abnormally susceptible to obesity (*16*). In contrast to these studies of single-gene heterozygous mutations, no studies have examined the specific pathological effects of multiple gene heterozygosity in complex mammalian systems and, in particular, for genes regulating the cellular response to DNA damage.

Atm and *Mrad9* play critical roles in the cellular response to DNA damage (*17, 18*) and are important for maintaining genomic integrity at least in part by regulating cell cycle checkpoints induced by radiation. Furthermore, Rad9 is phosphorylated by the ATM kinase activity after cells are irradiated (*19*). Recently, mouse embryo fibroblasts haploinsufficient for both *Atm* and *Mrad9* were shown to be more sensitive to radiation-induced transformation than either wild-type controls or those heterozygous for just one gene (*20*). On the basis of this and related observations, our group and others have suggested that differential radiosensitivity of some individuals might be explained by multiple heterozygosity for genes involved in cell cycle checkpoint control, DNA damage recognition, or DNA repair (*21–23*).

In contrast to these *in vitro* investigations, studies with

[1] During the final stages of this study and the preparation of this article, Dr. Basil V. Worgul passed away unexpectedly. We miss him as a good friend, long-time collaborator and valued colleague and dedicate this paper to his memory and to the continuation of the research he held so dear.

[2] Address for correspondence: Center for Radiological Research, Columbia University College of Physicians and Surgeons: 630 W. 168th St., New York, NY 10032; e-mail: lieberman@cancercenter.columbia.edu.

intact animals offer the opportunity to examine the effect of single- or multiple-gene heterozygosity on specific pathologies. In terms of radiation exposure, animal studies offer the opportunity to model the genetic basis of human radiosensitivity and subsequent health outcomes. One such animal model uses development of radiation cataracts in rodents as a way to examine radiosensitivity (*24–26*). Cataractogenesis as an experimental end point allows the study of radiation effects in a late-responding normal tissue (*12*) and might provide additional insights into the large and growing worldwide societal health issues concerning cataract-related blindness (*27, 28*).

It is well established that exposure of the lens to ionizing radiation causes cataracts (*30, 31*). The clinico-histopathological changes accompanying radiation cataractogenesis are similar in all vertebrate lenses, and at least four readily distinguishable stages are identifiable by slit-lamp biomicroscopy. They form a basis for the classification of cataract severity (*31*). Initial presentation of a radiation cataract usually involves a posterior superficial opacification of the lens, termed a posterior subcapsular cataract. While other environmental insults might also result in formation of posterior subcapsular cataracts, radiation-induced cataracts are generally associated with this form of lens opacification (*32, 33*). Its development is directly related to radiation dose and also depends on the rate at which damaged lens epithelial cells divide, aberrantly differentiate and migrate to the posterior pole (*34*). Although the precise inducing mechanism is not known, it is generally accepted that genomic damage results in altered cell division, transcription and/or abnormal lens fiber cell differentiation that leads to cataract development (*35, 36*).

Work in the current study addresses whether mice haploinsufficient for two genes involved in DNA damage repair and/or cell cycle checkpoint control, specifically *Atm* and *Mrad9*, might be more susceptible to the cataractogenic effects of ionizing radiation than $Mrad9^{+/+}$ $Atm^{+/+}$ animals or those haploinsufficient for only one of these genes. The investigations reveal that $Atm^{+/-}$ or $Mrad9^{+/-}$ animals develop spontaneous as well as radiation-induced cataracts with earlier onset and greater severity than wild-type controls, which lends support for the role of genetics in cataract development. Of note, mice heterozygous for both genes demonstrate an even greater predisposition to cataract formation.

These findings provide further insights into a genetic basis for radiation sensitivity in a highly organized and differentiated mammalian tissue in a mouse model that has great relevance to the human response to radiation exposure. The results of this study have important implications for radiosensitive subsets of the human population, especially in the context of individuals exposed to radiation such as the astronaut corps or patients undergoing radiotherapy. These findings are also likely to be useful for the development of guidelines for occupational and therapeutic radiation exposure, and in particular national radiation risk policy, by taking into account individual genetic variability.

MATERIALS AND METHODS

Mice

Atm and *Mrad9* heterozygous mice, as well as corresponding wild-type control animals, were constructed and maintained as described previously (*37, 38*). Heterozygous animals are haploinsufficient for their respective encoded proteins (*10, 38*). Mice heterozygous for both *Mrad9* and *Atm* were made by mating animals containing a single heterozygous gene defect. Expected frequencies of progeny with the genotypes $Mrad9^{+/+}$ $Atm^{+/+}$, $Mrad9^{+/-}$ $Atm^{+/-}$, $Mrad9^{+/+}$ $Atm^{+/-}$ and $Mrad9^{+/-}$ $Atm^{+/+}$ were obtained, indicating the lack of selective advantage with respect to the heterozygous status of these two genes. $Mrad9^{+/+}$ $Atm^{+/+}$ mice weighed more (approximately 10–20%) on average than their corresponding littermates. Mouse genotypes were determined using a PCR-based assay. Genomic DNA was isolated from tail fragments using DirectPCR (Tail) lysis reagent (Viagen Biotech Inc., Los Angeles, CA). To detect *Mrad9* heterozygotes, primers 5′-CCGGGTGAACCAATAAGGAA-3′ and 5′-AAGGAAGCAGGCATAGGCAG-3′ were used. PCR conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30s, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 2 min. To assess the status of the *Atm* gene in mice, primers M1: 5′-TGTAGATAGGTCAGCATTGGAT-3′; M10: 5′-GTCAAAATTCGATCTGCTGCT-3′; Neo1: 5′-GCTCTTTACTGAAGGCTCTTTAC-3′ were used. PCR conditions were 2 min at 95°C, followed by 30 cycles of 95°C for 1 min, 60°C for 2 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. PCR products were resolved in 2% agarose gels. *Mrad9* wild-type and mutant genotypes were identified as 672-bp and 482-bp bands, respectively. *Atm* wild-type and mutant genotypes were identified as 125-bp and 400-bp bands, respectively. One hundred and thirteen mice survived to the end of the study. Of these, 57 mice were irradiated in the right eye and 56 were unirradiated control animals. Both irradiated and unirradiated groups were composed of approximately equal numbers of each of the four genotypes.

Mice were cared for in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals. Experimental protocols, animal handling and animal care were approved by the Columbia University Institutional Animal Care and Use Committee (IACUC).

Irradiation

The right eyes of 28-day-old mice of each genotype were exposed to 50 cGy of 250 kVp X rays, as described previously (*12*). The shielded and thus unirradiated left eyes from X-ray-exposed animals, as well as unirradiated animals of each genotype, were used as controls. Since no difference was noted between data obtained from these two different types of controls, results from these two groups were combined.

Cataract Detection and Data Analyses

Mice were examined at a minimum of every 2–4 weeks for about 55 weeks postirradiation. Investigators who scored mice for cataract development were blinded with respect to irradiation status and genotype of the animals until the completion of the study. Lens opacification was monitored by slit-lamp biomicroscopy and analyzed using a modified version of the Merriam-Focht scoring criteria (*31, 39*). This 0–4 scoring range depends on the fact that radiation cataracts develop in a characteristically sequential fashion with the earliest changes, at stage 0.5, consisting of less than four dots, vacuoles or diffuse opacities around the central suture in the posterior subcapsular region, and progressing over time to stage 4, when there is a complete opacification of the lens. A score of 2.0 or higher is vision impairing. Since animals were not always examined every week, values for weeks where no assessment was made were filled in using the score obtained at the last observation.

Kaplan-Meier estimation (*40*) was used to obtain non-parametric max-

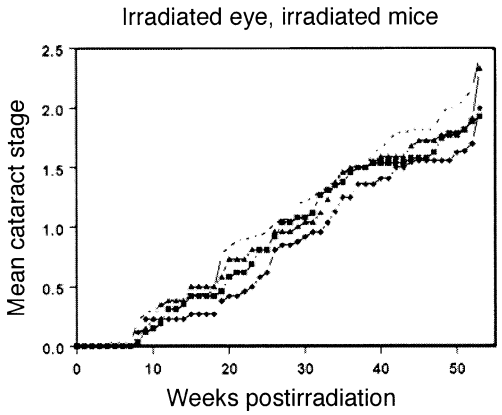

FIG. 1. Mean cataract stage as a function of time in the irradiated eyes of mice exposed to 50 cGy of X rays. Genotypes of mice: $Atm^{+/+}$ $Mrad9^{+/+}$ (diamonds); $Atm^{+/+}$ $Mrad9^{+/-}$ (squares); $Atm^{+/-}$ $Mrad9^{+/+}$ (triangles); $Atm^{+/-}$ $Mrad9^{+/-}$ (circles).

imum-likelihood estimates of the stage-specific cataract prevalence, or the probability that an animal will develop a cataract of a particular stage as a function of time after irradiation. Standard error of estimated prevalence was calculated using Greenwood's formula (*41*).

RESULTS

The right eyes of 28-day-old wild-type, *Atm* and/or *Mrad9* heterozygous mice were exposed to 50 cGy of X rays, and both the unirradiated and the exposed eyes were examined periodically for up to 55 weeks to determine the time of onset and degree of radiation-induced lens opacification. Figure 1 indicates that at any given week postirradiation, the mean cataract stage of double heterozygotes is higher than that of single heterozygotes and that, essentially at any time postirradiation, all mutant mice have a more severe mean cataract stage than corresponding wild-type controls. Thus acceleration of radiation-induced opacification is observed in all mice with mutant genotypes compared to wild-type control animals.

Additional information can be obtained when cataract prevalence is compared at each stage of development across genotypes (Fig. 2). At earlier stages of opacification, 0.5 and 1.0 (Fig. 2A, B), the rate of cataract progression was significantly greater in single or double heterozygotes than in wild-type control animals. In addition, relative to the single heterozygotes, irradiated double heterozygotes have a higher cataract prevalence at earlier times. When the prevalence of stage 2.0 cataract is compared in mice spanning the four genotypes, it is clear that double heterozygotes are the most radiosensitive followed by single heterozygotes and then wild-type animals (Fig. 2C). Thus, among all irradiated mice, those carrying heterozygous mutations for both *Atm* and *Rad9* developed each stage of lens opacification at a higher rate than single heterozygotes or wild-type control animals. Furthermore, the data suggest a slightly higher rate of cataract progression in the *Mrad9* heterozygotes than in the *Atm* heterozygotes. This is supported by data for stage 2.5 cataracts (Fig. 2D). Only the double heterozygous or single heterozygous *Mrad9* animals reach this vision-impairing, advanced stage of opacification, requiring almost 50 weeks, essentially half the life span of the animals. In addition, the double heterozygote exhibited a 5-week earlier onset of stage 2.5 cataracts relative to the *Mrad9* single heterozygote.

Compared to irradiated eyes, unirradiated eyes from mice of all genotypes developed each stage of lens opacification approximately 9 weeks later and at a slower rate (Figs. 3, 4). Unirradiated wild-type control animal eyes exhibited a significantly later onset for each stage of cataract compared to single or double heterozygous mutants, and no wild-type animals progressed to stage 2.0 by 55 weeks (Figs. 3C, 4). Unlike irradiated animals, however, no clear difference between single heterozygotes or $Atm^{+/-}$ $Mrad9^{+/-}$ double mutants was noted for either time of onset or rate of cataract progression (Fig. 4).

A comparison of mean cataract stage between unirradiated control and irradiated wild-type or double heterozygous mice is shown in Fig. 5. In exposed eyes, cataract onset begins at about 8 weeks in animals of both genotypes, although the double heterozygous mice ultimately reach a higher average cataract stage by 55 weeks postirradiation. In unexposed double heterozygous animals, however, it is interesting to note that the onset of cataracts begins earlier, at 19 weeks, compared to 25 weeks in wild-type animals. Cataracts in unirradiated mice of either genotype do not progress past stage 1.5 by 55 weeks.

Cataracts of every grade in mice of the same genotype appeared earlier in irradiated than unirradiated eyes. A characteristic latent period between radiation exposure time and cataract formation was observed. Mice never exposed to radiation developed cataracts with approximately the same severity and time of onset as the unirradiated control eyes of the irradiated animals, indicating that proximity to the radiation field had no effect on lens opacification and that shielding was effective (data not shown).

DISCUSSION

This study indicates that heterozygosity for *Mrad9* or *Atm*, two genes critical for multiple cellular responses to DNA damage, predisposes mice to the development of spontaneous as well as X-ray-induced lens opacification. Furthermore, mice heterozygous for both *Mrad9* and *Atm*, relative to single heterozygotes or wild-type control animals, were more sensitive to the cataractogenic effects of X radiation as demonstrated by a generally greater mean cataract stage, an earlier onset time and higher prevalence of most grades of cataract, and a greater likelihood of reaching a more severe cataract stage by the end of the study.

These results are somewhat surprising since Rad9 and Atm participate in similar biological functions, including cell cycle checkpoint control, apoptosis and the promotion of resistance to ionizing radiation [for reviews, see refs. (*17,*

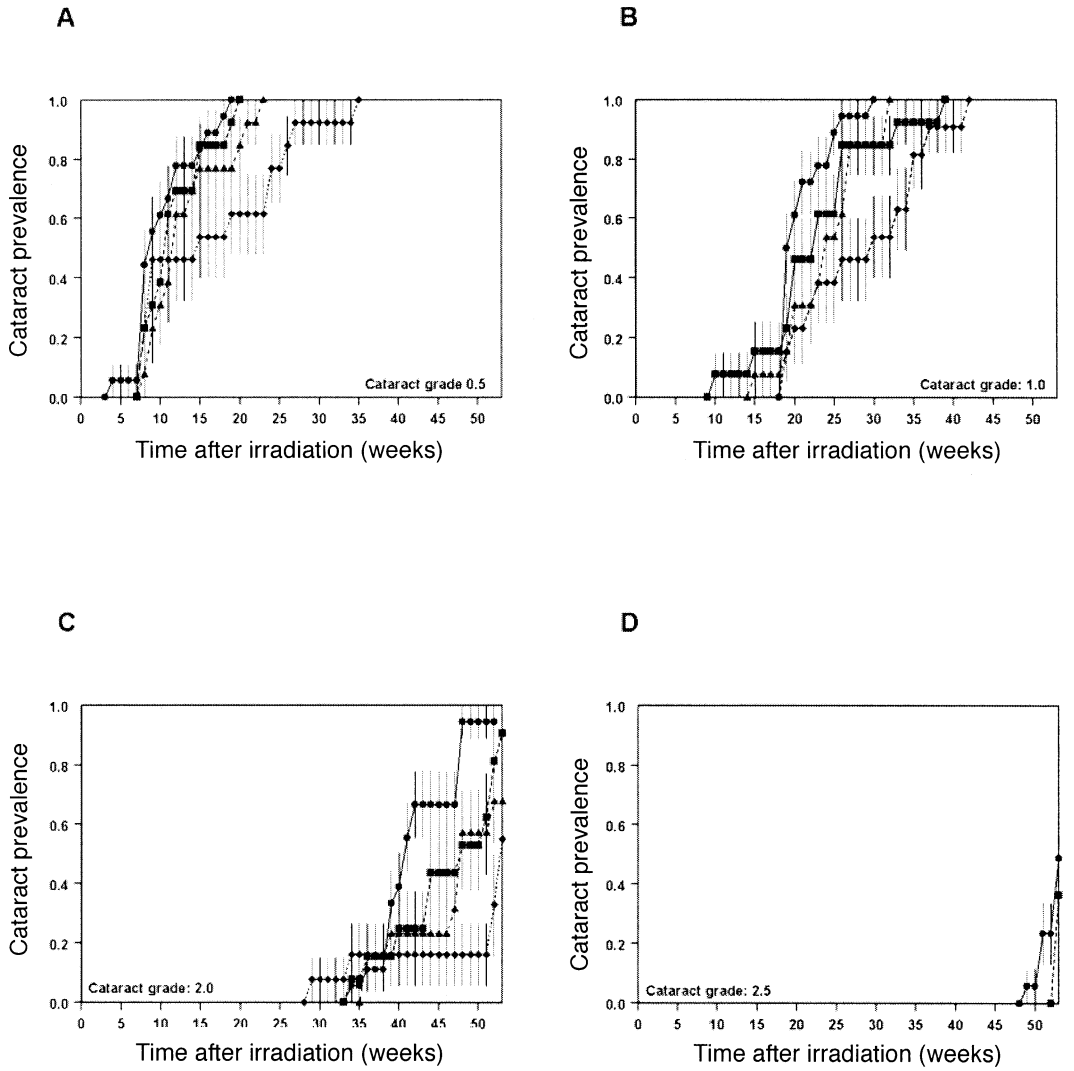

FIG. 2. Prevalence of cataracts as a function of time in the eyes of mice exposed to 50 cGy of X rays. Cataract grade: (panel A) 0.5; (panel B) 1.0; (panel C) 2.0; and (panel D) 2.5. Genotypes of mice: $Atm^{+/+}$ $Mrad9^{+/+}$ (diamonds); $Atm^{+/+}$ $Mrad9^{+/-}$ (squares); $Atm^{+/-}$ $Mrad9^{+/+}$ (triangles); $Atm^{+/-}$ $Mrad9^{+/-}$ (circles). The vertical bars are standard errors, calculated by using Greenwood's formula (*62*).

18)]. However, the activities of the proteins are not identical, because for example only mutations in *Mrad9* confer UV-radiation sensitivity in cell cultures (*37, 42*). Interestingly, ATM kinase activity can phosphorylate RAD9 in response to ionizing radiation exposure (*19*), establishing a molecular link between these proteins.

It is not clear which cellular functions of Rad9 influence cataract development. Among other roles, Rad9 modifies transcription of downstream target genes (*43*) and generally maintains genomic integrity (*37*). In a similar fashion, the activities of Atm critical for cataractogenesis also have not been determined.

Worgul and coworkers reported previously that *Atm* heterozygous mice are more prone to develop radiation-induced cataracts than wild-type control animals (*12*). The present study confirms and extends this finding by demonstrating that *Mrad9* heterozygotes also have enhanced sensitivity to radiation-induced cataract formation. More significantly, this study found that mice heterozygous for alterations in both genes have a greater likelihood for development of more severe grades of radiation-induced cataracts than animals with a deficiency in either *Mrad9* or *Atm* alone.

These results demonstrate, for the first time, the ability of two different heterozygous gene mutations to interact in a manner that increases the frequency of cataract formation. While the mechanism responsible for the susceptibility of these mutant mice to radiation-induced cataract formation is unknown, based on the established functions of Atm and Rad9, it is likely that DNA damage recognition, cell cycle checkpoint control and/or DNA repair is involved.

A number of reports suggest that oxidative stress is a major early or initiating event in the development of cataracts induced by a variety of different agents (*44–49*). The

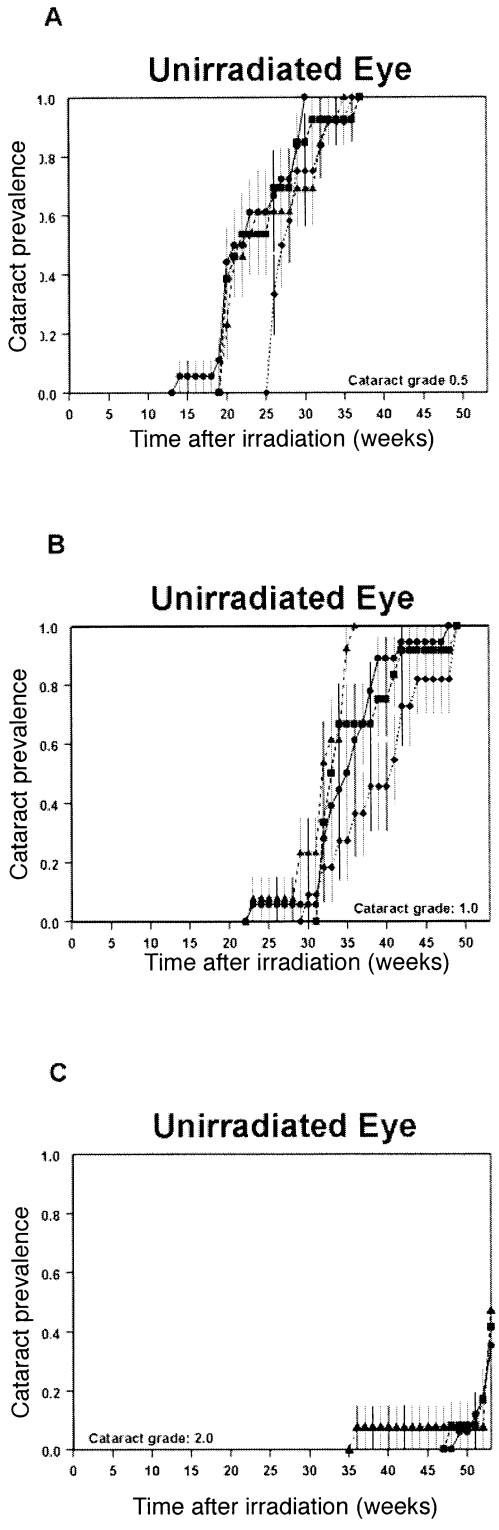

FIG. 3. Prevalence of cataracts as a function of time in the unirradiated eyes of mice. Cataract grade: (panel A) 0.5; (panel B) 1.0; and (panel C) 2.0. Genotypes of mice: $Atm^{+/+}$ $Mrad9^{+/+}$ (diamonds); $Atm^{+/+}$ $Mrad9^{+/-}$ (squares); $Atm^{+/-}$ $Mrad9^{+/+}$ (triangles); $Atm^{+/-}$ $Mrad9^{+/-}$ (circles). The vertical bars are standard errors, calculated by using Greenwood's formula (*62*).

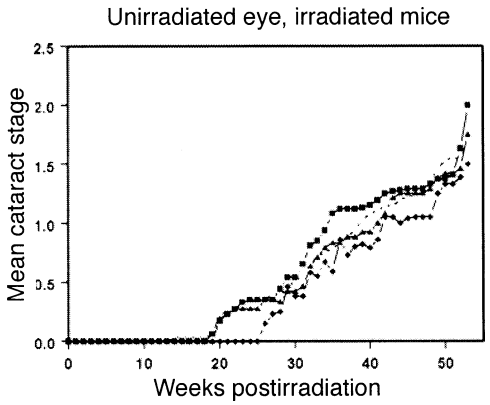

FIG. 4. Mean cataract stage as a function of time in the unirradiated eyes of mice exposed to 50 cGy of X rays. Genotypes of mice: $Atm^{+/+}$ $Mrad9^{+/+}$ (diamonds); $Atm^{+/+}$ $Mrad9^{+/-}$ (squares); $Atm^{+/-}$ $Mrad9^{+/+}$ (triangles); $Atm^{+/-}$ $Mrad9^{+/-}$ (circles).

accumulation of DNA as well as nuclear and mitochondrial debris is associated with the presence of reactive oxygen species (ROS) at sites of age-related cortical cataracts in mice (*50*). In human lenses, oxidation of lens constituents is a consistent finding (*51–53*). Experiments with lens organ and cell cultures have demonstrated that stress caused by peroxide results in rapid metabolic and cellular changes similar to those observed in human cataracts (*54–59*). Changes in cellular redox potential, membrane function, mitochondrial viability and DNA damage are known to be the earliest events that immediately follow oxidative stress (*55, 59, 60, 61*).

Since ionizing radiation causes increased oxidative stress and subsequent ROS production, it is likely that haploinsufficiency for Rad9 and Atm reduces the ability of lens cells to repair the accompanying DNA damage, thus leading to an increase in cataract frequency. Consistent with this possibility, it was demonstrated previously that mouse cells heterozygous for mutations in both *Mrad9* and *Atm* have different kinetics for repairing radiation-induced DNA double-strand breaks than cells bearing a mutation in only one of those genes (*20*).

In this study, we investigated the effects of multiple haploinsufficiency on a specific pathological, biological response in a highly organized mammalian tissue. The findings further underscore the importance of genetic heterozygosity in the development of pathology and specifically indicate that haploinsufficiency in RAD9, ATM or other proteins with similar DNA damage recognition or response functions could predispose humans to lens opacification.

With respect to radiation biology, this cataract model is the first higher-level organ system in which it is demonstrated that heterozygosity for *Atm* or *Rad9* alters the late response of a normal tissue to radiation exposure. More importantly, the identification of genes relevant for radiation-induced cataract development provides broader insight into more general mechanisms and risks for other aspects of radiosensitivity. For example, studies such as these may

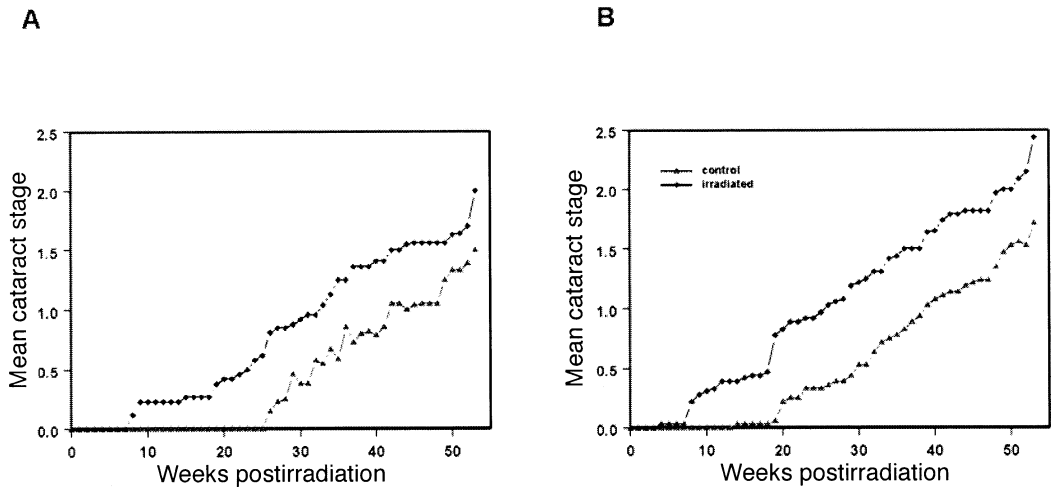

FIG. 5. Mean cataract stage as a function of time in unirradiated or irradiated eyes of mice. Genotypes of mice: (panel A) $Atm^{+/+}$ $Mrad9^{+/+}$; (panel B) $Atm^{+/-}$ $Mrad9^{+/-}$. Unirradiated (triangles); exposed to 50 cGy of X rays (diamonds).

facilitate identification of subsets of the human population genetically predisposed to radiation effects, not limited to cataractogenesis, and may have particular relevance for radiotherapy patients, astronauts or other individuals exposed to elevated levels of ionizing radiation.

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