In vivo Recombination After Chronic Damage Exposure Falls to Below Spontaneous Levels in "Recombomice"

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Abstract

All forms of cancer are initiated by heritable changes in gene expression. Although point mutations have been studied extensively, much less is known about homologous recombination events, despite its role in causing sequence rearrangements that contribute to tumorigenesis. Although transgenic mice that permit detection of point mutations have provided a fundamental tool for studying point mutations *in vivo*, until recently, transgenic mice designed specifically to detect homologous recombination events in somatic tissues *in vivo* did not exist. We therefore created fluorescent yellow direct repeat mice, enabling automated detection of recombinant cells *in vivo* for the first time. Here, we show that an acute dose of ionizing radiation induces recombination in fluorescent yellow direct repeat mice, providing some of the first direct evidence that ionizing radiation induces homologous recombination in cutaneous tissues *in vivo*. In contrast, the same total dose of radiation given under chronic exposure conditions suppresses recombination to levels that are significantly below those of unexposed animals. In addition, global methylation is suppressed and key DNA repair proteins are induced in tissues from chronically irradiated animals (specifically AP endonuclease, polymerase β, and Ku70). Thus, increased clearance of recombinogenic lesions may contribute to suppression of homologous recombination. Taken together, these studies show that fluorescent yellow direct repeat mice provide a rapid and powerful assay for studying the recombinogenic effects of both short-term and long-term exposure to DNA damage *in vivo* and reveal for the first time that exposure to ionizing radiation can have opposite effects on genomic stability depending on the duration of exposure. (Mol Cancer Res 2004;2(10):567–73)

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Introduction

Chemicals and radiation pose a constant threat to genomic integrity. To prevent DNA damage from leading to permanent changes in sequence information, cells have evolved sophisticated DNA repair systems. For example, when damage occurs to just one strand of the DNA duplex, the complementary strand can be used as a template for base excision repair (BER) or nucleotide excision repair (1). However, when both strands are damaged, such as by a double-strand break, another source of sequence information is required for accurate repair. Homologous recombination allows cells to use the undamaged sister chromatid or the homologous chromosome as a template for repair and also permits accurate reinsertion of a broken end following collapse of the replication fork (for excellent reviews of homologous recombination, see refs. 2-5).

Although homologous recombination is important for safeguarding genome integrity, transfer of genetic information can be risky. For example, recombination between misaligned sequences can lead to insertions, inversions, translocations, and deletions, and recombination between homologous chromosomes can cause loss of heterozygosity along vast stretches of chromosomes. Indeed, dozens of known carcinogens are recombinogens (6-8). Furthermore, people who are born predisposed to spontaneously high levels of recombination are prone to cancer (e.g., BLM and ATM; refs. 1, 9-15). Thus, whether caused by exposure or by an inherited predisposition, homologous recombination events are a form of genetic instability that contributes to cancer.

Although much is known about DNA damage–induced point mutations, much less is known about recombination events, in part because of the lack of effective systems for direct detection of recombinant cells in somatic tissues. We therefore developed "recombomice" in which recombinant cells fluoresce. Although spontaneous mitotic homologous recombination events at the fluorescent yellow direct repeat (FYDR) recombination substrate are relatively rare (16), recent advances in flow cytometry make it possible to quantify recombinant cells that arise *in vivo*. Here, we describe the first application of the FYDR recombomice in studies of ionizing radiation–induced homologous recombination *in vivo*.

There is widespread concern about the possible adverse health effects of ionizing radiation. Although most studies of the biological effects of ionizing radiation have employed single acute exposures, most clinically and environmentally relevant exposures occur under chronic conditions. Given that homologous recombinational repair is most active during S phase (17, 18) and that not all of the cells in a tissue are in

S phase at any particular point in time, chronic exposure might be more recombinogenic than an acute exposure, since over time a higher number of S-phase cells would be exposed to damage. On the other hand, chronic exposure to ionizing radiation may have a protective effect. It has been shown that exposure to low doses of ionizing radiation induce an adaptive response, rendering cells resistant to the toxic effects of subsequent exposures (reviewed in ref. 19). Most studies of the adaptive response have focused on suppression of toxicity, but a few described studies aimed at revealing the effects of the adaptive response on homologous recombination. For example, several studies show that exposure to low-dose ionizing radiation causes cells to become less susceptible to sister chromatid exchanges (SCEs) induced by a subsequent high-dose exposure (20-23). However, in all of these reports, adaptive protection was slight (~ 10-30% fewer SCEs induced by the challenging dose). Furthermore, to our knowledge, there are no studies reporting the effects of long-term chronic ionizing radiation on baseline levels of homologous recombination. We therefore set out to determine the effects of chronic irradiation on homologous recombination *in vivo*.

Here, we describe the first application of the FYDR recombomice for studies of DNA damage–induced recombination *in vivo*. We found that, although an acute high dose of radiation induces homologous recombination *in vivo*, the same total dose given daily over the course of several weeks does not induce recombination. Indeed, conditions of chronic irradiation suppress recombination to levels that are far below those of control unexposed animals. In addition, we found that the levels of several key DNA repair enzymes involved in BER and nonhomologous end joining are significantly induced under chronic irradiation conditions. Taken together, these results are consistent with an adaptive response associated with global changes in DNA repair capacity and increased genomic stability.

Results and Discussion

The FYDR mice carry a transgenic direct repeat recombination substrate composed of two different cassettes, each lacking essential sequences for expression of enhanced yellow fluorescent protein (EYFP). The 5′ *eyfp* expression cassette is missing essential coding sequences that are present in the 3′ *eyfp* cassette (Fig. 1A). Therefore, mitotic homologous recombination can reconstitute full-length *EYFP* coding sequence and cause a fluorescent phenotype. To determine the spontaneous frequency of recombinant cells in FYDR mice, cutaneous tissue was isolated from 23 mice, and cells were disaggregated and analyzed by flow cytometry. Recombinant cells were detected in cells from about half of the mice (Fig. 1C, *left*). As expected, the frequency of recombinant cells varied among individual mice (16), which reflects the probability of a recombination event occurring at different times during growth (24). Based on the MSS Maximum Likelihood Method (see Materials and Methods), we calculated that the spontaneous rate of recombination is ~2.1 recombination events per 10^6 cell divisions, which is comparable with recombination rates reported in cultured mammalian cells harboring analogous recombination

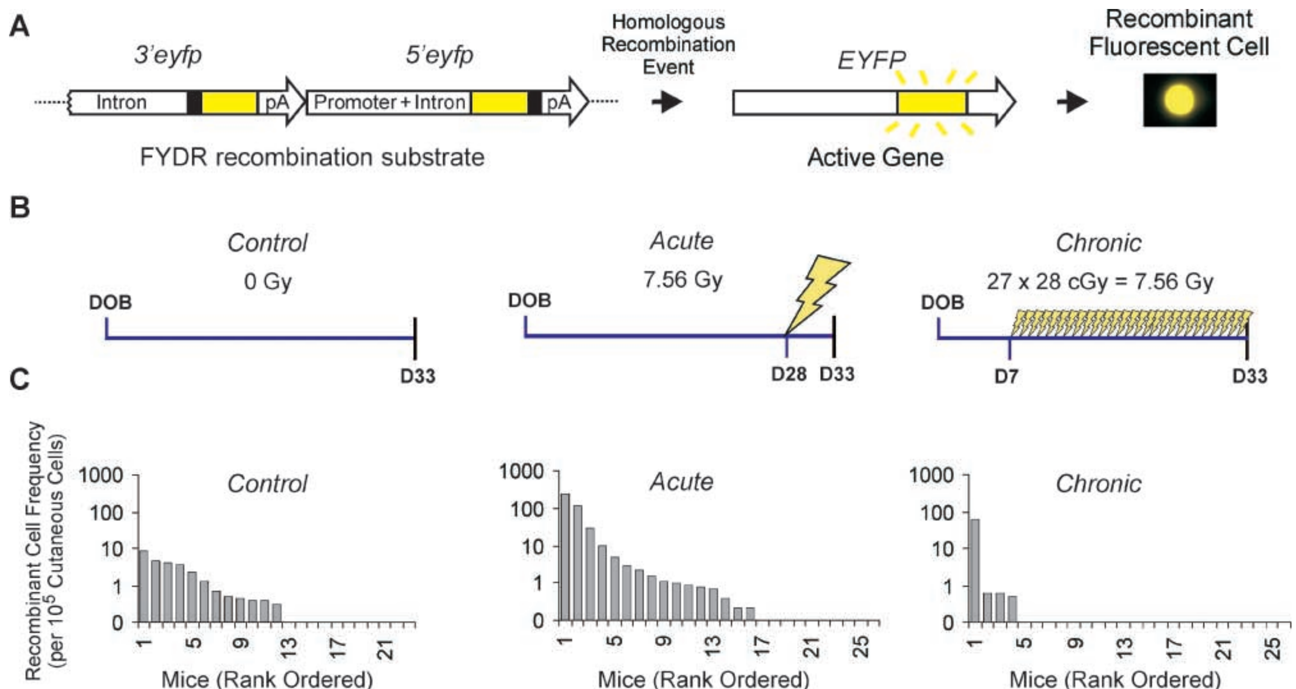

FIGURE 1. Radiation induced homologous recombination in FYDR mice. **A.** Arrangement of the FYDR recombination substrate. *Large arrows,* expression cassettes; *yellow,* coding sequences; *black boxes,* positions of deleted sequences. Note that deletion sizes are not to scale. *Far right,* image of a recombinant fluorescent FYDR cell. **B.** Treatment conditions for three mouse cohorts. Animals in the control cohort were sham treated, those in the acute cohort were exposed to 7.56 Gy at 28 days, and those in the chronically irradiated cohort were exposed daily to 28 cGy from 7 to 33 days. *DOB,* date of birth. **C.** Recombinant cell frequencies in cutaneous tissues of control, acute, and chronically irradiated animals.

substrates (e.g., refs. 25-27). Thus, the FYDR mice can be used to estimate the rate of recombination *in vivo* and could potentially be useful for monitoring the effects of environmental exposures *in vivo*.

It is well established that ionizing radiation induces recombination at direct repeat substrates in mammalian cells and induces SCEs in mice (8, 28-31). To learn about the potential utility of the FYDR mice for detecting damage-induced recombination *in vivo*, we exposed a cohort of FYDR mice to an acute dose of radiation and quantified recombinant cells in cutaneous tissues. Exposure to 7.5 Gy (LD_{50} at 30 days postirradiation) significantly increased the frequencies of recombinant cells (Fig. 1C; $P \leq 0.05$, Mann-Whitney test). Indeed, the average frequency increased by over 10 fold, from 1.1 to 15.1 per 10^5 cells. To our knowledge, these are the first studies to show that irradiation induces recombination in cutaneous tissue *in vivo*, thus demonstrating the utility of the FYDR mice for detecting damage-induced recombination in animals.

To reveal the potential recombinogenic effects of chronic exposure conditions, 26 FYDR mice were exposed to 28 cGy/d until they reached the same total dose as under the acute conditions (mice received 27 daily exposures; Fig. 1B, *right*). There were no overt signs of toxicity or any significant effects on cell proliferation (as studied by Ki67 immunohistochemistry; data not shown). Unexpectedly, rather than inducing homologous recombination, chronic irradiation suppressed the frequency of recombinant cells to levels that are significantly below those of control animals (Fig. 1C; $P < 0.05$, Mann-Whitney test). To our knowledge, these are the first studies to suggest that chronic exposure to ionizing radiation can suppress sequence rearrangements. However, it remained formally possible that chronic irradiation was simply suppressing expression of *EYFP*, rendering recombinant cells undetectable by flow cytometry.

To determine if the recombination substrate had been inactivated by chronic irradiation, the recombination rate was evaluated in primary mouse adult fibroblasts (MAFs) derived from chronically irradiated mice. We found that MAFs cultured from mice in the chronic cohort were not significantly different from control MAFs in their ability to give rise to recombinant fluorescent cells *ex vivo* (Fig. 2A), thus demonstrating that the recombination substrate could not have been permanently inactivated. However, it remained possible that *EYFP* expression was silenced *in vivo* by chronic irradiation and that silencing had been reversed in culture. Therefore, we measured the transcript levels expressed from the FYDR recombination substrate in samples of cutaneous tissue directly isolated from mice in the control and irradiated cohorts. Radiation did not significantly affect transcription from this locus (Fig. 3A), indicating that the reduced appearance of fluorescent recombinant cells in the chronically irradiated mice is not due to suppression of gene expression and is therefore likely to be caused by suppression of homologous recombination.

To assess the magnitude of the suppression *in vivo*, we calculated the *in vivo* recombination rates among control and chronically irradiated mice and found that chronic irradiation suppressed the recombination rate *in vivo* by ~8-fold (Fig. 2B; for rate calculations, see Materials and Methods). This result

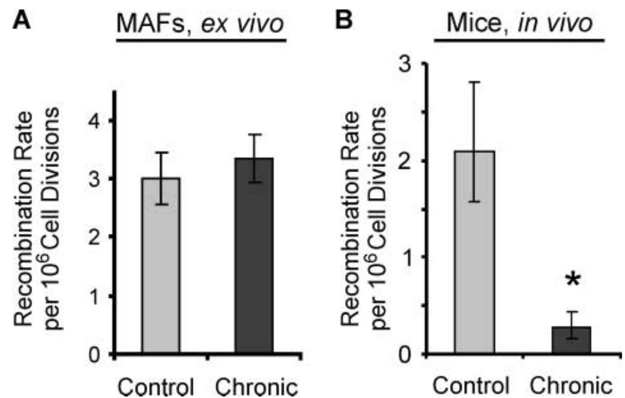

FIGURE 2. Recombination rates in cells and animals. **A.** MAFs isolated from mice in the control and chronically irradiated cohorts (calculated by the P_0 method; ref. 24). Due to the toxicity associated with acute exposure to 7.56 Gy, MAFs could not be cultured from mice in the acute cohort. *Columns,* mean of three to four independent experiments; *bars,* SE. **B.** Calculated recombination rates *in vivo* based on MSS Maximum Likelihood Method (62). The recombination rate among acutely exposed animals has been omitted, because the toxicity associated with the acute exposure impinges on the number of cell divisions postirradiation to an unknown extent. *Columns,* recombination rates; *bars,* SE [calculated as described previously (62)]. *, $P < 0.05$, Student's t test.

contrasts with the observation that the recombination rate in MAFs isolated from control and chronically irradiated mice were similar (Fig. 2A). Two possible explanations for this discrepancy are (*a*) the *in vitro* conditions do not accurately reflect the conditions *in vivo* and (*b*) the suppressive effects of chronic irradiation on recombination are transient (e.g., the recombination rate had reverted to normal when the cells were expanded for rate analysis in culture). To determine if the effects of chronic irradiation *in vivo* can be recapitulated *in vitro*, MAF cultures were created from unexposed FYDR mice and chronically irradiated during expansion in culture. Consistent with the effects of chronic exposure *in vivo*, chronic irradiation of cultured cells significantly suppressed the frequency of recombinant cells ($P < 0.05$, Mann-Whitney test), and the rate of homologous recombination was significantly lower than that of unexposed cells (Fig. 4A). [Note that there were no overt signs of cytotoxicity, and the population doubling time was not affected by chronic irradiation (data not shown)]. To determine if chronic irradiation similarly suppresses recombination in embryonic cells, we measured the frequency of recombinant cells among FYDR mouse embryonic fibroblasts (MEF). As for the MAFs, chronic irradiation similarly suppressed homologous recombination in FYDR MEFs (Fig. 4B). Thus, chronic irradiation suppresses homologous recombination at the FYDR substrate in fibroblasts from both adult and embryonic tissues. To determine if recombination is suppressed throughout the genome (rather than at this particular locus), we measured the frequency of SCEs in metaphase spreads. Consistent with the observation that radiation suppresses recombination at the FYDR recombination substrate, four daily exposures to 28 cGy significantly suppressed the frequency of SCEs in MAFs (Fig. 4C). Thus, chronic irradiation causes a general reduction in the frequency and rate of homologous recombination events both *in vivo* and *in vitro*.

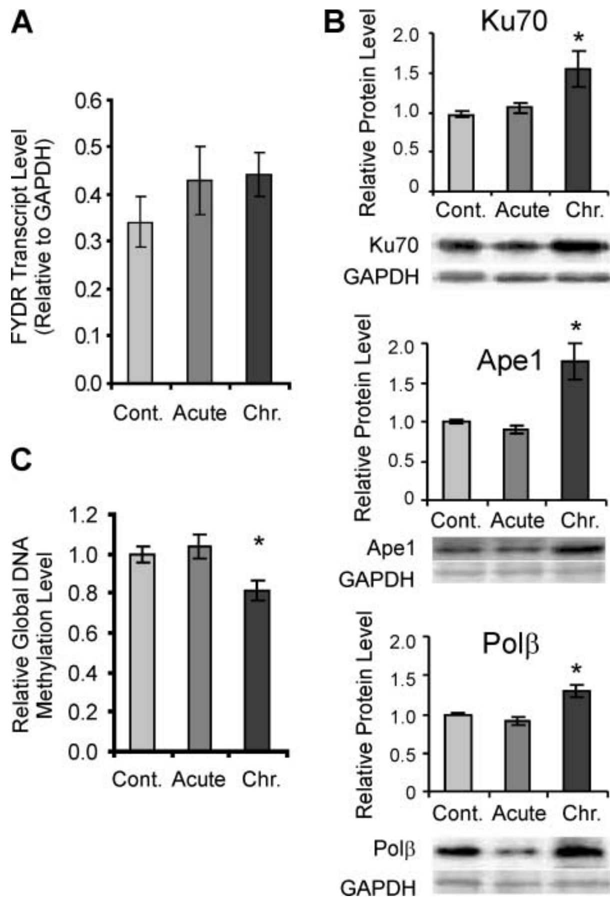

FIGURE 3. Effects of radiation on gene expression and methylation *in vivo*. **A.** Levels of RNA transcripts expressed from the FYDR recombination substrate as measured by real-time PCR. Averages relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). **B.** Lysates from cutaneous tissue were immunoblotted using antibodies against Ku70, Polβ, Ape1, and GAPDH as a loading control. Protein levels relative to those of control animals. Representative Western blots from among eight independent experiments. **C.** Global genome methylation level as measured by cytosine extension (relative to control animals). *Cont.,* control mice; *Chr.,* chronic mice. *Columns,* mean; *bars,* SE. *, $P < 0.05$, Student's t test.

Exposure to ionizing radiation is known to induce an adaptive response that is associated with increased levels of DNA repair in mammalian cells (32-37). Thus, one of the underlying causes of suppressed recombination in chronically irradiated animals could be that there are increased levels of DNA repair proteins that promote clearance of recombinogenic strand breaks and abasic sites. As a first step toward revealing the molecular basis for radiosuppression of homologous recombination, we therefore measured the levels of several DNA repair proteins that have been shown previously to suppress homologous recombination, namely, Ku70, AP endonuclease (Ape1, also known as Hap1/Ref1/Apex), and polymerase β (Polβ; refs. 38-40).

In mammals, most double-strand breaks are repaired by nonhomologous end joining (41, 42). Ku70 is essential for nonhomologous end joining and is induced in mammalian cells exposed to ionizing radiation (32, 43). We therefore measured the levels of Ku70 in cutaneous samples from mice in the control, acute, and chronically irradiated cohorts. Although we did not detect induction of Ku70 in cutaneous tissues from the acute cohort, there was a significant increase in the levels of Ku70 within tissues of chronically irradiated animals (Fig. 3B), suggesting that enhanced nonhomologous end joining may help prevent double-strand break–induced homologous recombination.

In addition to double-strand breaks, ionizing radiation also increases the levels of oxidized bases (1). Radiation-induced oxidized bases are rapidly removed by DNA glycosylases to

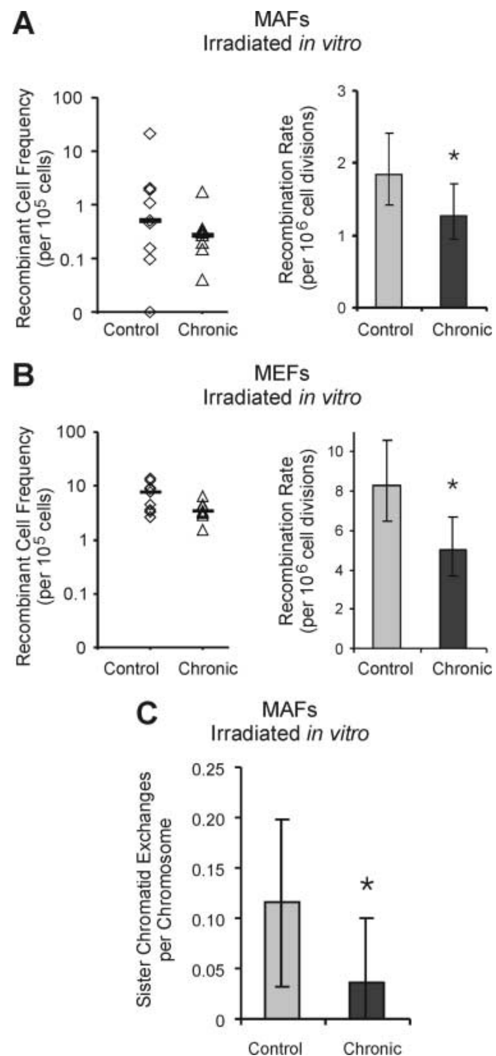

FIGURE 4. Chronic irradiation suppresses recombination in cultured cells. **A.** Recombinant cell frequencies and recombination rates for control and chronically irradiated MAFs. Each MAF culture was derived from the ear tissue of an independent mouse. **B.** Recombinant cell frequencies and recombination rates for control and chronically irradiated MEFs derived from a FYDR embryo. **A** and **B.** Cells were exposed in culture to 28 cGy/d or sham treated for 6 days. *Points,* recombinant cell frequencies; *horizontal bars,* median frequencies. *Columns,* recombination rates; *bars,* SE [calculated using the MSS Maximum Likelihood Method; SE was calculated as described previously (62)]. **C.** SCE frequencies in MAFs exposed to 28 cGy/d for 4 days. *Columns,* mean among 18–20 independent metaphase spreads; *bars,* SD. *, $P < 0.05$, Student's t test.

yield abasic sites that are subsequently repaired by downstream BER enzymes (44). It is well established that conditions that result in increased levels of BER intermediates, such as abasic sites and single-strand breaks, are recombinogenic (39, 40, 45-47). However, if BER enzymes are induced in a coordinated fashion, then recombinogenic BER intermediates may be cleared more efficiently, thus suppressing homologous recombination. In mammals, Ape1 and Polβ are critical for creating an extendable 3′OH group and a ligatable 5′ phosphate during BER (44). Previous studies have shown that ionizing radiation induces expression of Ape1 (35, 48), and we show here that Ape1 is induced in the chronically exposed animals, suggesting that Ape1 helps to clear recombinogenic abasic sites (Fig. 3B). In contrast to Ape1, there are several studies showing that ionizing radiation does not induce Polβ (33, 48, 49). Consistent with these studies, we did not detect induction of Polβ following an acute exposure (Fig. 3B). However, we did observe that Polβ is induced under chronic exposure conditions (Fig. 3B), which suggests that BER proteins may be coordinately up-regulated under conditions of chronic irradiation. In addition to being formed by exposure to irradiation, double-strand breaks and oxidative base lesions are also formed spontaneously. Thus, one possible model for suppression of recombination in chronically irradiated animals is that nonhomologous end joining and BER reduce the number of recombinogenic lesions to below spontaneous levels.

Gene silencing is often controlled by methylation at CpG islands, and it is known that DNA damage can alter methylation patterns (50, 51). We therefore compared the levels of global methylation in tissue isolated from the control and irradiated cohorts. Figure 3C shows that chronic irradiation, but not acute radiation, significantly reduces the levels of global methylation in the cutaneous tissue of mice. This shift in global methylation suggests that there may be a widespread shift in gene expression patterns. Thus, in addition to Ku70, Polβ, and Ape1, additional changes in gene expression may also affect the levels of homologous recombination.

Genetic and environmental factors that lead to increased levels of recombination are associated with an increased risk of cancer and accelerated aging (6-8, 14, 15, 29, 52-54). Although exposure to acute doses of ionizing radiation clearly leads to cancer (55), there is evidence that low-dose exposure to ionizing radiation can suppress neoplastic transformation and enhance longevity (56, 57). The results presented here provide some of the first evidence that chronic exposure to ionizing radiation causes the rate of recombination to decrease to levels that are significantly below that of unexposed control animals. Although further studies are needed to assess the effects of chronic irradiation on other end points of genomic stability (such as point mutations), these results suggest that protective responses induced by chronic irradiation may provide beneficial suppression of deleterious sequence rearrangements.

One third of people alive today are likely to get cancer (58), and according to the National Cancer Institute, about half of cancer patients today receive some form of radiotherapy. Importantly, radiation therapy is given under conditions that are quite similar to those used in these experiments (patients generally receive daily doses of 1.5-3 Gy for several weeks; in these studies, animals were exposed to daily doses of 0.28 Gy for several weeks). Therefore, these results suggest that radiation therapy may similarly induce expression of repair proteins and suppress recombination. Indeed, previous studies by Little and colleagues have shown that following daily clinical doses of ionizing radiation, gliomas become radioresistant (59), and a correlation between radiosensitivity and adaptation has also been reported for other cancers as well (e.g., refs. 60, 61). Despite these important results, the possibility that an adaptive response takes place during radiotherapy has received little attention in the clinic. The studies presented here call attention to the fact that daily radiation at doses that are not far below those used in the clinic leads to global changes in methylation, induction of key repair proteins, and suppression of recombination, all of which are likely to impinge on cellular sensitivity to subsequent doses of radiation and chemotherapeutics.

The FYDR mice provide a novel system for rapid and automated detection of DNA damage–induced sequence rearrangements *in vivo* that can be used to address fundamental biological questions about how mammals respond to DNA damage. The results of these studies clearly indicate that acute and chronic exposure conditions can result in opposite effects and underscore the limitations of studies using single acute exposures. The FYDR mice thus provide a broadly applicable tool for genotoxicity testing under conditions that accurately reflect the human experience in terms of occupational, environmental, and clinical exposures.

Materials and Methods

Irradiation of Animals

Mice heterozygous for the FYDR recombination substrate were randomly assigned to different treatment groups. Animals were housed in a virus-free facility and given food and water *ad libitum*. The "chronic" group received 28 cGy (2 cGy/s) whole-body X-rays (90 kV, 5 mA) applied daily for 27 consecutive days until reaching a total dose of 7.56 Gy. The "acute" group received a single dose of 7.56 Gy (2 cGy/s) on day 28. Control mice were sham treated. All animals were humanely sacrificed at the age of 33 days on completion of the treatment protocol. Cutaneous tissue was isolated immediately on sacrifice and processed for subsequent molecular studies (see below), fixed in 4% paraformaldehyde for immunohistochemical staining, or disaggregated for analysis of recombinant cell frequency. Concomitantly, cells were isolated from ear tissue of four mice from each cohort and cultured *ex vivo* to determine the recombination rate *ex vivo*.

Estimates of Recombination Frequencies and Rates

The frequency of recombinant cells in disaggregated cutaneous tissues was measured by flow cytometry as described previously (16). The frequencies of recombinant cells per cutaneous tissue sample (Fig. 1C) were used to calculate the *in vivo* recombination rates. The *in vivo* recombination rates were calculated using the MSS Maximum Likelihood Method, as described previously (62), under the assumption that the number of cell divisions can be approximated by the number of cells analyzed by flow cytometry.

MAFs were isolated from ear tissue of unexposed and irradiated FYDR mice and expanded in culture for 3 days. Cells were then plated into 22 to 24 independent cultures and expanded an additional 5 to 7 days. Recombinant cell frequencies were measured by flow cytometry and the recombination rate *ex vivo* was calculated using the P_0 method (24).

For *in vitro* irradiation studies, MAFs and MEFs were isolated from unexposed FYDR mice and embryos, respectively. MAF cultures were derived from independent mice, whereas MEF cultures were created from a single homogenous population of cells. The frequency of recombination among 9 to 10 independent MAF and MEF cultures was determined by flow cytometry after six daily exposures to 28 cGy using a Cs-137 Gamma Cell G40 (63 rads/min). The recombination rates were calculated using the MSS Maximum Likelihood Method (62). For SCEs, cultured MAFs were exposed to 28 cGy for 4 days (or sham treated), and BrdUrd was added to the medium 6 hours after the last exposure. After 48 hours, colcemid was added and cells were incubated for an additional 14 hours prior to isolation of mitotically arrested cells. SCEs were stained as described previously (39) and counted in a blinded fashion.

RNA Preparation, Reverse Transcription, and Real-time PCR

For RNA preparation, cutaneous tissues were sampled on sacrifice and immediately equilibrated in RNAlater solution (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD). RNA samples were treated with DNase I (Invitrogen, San Diego, CA) and RNA was further purified using the RNeasy total RNA cleanup protocol (Qiagen). The RNA yields were measured using RiboGreen assay (Molecular Probes, Eugene, OR). Reverse transcription was done using RevertAid H Minus First-Strand cDNA Synthesis Kit (Fermentas, Hanover, MD). Primers that amplify a 112 bp segment of coding sequence that is present in both unrecombined FYDR substrate and full-length *EYFP* were used to evaluate the levels of transcripts expressed from the FYDR locus.

Real-time PCR was done in a total volume of 25 μL using 1 μL of the first-strand cDNA synthesis mixture as a template, 300 nmol/L of primers, and 12.5 μL of 2× SYBRGreen PCR Master Mix (Applied Biosystems, Foster City, CA). Duplicate reactions were carried out with 1:3 and 1:15 dilutions of the first-strand cDNA synthesis mixture. A SmartCycler (Cepheid, Sunnyvale, CA) was used to perform PCR, and fluorescence was quantified against standards. Wells containing SYBRGreen PCR master mix and primers without sample cDNA were used as negative controls and emitted no fluorescence. Levels of FYDR transcripts were standardized against glyceraldehyde-3-phosphate dehydrogenase (levels were measured in parallel). Primer sequences are available on request.

Quantification of Global DNA Methylation

Total DNA was prepared from cutaneous tissues using a Qiagen DNeasy kit. A cytosine extension assay was done to measure the relative levels of DNA methylation (51).

Western Immunoblotting

Cutaneous tissue was snap frozen immediately after isolation. Protein samples were sonicated in 1% SDS and boiled for 10 minutes. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were incubated with antibodies against Ku70 (BD Biosciences, Mountain View, CA), Ape1/Ref1 (Biomira, Cranbury NJ), Polβ (Novus Biologicals, Littleton, CO), and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was revealed by incubation with horseradish peroxidase–conjugated secondary antibodies (Amersham, Piscataway, NJ) and the Enhanced Chemiluminescence Plus detection system (Amersham). Polyvinylidene difluoride membranes were stained with Coomassie blue (Bio-Rad, Hercules, CA) and the intensity of the M_r 50,000 protein band was assessed as a loading control. Signals were quantified using NIH Image 1.63 Software and normalized relative to both glyceraldehyde-3-phosphate dehydrogenase and the M_r 50,000 protein band, which gave internally consistent results (values relative to M_r 50,000 are plotted).

Statistical Analysis

Statistical analysis was done using MS Excel 2000, Analyze-It, JMP5, and Mathematica software packages.

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