Disruption of Brca2 increases the spontaneous mutation rate in vivo: synergism with ionizing radiation

Andrew N.J. Tutt, Conny Th.M. van Oostrom, Gillian M. Ross, Harry van Steeg & Alan Ashworth+

The Breast Cancer Research Centre, Institute of Cancer Research, Fulham Road, London SW3 6JB, UK and National Institute of Public Health and the Environment, Laboratory of Health Effects Research, RIVM/LEO, Bilthoven, The Netherlands

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The breast cancer predisposition gene BRCA2 encodes a protein involved in the repair of DNA double-strand breaks, which arise spontaneously and following exposure to ionizing radiation (IR). To develop a mouse model that examines the effect of BRCA2 mutation and IR exposure on in vivo somatic mutation acquisition, we crossed mice with targeted disruption of Brca2 with a LacZ transgenic mutation reporter strain. Loss of both wild-type Brca2 alleles caused a 2.3-fold increase, equivalent to an extra 100 mutations per cell, in the in vivo acquisition of spontaneous somatic mutation by 2 weeks gestation. IR (4 Gy) had a disproportionate effect on animals homozygous for Brca2 disruption, inducing 3.4-fold more mutations compared with wild-type animals. These data provide the first evidence that loss of Brca2 increases in vivo somatic mutation acquisition and synergizes with IR exposure, with potential attendant implications for mammographic screening and therapeutic IR in mutation carriers.

INTRODUCTION

Human cancers require the acquisition of a number of somatic mutations in key oncogenes and tumour suppressors to achieve a malignant phenotype (Lengauer et al., 1998); abnormal DNA repair and consequent genomic instability can accelerate this process (Loeb, 1991). Germline mutation in BRCA2 considerably increases the risk of breast, ovarian and other cancers (Rahman and Stratton, 1998), and the BRCA2 protein has been implicated in the repair of DNA double-strand breaks (DSBs) by homologous recombination (Kerr and Ashworth, 2001). Cell-based assays have shown that BRCA2 is required for the error-free repair of DNA DSBs by gene conversion (Moynahan et al., 2001; Tutt et al., 2001). Loss of BRCA2 appears to result in the use of an alternative error-prone single-strand annealing (SSA) pathway (Karran, 2000; Tutt et al., 2001). However, it is not yet known whether these in vitro results, suggesting an increase in mutation frequency caused by loss of BRCA2, are applicable in vivo. DNA DSBs arise spontaneously during DNA replication and are also induced by ionizing radiation (IR). Carriers of BRCA2 mutations are subjected to IR during both screening mammography and adjuvant radiotherapy for breast cancer, raising concern about excessively increased mutation induction by IR (Bennett, 1999; Friedenson, 2001). Here we use in vivo mutation reporter mice (Boerrigter et al., 1995) carrying a targeted disruption of Brca2 (Connor et al., 1997) to model this question. We found that Brca2 heterozygotes undergo no elevated spontaneous or IR-induced mutation frequency compared with wild-type animals. However, mice homozygous for Brca2 disruption had a substantial increase in spontaneous mutation frequency, which is considerably exacerbated by IR.

RESULTS

Somatic mutation reporter mouse model

We have described previously a mouse model to examine the in vivo phenotype of a truncating mutation (Brca2Tr2014, hereafter referred to as Brca2Tr; Connor et al., 1997), which mimics cancer-predisposing mutations in humans. This mutation truncates the protein just before the final BRC repeat in exon 11.

+Corresponding author. Tel: +44 20 7970 6058; Fax: +44 20 7878 3858; E-mail: alana@icr.ac.uk

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at a similar site to the common Ashkenazi 6174delT BRCA2 truncation. Mice homozygous for the Brca2Tr mutation are prone to tumorigenesis. We used in vitro cell culture systems to show an increase in error-prone DNA DSB repair after disruption of Brca2 (Tutt et al., 2001). To establish whether this defect seen in vitro is associated with increased somatic mutation in vivo, we have crossed mice heterozygous for the Brca2Tr mutation with a LacZ-plasmid transgenic mutation reporter mouse (Boerrigter et al., 1995; Figure 1). This system can detect spontaneous and IR-induced somatic mutations including the large genomic deletions, insertions or translocations (Gossen et al., 1995) predicted to occur from DSB misrepair using non-homologous end-joining (NHEJ) or SSA (Jasin, 2000). We generated Brca2Tr/Wt mice that were additionally homozygous for the pUR288 transgene mutation reporter concatamer at each of two chromosomal loci, then intercrossed these mice to examine the effect of the truncating mutation in Brca2 on spontaneous somatic mutation frequency using the LacZ reporter genes (Figure 1).

Fig. 1. Schematic diagram of a LacZ plasmid transgenic mouse model for analysis of the effect of Brca2 loss of function on in vivo somatic mutation frequency. Mice are shown of the three potential Brca2 genotypes either treated or untreated with IR. These mice are also homozygous for two pUR288 LacZ plasmid concatamer chromosome integration sites on chromosomes 3 and 4 (marked as black chromosomes). Each concatamer contains ~10 copies of pUR288. The ampicillin’ gene is shown as a blue box, the bacterial origin of replication as a yellow box and the LacZ reporter gene in red. HindIII sites between the plasmids are marked (H). Flanking regions of genomic DNA are represented by the green line. Individual linear plasmids are shown after release from genomic DNA by HindIII digestion. The central plasmid has a point mutation within LacZ represented by a green dot. The plasmid on the right-hand side has suffered a deletion or genomic rearrangement removing the 3' LacZ sequence with a break point extending into flanking genomic DNA. This removes the original HindIII site and thus HindIII digestion recovers a LacZ mutant plasmid of different size to the wild type. Single reporter plasmids are recovered from genomic DNA, transformed into E. coli and mutation frequency determined by comparing colony numbers on mutant selection (P-gal) and non-selective rescue efficiency (X-gal) plates.
Effect of loss of Brca2 on spontaneous in vivo somatic mutation acquisition

Most Brca2\textsuperscript{Tr/Tr} mice die at birth (Connor et al., 1997), so to generate sufficient samples for analysis we examined the mutation frequency in littermate Brca2\textsuperscript{Wt/Wt}, Brca2\textsuperscript{Tr/Tr} and wild-type embryos at day 14.5 gestation (Figure 2A). We found that loss of both wild-type Brca2 alleles causes a rapid and significant increase in spontaneous somatic mutation frequency at this embryonic stage: Brca2\textsuperscript{Tr/Tr}, 8.9 ± 0.4 \times 10^{-5}; Brca2\textsuperscript{Wt/Wt}, 3.9 ± 0.6 \times 10^{-5} (P = 0.0018). This is equivalent to the somatic mutational load acquired by a 2-year-old wild-type mouse (Dolle et al., 1997). Based on a LacZ reporter gene target size of 3 kb and a genome size of 6 \times 10^9 bp, this equates to an additional 100 mutations in every cell after only 2 weeks gestation. As un repaired DNA DSBs within the reporter gene will not be recovered from genomic DNA by this assay, the observed increase in mutation frequency therefore represents completed DNA repair by an error-prone mechanism. Heterozygosity for this Brca2 mutation has no effect on somatic mutation frequency: Brca2\textsuperscript{Tr/Wt}, 3.34 ± 0.3 \times 10^{-5}; Brca2\textsuperscript{Wt/Wt}, 3.9 ± 0.6 \times 10^{-5} (P = 0.42). This suggests that at this embryonic stage, at least, there is no mutator phenotype associated with loss of one wild-type copy of Brca2 and that loss of the second wild-type allele drives the rapid acquisition of somatic mutation throughout the genome.

Characterization of in vivo mutations induced by Brca2 mutation

A high proportion of the infrequent spontaneous somatic mutations in wild-type LacZ-plasmid transgenic reporter mice arise from deletions and genomic rearrangements rather than point mutations (Dolle et al., 2000). This type of mutation can arise from DNA DSB misrepair events by error-prone NHEJ and SSA repair pathways (Dolle et al., 2000). IR also increases this class of mutation (Gossen et al., 1995). We have shown that loss of wild-type Brca2 results in increased use of error-prone SSA to repair a chromosomal DSB in a cell culture system (Tutt et al., 2001). Therefore, we asked whether somatic mutations induced by loss of Brca2 in vivo are of the deletion/rearrangement type by analysing mutations present in the LacZ reporter plasmids recovered in the experiments described above (Figure 2A and B). Deletions, insertions and translocations, such as those induced by IR, cause restriction fragment size changes (Gossen et al., 1995), whereas point mutations, such as those induced by ethyl nitrosourea, do not (Dolle et al., 1996). We found no significant differences in mutation frequency and mutation type between the experimental groups and the wild-type controls.
difference \( P = 0.75 \) between the proportion of reporter plasmid mutants with detectable size changes recovered from Brca2\(^{Tr}\) embryos (76%; 51/67) compared with Brca2\(^{Wt/Wt}\) embryos (71%; 22/31) and Brca2\(^{Wt/Tr}\) embryos (78%; 43/55). We then used a PCR assay to distinguish size change mutations due to gross deletions/rearrangements involving flanking mouse genomic DNA from deletions internal to LacZ (Dolle et al., 2000). Interestingly, deletions/rearrangements involving the mouse genome are more common in Brca2\(^{Wt/Tr}\) recovered mutants (96%; 49/51) than in Brca2\(^{Wt/Wt}\) and Brca2\(^{Wt/Tr}\) control mutants (78%; 51/65) \( P = 0.0065 \). Thecharacter of these mutations is compatible with use of error-prone repair of DNA DSBs and shows that tissues lacking Brca2 acquire somatic mutation via this mechanism at a greater rate than in tissues with wild-type Brca2 (Figure 2C).

In vivo somatic mutation induction by exposure to IR

IR induces, amongst other lesions, DNA DSBs and is recognized as being carcinogenic to breast tissue (Goss and Sierra, 1998). An increase in error-prone repair of IR-induced DNA DSBs due to abnormal function of BRCA2 would be predicted to increase radiation-induced mutagenesis. This is of clinical interest, as carriers of BRCA2 are exposed to IR during mammography and adjuvant radiation therapy associated with breast conserving surgery for ductal carcinoma in situ and invasive breast cancer.

To examine the effect of Brca2 mutation on IR-induced somatic mutations, we compared littermate Brca2\(^{Wt/Wt}\), Brca2\(^{Wt/Tr}\) and Brca2\(^{Tr/Tr}\) embryo sets irradiated with 4 Gy X-rays 24 h prior to embryo harvest on day 14.5 gestation. We found that 4 Gy induces a significant increase over the spontaneous mutation frequency in wild-type (10.6 ± 1 × 10\(^{-5}\) versus 3.9 ± 0.6 × 10\(^{-5}\); \( P = 0.0013 \)), Brca2\(^{Wt/Tr}\) (10.6 ± 0.6 × 10\(^{-5}\) versus 3.34 ± 0.3 × 10\(^{-5}\); \( P < 0.0001 \)) and Brca2\(^{Tr/Tr}\) embryos (31.6 ± 2.9 × 10\(^{-5}\) versus 8.93 ± 0.4 × 10\(^{-5}\); \( P = 0.0004 \); Figure 3A). The induction of mutations (in absolute numbers) above the spontaneous background is therefore ∼3-fold greater in Brca2\(^{Tr/Tr}\) embryos than in the wild type (22.7 ± 2.5 × 10\(^{-5}\) versus 6.7 ± 1 × 10\(^{-5}\); \( P < 0.0001 \); Figure 3B). We estimate that loss of Brca2 in vivo results in an extra 320 somatic mutations in every cell as a result of misrepair of 4 Gy IR-induced damage. We also asked whether any subtle mutator phenotype associated with haploinsufficiency due to mutation in one Brca2 allele, not revealed in untreated Brca2\(^{Tr}\) embryos, might become apparent under the stress of DNA damage induced by 4 Gy of IR. In fact, we found no increase in radiation-induced mutagenesis in Brca2\(^{Wt/Tr}\) embryos compared with the wild type (7.2 ± 0.8 × 10\(^{-5}\) versus 6.7 ± 1 × 10\(^{-5}\); \( P = 0.68 \)). This further supports the contention that mutation or loss of both copies of Brca2 is required before a mutator phenotype is acquired, after which Brca2-deficient tissues then suffer a greater number of somatic mutations per unit dose of IR.

Characterization of IR-induced in vivo mutations

Characterization of the size of recovered mutant plasmids reveals that increased mutation induction by IR in Brca2\(^{Tr}\) embryos is predominantly via a deletion/rearrangement mechanism (86%; 32/37). We found that although the mutation spectrum is similar to that in wild-type embryos (76%; 16/21) and Brca2\(^{Wt/Tr}\) embryos (74%; 32/43; \( P = 0.39 \)), the frequency at which these

Fig. 3. Analysis of the effect of IR on in vivo somatic mutation frequency and mutation type. (A) The bar chart shows the somatic mutation frequency in the LacZ pUR288 reporter in 4 Gy irradiated Brca2\(^{Wt/Wt}\) \( (n = 8) \), Brca2\(^{Wt/Tr}\) \( (n = 9) \) and Brca2\(^{Tr/Tr}\) \( (n = 6) \) day E14.5 embryos compared with the unirradiated embryos of the same genotype. Error bars represent SEM. (B) Scatter plot showing the frequency of somatic LacZ mutation in the pUR288 reporter induced over the spontaneous background in 4 Gy-irradiated Brca2\(^{Wt/Wt}\) \( (n = 8) \), Brca2\(^{Wt/Tr}\) \( (n = 9) \) and Brca2\(^{Tr/Tr}\) \( (n = 6) \) day E14.5 embryos. (C) The bar chart shows the frequency of size-change and no-change LacZ mutants in 4 Gy-irradiated Brca2\(^{Wt/Wt}\) \( (n = 8) \), Brca2\(^{Wt/Tr}\) \( (n = 9) \) and Brca2\(^{Tr/Tr}\) \( (n = 6) \) day E14.5 embryos. Error bars represent the SEM.
somatic mutations occur is lower than in Brca2<sup>Wt/Tr</sup> embryos (Figure 3C).

DISCUSSION

Homozygous mutation in Brca2 has been shown in cell-based in vitro assays to decrease error-free DNA DSB repair by homology-directed gene conversion (Moynahan et al., 2001) and increase error-prone homology-directed repair (Tutt et al., 2001). Here we have used an in vivo mouse mutation reporter model, carrying a Brca2-truncating mutation, similar to those found in humans, to show that loss of both wild-type Brca2 alleles causes rapid spontaneous acquisition of genome-wide somatic mutations. These are predominantly deletion/rearrangement mutations consistent with misrepair of DNA DSBs arising during DNA replication. We have also shown that the loss of both wild-type Brca2 alleles from tissues in vivo causes an increase in the number of somatic mutations induced by a given dose of IR. Loss of the remaining wild-type Brca2 allele is presumed to be a very early event in familial breast cancer development and is found to occur in pre-invasive cancer, and even in histologically normal luminal epithelial tissue from carriers of the BRCA2 mutation (S. Lakhani, personal communication). Our data suggest that, if these results are applicable to human breast epithelial cells, IR may induce genome-wide somatic mutations at a disproportionately high rate in these BRCA2-deficient tissues in carriers of the BRCA2 mutation, thus providing the genomic diversity for selection of a more malignant phenotype. We also show that heterozygous tissues retaining a functional Brca2 allele do not acquire radiation-induced somatic mutation more frequently than the wild type, suggesting that the ‘normal’ tissues of a human BRCA2 mutation carrier may acquire IR-induced somatic mutations no faster than a non-carrier. However, these tissues may remain at risk as a result of random inactivation of the single remaining wild-type BRCA2 allele by IR and consequent induction of a mutator phenotype.

There are two sources of iatrogenic IR to which carriers of BRCA2 mutation are commonly exposed: screening mammography and radiation therapy used as an adjuvant to breast cancer induction (Hall, 2000). The nature of this dose–response curve for tissues lacking BRCA2 remains to be established. It is likely that BRCA2-deficient tissues within the treated breast that receive the full dose (50 Gy) will be protected by this effect. The untreated breast is exposed to a lower dose of scattered irradiation (2–6 Gy; Tercilla et al., 1989) and, consequently, BRCA2-deficient cells that have acquired multiple IR-induced mutations may survive to form tumours. Studying the effect of scattered irradiation on long-term contralateral breast cancer risk in BRCA2 mutation carriers requires a long follow-up and is currently unknown. If found to be significant, radiotherapy techniques that minimize the radiation dose to the untreated breast or the selective use of more extensive resection or mastectomy without radiation therapy may be preferred by some women. Our results, obtained in an embryonic mouse model of BRCA2 mutation showing a 3-fold increase in in vivo radiation mutagenesis, suggest that these issues should be further investigated in both tissue-specific Brca2 ‘knockout’ mouse models and in the clinic.

METHODS

Generation of Brca2<sup>Wt/Tr</sup> LacZ pUR288 mutation reporter strain. C57BL/6-TgNLacZpl/60vij mice were obtained from the Jackson Laboratory and were crossed with C57BL/10 Brca2<sup>Wt/Tr</sup> mice (Connor et al., 1997). These mice were then back-crossed to the C57BL/6-TgNLacZpl/60vij mutation reporter strat and homozygosity for both pUR288 concatamer integration sites was confirmed by test breeding to wild type and genotyping with primer sets specific for the two pUR288 cloned integration sites. For generation of embryos, Brca2<sup>Wt/Tr</sup> C57BL/6-TgNLacZpl/60vij mice were intercrossed and the females killed on day E14.5 gestation measured from the ‘plug’ date.

Mouse irradiation and embryo harvest. For irradiation experiments, female mice received 4 Gy whole-body irradiation on day E13.5 gestation from a Pantak 250 kV X-ray set at 90 cm focus skin distance at a dose rate of 0.75 Gy/min. Embryos were harvested 24 h later. For all experiments, embryos were harvested on day E14.5, washed in phosphate-buffered saline at 4°C and were snap frozen on dry ice and stored at −80°C. Genomic DNA was prepared by phenol–chloroform extraction.

Plasmid rescue and mutant frequency determination. Complete protocols for this system have been described elsewhere (Vijg, 1996). Briefly, between 10 and 20 μg of genomic DNA were digested with HindIII for 1 h in the presence of magnetic beads (Dynal) pre-coated with lac-lacZ fusion protein. The beads were washed three times to remove the unbound mouse genomic DNA. Plasmids were subsequently eluted from the beads with isopropl-o-thiogalactoside. After circularization with T4 DNA ligase, plasmids were used to electrotransform Escherichia coli C (ΔlacZ, galE-) cells by electroporation. Transformations were plated on titration plates containing X-gal and LacZ mutant selection plates containing P-gal. Mutant frequencies were determined as the ratio of the number of colonies on the selective plates versus titration plates. Each determination point is based on at least 100 000 recovered plasmids. The in vitro background mutant frequency of this system is ∼1 × 10<sup>−5</sup> (Dolle et al., 1999). Identification of size-change mutations was performed by digestion of rescued LacZ mutant pUR288 plasmids with Aval and PstI. To screen for size-change mutants with a breakpoint in the mouse genome, a PCR amplification was performed by using a forward primer specific for the last 18 bp
of lacZ before the HindIII site (pUR3285-F) in combination with a reverse primer after the HindIII site (pUR3421-R; Dolle et al., 2000). This procedure results in a 137 bp product only when both breakpoints are in the lacZ gene. As a positive control, a primer set (pUR4071-F and pUR4181-R) specific for the AmpR gene was included in the same reaction.

**Statistical analyses.** Mutation frequencies were compared using a two-tailed unpaired Student’s t-test. Proportions of recovered reporter plasmids containing restriction fragment size changes or with mouse genome rearrangements were compared using two-sided analysis of contingency tables by Fisher’s exact test or the Chi squared test. All statistical comparisons were performed using Graphpad Prism software.

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**A.N.J. Tutt et al.**