CHAPTER 10

RADIATION-INDUCED GENOMIC INSTABILITY IN THE OFFSPRING OF IRRADIATED PARENTS

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Abstract: So far, mutation induction in the germline of directly exposed parents has been regarded as the main component of the genetic risk of ionising radiation. However, recent data on the delayed effects of exposure to ionising radiation challenge for the existing paradigm. The results of some publications imply that exposure to ionising radiation results in elevated mutation rates detectable not only in the directly irradiated cells, but also in their non-irradiated progeny. Here I review the data on transgenerational instability showing that radiation-induced instability in the germline of irradiated parents manifests in their offspring, affecting their mutation rates and some other characteristics. This paper summarises the data on increased cancer incidence and elevated mutation rates in the germline and somatic tissues of the offspring of irradiated parents. The possible mechanisms of transgenerational instability are discussed.

Keywords: instability; mutation; radiation; germline; genetic risk; mouse

Introduction

The effort to predict the long-term genetic effects of ionising radiation for humans has certainly been one of the most important issues of radiation biology in the past years. However, despite the vast amount of experimental data describing the phenomenon of mutation induction in the directly exposed somatic and germ cells (UNSCEAR, 2001), the results of some recent studies clearly show that the genetic risks of ionising radiation may be far greater than previously thought. For example, the results of numerous in vitro studies have shown that mutation rates in the progeny of irradiated cells remains elevated over a considerable period of time after the initial exposure

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(Morgan, 2003a). The manifestation of radiation-induced genomic instability has also been reported in vivo (Morgan, 2003b). It should be stressed that these data challenge the existing paradigm in radiation biology which regards mutation induction in the directly exposed somatic and germ cells as the main component of genetic risk for humans (UNSCEAR, 2001). As mutation rates in the non-exposed progeny of irradiated cells remain considerably elevated over many cell divisions after irradiations, radiation-induced genomic instability can be regarded as a risk factor for radiation-induced carcinogenesis. It is well established that carcinogenesis is a multistep process in which somatic cells acquire mutations in a specific clonal lineage (Loeb et al., 2003). However, the pattern of accumulation of multiple mutations in the irradiated cells over a clinically relevant time period still remains unclear. It was therefore suggested that ongoing genomic instability could result in the accumulation of mutations over a certain period of time after irradiation which, together with mutations directly induced in the irradiated cells, may significantly enhance radiation carcinogenesis (Little, 2000; Goldberg, 2003; Huang et al., 2003).

The issue of the delayed effects of radiation is also highly relevant to the understanding of the mechanisms underlying a therapy-induced second malignancy (Goldberg, 2003; Sigurdson and Jones, 2003). The development of effective radiotherapy and chemotherapy regimes for the treatment of cancer has recently resulted in a dramatically increased number of long-term survivors. Given that many treatments used for cancer, including ionising radiation, are mutagenic, the impressive increase in cure and survival rates has been accompanied by a worrisome increase in the incidence of therapy-related second malignancies (Garwicz, 2000; UNSCEAR, 2000). With respect to cancer patients, the tissue at risk for the induction of secondary malignancy is normal tissue that has been exposed to ionising radiation, but not killed. It appears that therapy-related genomic instability can follow radiation therapy or chemotherapy and may thus contribute to the development of cancer in normal tissue. However, the extent to which genomic instability plays a role in the development of therapy-induced second malignancy still remains unknown.

Apart from the studies on mutation rates in somatic cells, considerable progress has been made in the analysis of radiation-induced instability in the mammalian germline, where the effects of radiation exposure were investigated among the offspring of irradiated parents (reviewed in Dubrova, 2003; Nomura 2003; Barber and Dubrova, 2006). These transgenerational studies were designed to test the hypothesis that radiation-induced instability in the germline of irradiated parents could manifest in the offspring, affecting their mutation rates and some other characteristics. The aim of this paper is to review a number of publications addressing transgenerational instability in mice and other laboratory animals. Given that a considerable number of

publications have characterised the transgenerational changes using a variety of phenotypic traits, this review mainly describes the progress made in the analysis of transgenerational changes in cancer predisposition and mutation rates.

Cancer Predisposition

The in-depth analysis of the incidence of cancer in the offspring of irradiated parents was initiated by the findings showing clustering of childhood leukaemia in the vicinity of the Sellafield nuclear reprocessing plant (Gardner et al., 1990) and a substantial increase in the incidence of tumours in the non-exposed first-generation offspring (F_1) of male mice exposed to X-rays or uretane (Nomura, 1982). It should be noted that the Nomura's data were not confirmed in the later studies (Cattanach et al., 1995, 1998), the results of which indicated that this phenomenon could partially be attributed to the seasonal variation in tumour incidence in mouse colonies. However, the results of some publications suggest that although the incidence of cancer among the offspring of exposed parents does not exceed that of the control, the morphology of the tumours in the offspring differs. For example, it has been shown that the mean number of lung adenomas per tumour in the offspring of parents pre-natally exposure to benz(*a*)pyrene remains persistently elevated over several generations (Turusov et al., 1990).

A substantial contribution to the analysis of transgenerational cancer predisposition has been made in more recent studies where the offspring of irradiated parents were exposed to carcinogens (Nomura, 1983; Vorobtsova, 1993; Lord et al., 1998; Hoyes et al., 2001). In contrast to the data obtained on the non-treated offspring, the results of these studies showed an elevated incidence of cancer among the carcinogen-challenged offspring of irradiated males (Fig. 1a).

The pattern of malignancy among the treated offspring of irradiated males was also modified (Lord et al., 1998a, b). Thus the treatment shortened latent period for the leukaemia and resulted in a switch from the predominant thymic lymphoma in the controls to a predominance of leukaemia in the offspring of irradiated males.

Somatic Mutation Rates

Given carcinogenesis is a process of accumulation of mutations in somatic cells (Loeb et al., 2003), the data showing a substantially elevated cancer risk in the offspring of irradiated parents indicates that somatic mutation rates in these animals may also be increased. Indeed, the results of some studies provide a strong evidence for transgenerational increases in somatic mutation rates.

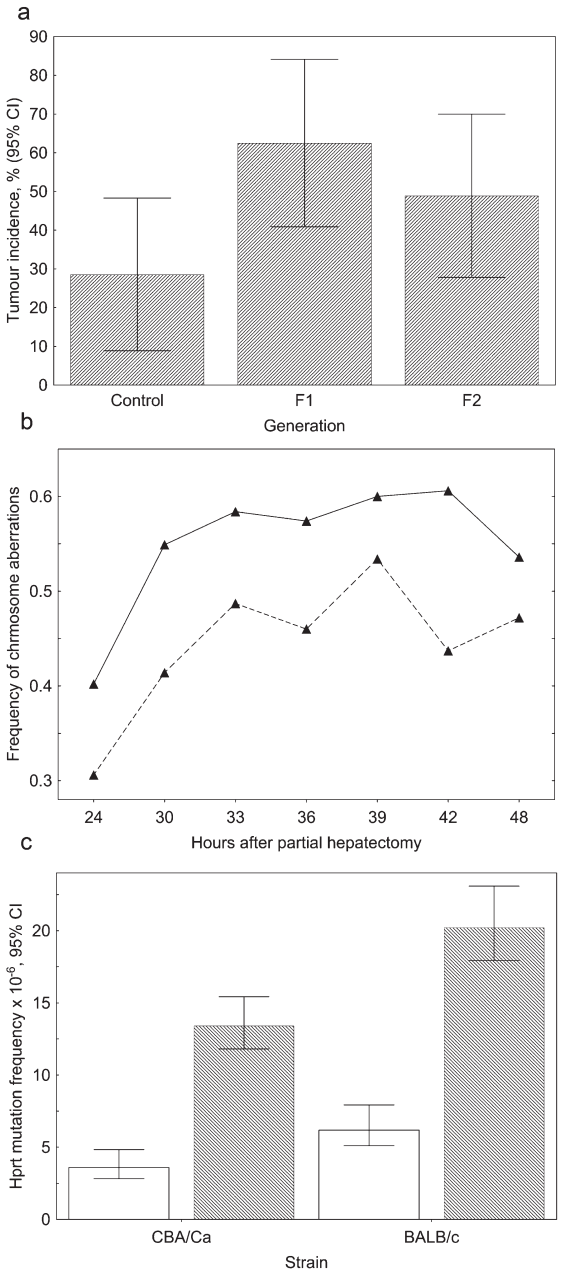

Fig. 1. Transgenerational changes in somatic tissues of the offspring of irradiated parents. (a), Promotion of skin tumours by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the offspring of irradiated male mice (data from Vorobtsova et al., 1993). (b), Frequency of chromosome aberrations in liver of controls (dashed line) and F_1 offspring (solid line) of irradiated male rats (data from Vorobtsova, 2000). (c), Frequency of *hprt* mutations in controls (open bars) and F_1 offspring of irradiated male mice (hatched bars, data from Barber et al., 2006). The 95% confidence intervals (CI) are shown.

To date, the frequency of somatic mutations in the offspring of irradiated parents has been analysed using a variety of endpoints. Thus, the study by Vorobtsova (2000) provided a convincing evidence for elevated frequency of chromosome aberrations in the liver of F_1 offspring of irradiated male rats (see Fig. 1b). These data were later confirmed by studying the frequency of chromosome aberrations in the liver and as well as in some other rat tissues (Kropacova, 2002; Slovinska et al., 2004; Sanova et al., 2005). An elevated frequency of chromosome aberrations was also found in the bone marrow tissue of F_1 offspring of irradiated male mice (Lord et al., 1998a, b). These results are further supported by the data showing an elevated frequency of micronuclei in the offspring of irradiated male mice (Fomenko et al., 2001). Given the micronuclei contain the fragments of mis-repaired or damaged chromosomes (Fenech et al., 1999), the observed increase is therefore attributed to chromosomal instability.

Transgenerational changes in somatic mutation rates were also observed by studying the frequency of mutations at some protein-coding genes. Thus, Luke et al. (1997) showed an elevated frequency of mutations at the *lac*I transgene in the F_1 offspring of irradiated male mice. The most compelling data addressing somatic instability in the F_1 offspring of irradiated male mice were obtained from the analysis of somatic reversions of the pink-eyed unstable mutation (p^{un}). The mouse p^{un} mutation is caused by DNA sequence duplication within the *pink-eyed dilute* locus and results in reduced coat and eye coloration. This duplication is highly unstable and spontaneous reversions caused by deletion of one of the duplicated sequences are quite frequent (Brilliant et al., 1991; Gondo et al., 1993). Using this endpoint, Shiraishi et al., 2002 demonstrated that the frequency of p^{un} somatic reversions in the first-generation offspring of irradiated male mice was significantly elevated. The authors studied transgenerational effects in the first-generation offspring of two reciprocal matings of irradiated fathers – $\male p^{un} \times \female p^{j}$ and $\male p^{j} \times \female p^{un}$ – and found that the frequency of somatic reversions was equally elevated in the offspring of both reciprocal matings. Given that reversions only occur at the p^{un} allele, the data showing elevated frequency of somatic reversions in the offspring of $\male p^{j} \times \male p^{un}$ mating therefore demonstrate that transgenerational instability is manifested at the alleles derived from both irradiated and non-irradiated parents. These results were supported in recent studies on somatic mutation in the offspring of irradiated fish (Shimada and Shima, 2004; Shimada et al., 2005).

We have recently analysed the frequency of thioguanine-resistant mutations at the hypoxanthine guanine phosphoribosil transferase (*hprt*) locus in the spleenocytes of F_1 offspring of irradiated male mice (Barber et al., 2006). A highly significant ~3.5-fold increase in the frequency of *hprt* mutations was found in both strains of mice (Fig. 1c). Given that in the male offspring of irradiated males the X-linked *hprt* locus is transmitted from the

non-exposed mothers, these data further confirm the abovementioned conclusion that transgenerational changes in mutation rates equally affect both alleles derived from the irradiated fathers and the unexposed mothers, thus implying a genome-wide destabilisation after fertilisation.

Germline Effects

The first evidence for the transgenerational increases in germline mutation rates was obtained by Luning et al., 1976. By analysing the frequency dominant lethal mutations in the germline of directly irradiated male mice and their first-generation offspring, the authors demonstrated that mutation rates in the germline of directly exposed parents and their non-irradiated offspring were equally elevated. Similar data were later obtained from the analysis of the F_1 offspring of male rats treated by cyclophosphamide (Hales et al., 1992). The results showing decreased proliferation of early embryonic cells and increased frequency of malformations in the F_2 offspring of irradiated parents are also consistent with these observations (Wiley et al., 1997; Pils et al., 1999). It should be noted that further analysis of transgenerational instability requires a sensitive technique capable of detecting relatively modest changes in mutation rate.

We have previously developed a new sensitive technique for monitoring mutation induction in the mouse germline by ionising radiation and chemical mutagens (Dubrova et al., 1993, 1998, 2000; Vilarino-Guell, 2003). This technique employs highly unstable expanded simple tandem repeat (ESTR) loci which consist of homogenous arrays of relatively short repeats (4–6 bp) and show a very high spontaneous mutation rates both in germline and somatic cells (Kelly et al., 1989; Gibbs et al., 1993; Bois et al., 1998; Yauk et al., 2002).

In our early studies, we have used this technique to evaluate ESTR mutation rates in the germline of F_1 and F_2 offspring of irradiated male mice (Dubrova et al., 2000; Barber et al., 2002). The analysis of the F_1 offspring of a male mouse exposed to fission neutrons showed that their germline mutation rates did not return to the mutation rates seen in unexposed individuals, but remained similar to those observed in directly exposed males (Dubrova et al., 2000). The increase was observed in most F_1 offspring and was in part attributable to increased mutational mosaicism in the germline, therefore indicating that transgenerational destabilisation should occur either immediately after fertilisation or on the very early stages of the developing F_1 germline.

To verify these results and to gain some insights on the mechanisms of transgenerational instability in the mouse germline, we later analysed ESTR mutation rates in the germline of first- and second-generation offspring of inbred male CBA/H, C57BL/6 and BALB/c mice exposed to either high-LET

fission neutrons or low-LET X-rays (Barber et al., 2002). Figure 2a presents the main result of this study, showing that paternal exposure to ionising radiation results in increased mutation rates in the germline of two subsequent generations of all inbred strains, demonstrating that transgenerational instability is not restricted to one particular inbred strain of mice. Our data also revealed inter-strain differences in the transgenerational effects, demonstrating that ESTR mutations rates in the F_1 and F_2 germline of BALB/c and CBA/H mice were significantly higher than in those of C57BL/6 mice. These data are consistent with the results of previous studies showing that that BALB/c and CBA mice are significantly more radiosensitive, and display higher levels of radiation-induced genomic instability in somatic cells than C57BL/6 mice (Roderick, 1963; Watson et al., 1997; Mothersill et al., 1999; Ponnaiya et al., 1997). The high level of radiation-induced genomic instability observed in BALB/c mice could potentially be explained by the strain-specific amino-acid substitutions affecting the activity of the $p16^{ink4a}$ cyclin-dependent kinase inhibitor and the catalytic-subunit of the DNA-dependent protein kinase (Zhang et al., 1998; Yu et al., 2001). Given the wide range of inherited variation in DNA repair capacity in humans (Mohrenweiser et al., 2003), there is potential that the same phenomenon may also exist in humans.

In this study we also compared the transgenerational effects of paternal exposure to high-LET fission neutrons and low-LET X-rays. It is well established that high-LET radiation produces highly complex and localised initial DNA damage, which is different to the sparse damage produced by low-LET radiation, resulting in the unique final biological effects of these different radiation sources (Goodhead, 1988). However, it appears that exposure to both types of radiation is capable of inducing genomic instability in somatic cells, though some studies have failed to detect the effects of low-LET exposure (Limoli et al., 2000).

It should be noted that our results indicated that most of the offspring of irradiated males showed elevated mutation rates in their germline, therefore providing important evidence for the involvement of epigenetic mechanisms in transgenerational instability. However, these data were obtained using a pedigree-based approach, which has low statistical power for the detection of mutation rate heterogeneity between individuals due to the relatively small litter size in mice. Our study also raised the possibility of transgenerational increases in ESTR somatic mutation rates. To establish whether ESTR mutation rates are equally elevated in the germline and somatic tissues of first-generation (F_1) offspring of irradiated males, we have used a single-molecule PCR approach which allows the recovery of large numbers of de novo mutants from a single individual and hence provides robust estimates of individual mutation rates (Yauk et al., 2002).

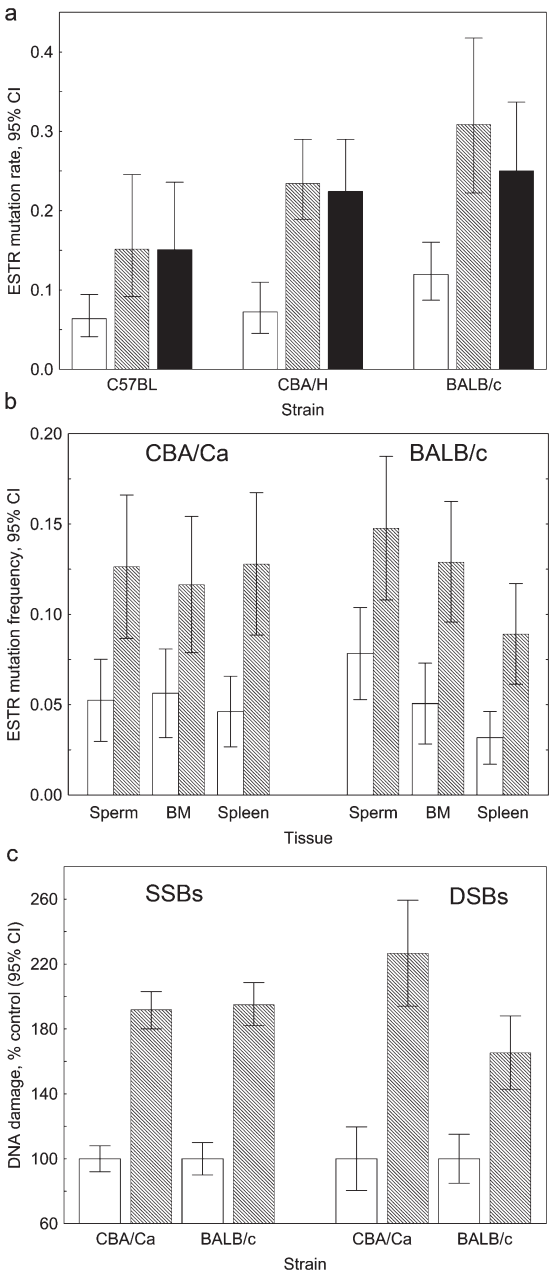

Fig. 2. Transgenerational changes in the germline and somatic tissues. (a), ESTR mutation rate in the germline of controls (open bars), F_1 (hatched bars) and F_2 (black bars) offspring of irradiated male mice (data from Barber et al., 2002). (b), ESTR mutation frequencies in the germline and somatic tissues of controls (open bars) and F_1 offspring (hatched bars) of irradiated males (data from Barber et al., 2006). (c), Endogenous DNA damage (single-strand, SSBs and double-strand, DSBs DNA breaks) in controls (open bars) and F_1 offspring (hatched bars) of irradiated male mice males (data from Barber et al., 2006).

The frequency of ESTR mutation was established in DNA samples prepared from sperm, bone marrow (BM) and spleen from the same animal (Barber et al., 2006). A statistically significant increase in the mean mutation frequency was found in all tissues of the offspring of irradiated males (Fig. 2b). These data confirmed our previous results obtained in the germline of F_1 offspring of irradiated males, using the more traditional pedigree-based approach (Dubrova, 2000; Barber, 2002) and showed that transgenerational genomic instability at ESTR loci was also manifested in somatic tissues. Most importantly, we observed that the frequency of ESTR mutation was elevated in the germline and somatic tissues of all the offspring of irradiated males (Barber et al., 2006).

Mechanisms

Cellular response to ionising radiation is a multistep process which includes the recognition of DNA damage, its repair, cell cycle arrest and apoptosis (Friedberg et al., 2006; Sancar et al., 2004; Bakkenist and Kastan, 2004). To date, the majority of pathways involved in the mammalian cellular response to radiation have been characterised. It should be stressed that the data on the delayed effects of exposure to ionising radiation represent a serious challenge to the existing paradigm. Although the mechanism(s) underlying the phenomenon of radiation-induced genomic instability still remain unknown, the results of some publications show that the ability of cells to exhibit elevated mutation rates cannot be ascribed to the conventional mechanisms of mutator phenotype and is most likely related to the epigenetic events (Lorimore et al., 2003; Morgan, 2003a,b). Such a conclusion is based on two sets of experimental data, showing that: (i) radiation-induced genomic instability persists over a long period of time after the initial exposure; (ii) the number of cells/organisms manifesting radiation-induced genomic instability is too high to be explained by the conventional mechanisms of direct targeting of DNA-repair and related genes. Our data showing that transgenerational changes affect the majority of the offspring of irradiated parents (Barber et al., 2006), together with the results of recent publication on transgenerational effects of paternal exposure to endocrine disruptors (Anway et al., 2005), further support this hypothesis.

As already mentioned, the data on transgenerational instability at the p^{un} and *hprt* loci showed that somatic mutation rates in the F_1 offspring are equally elevated at the alleles derived from the irradiated fathers and non-irradiated mothers (Shiraishi et al., 2002; Barber et al., 2006). Similar transgenerational data were obtained by studying the mouse ESTR loci (Dubrova et al., 2000; Barber et al., 2002; Niwa and Kominami, 2001). Taken together, these results show that an increased mutation rate in the offspring of irradiated males results from a genome-wide elevation of mutation rate.

In a number of publications the issue of stage-specificity of transgenerational changes has been addressed. These data provide some important clues onto the mechanisms of radiation-induced genomic instability. Thus, our data were obtained on the descendants conceived 3 and 6 weeks after the initial paternal exposure to ionising radiation (Barber et al., 2002, 2006; Dubrova et al., 2000). Given that these stages of the mouse spermatogenesis are transcriptionally active, their exposure to radiation could result in an accumulation of certain classes of RNA in the paternal germ cells which, being transmitted to the fertilised egg, may affect gene expression and stability in the developing embryo. If transgenerational instability is attributed to the zygotic transfer of RNA (Rassoulzadegan et al., 2006), then the offspring conceived just few days after paternal irradiation, from transcriptionally inert sperm cells (Rousseaux et al., 2005), should be genetically stable.

However, several recent publications report transgenerational changes in the offspring of male mice irradiated during the late post-meiotic stages of spermatogenesis, where gene expression is practically shut down (Vorobtsova et al., 1993, 2000; Shiraishi et al., 2002). Given that pre-mutational radiation-induced lesions in sperm DNA are effectively recognised and repaired within a few hours of fertilisation (Matsuda and Tobari, 1989; Derijck et al., 2006), it would therefore appear that radiation-induced damage to sperm DNA could trigger a cascade of events in the zygote, including profound changes in the expression of DNA repair genes in the pre-implantation embryo (Harrouk et al., 2000; Shimura et al., 2002) and alterations in DNA methylation and histone acetylation (Barton et al., 2002). The presence of such dramatic changes at fertilisation could also result in delayed effects, which may influence the stability of the developing embryo. The results showing an unusually high level of mutational mosaicism in the germline and somatic tissues of F_1 mice (Wiley et al., 1997; Niwa and Kominami, 2001) suggest that the destabilisation could occur at the very early stages of development.

Given that the results of transgenerational studies, together with the data on radiation-induced genomic instability in vitro clearly indicate that these phenomena are attributed to epigenetic events, we and other hypothesised that DNA methylation may be regarded as a strong candidate for such an epigenetic signal resulting in transgenerational mutagenesis (Wiley et al., 1997; Anway et al., 2005). DNA methylation and histone modification represent the main mechanisms by which DNA is epigenetically marked (Jones and Baylin, 2002). Methylation is known to survive the reprogramming of DNA methylation during spermatogenesis and early development (Holliday, 1997; Reik and Walter, 2001; Rakyan et al., 2001) and can to be transmissible through many cell divisions (Roemer et al., 1997). Alterations in the pattern of DNA methylation might affect genes responsible for maintaining genomic integrity and influence the recognition of DNA damage or its

repair. For example, promoter methylation switches off the transcription of the *hMLH1* mismatch repair gene colorectal carcinomas and results in microsatellite instability (Jones and Baylin, 2002). The transgenerational increases can also be attributed to the change in the expression patterns of genes involved in DNA repair in the offspring of irradiated males. Indeed, recent data showed persistently altered pattern of expression of some genes in the offspring of irradiated male mice (Nomura et al., 2004; Baulch et al., 2001; Vance et al., 2002).

It should be stressed that the altered expression of DNA repair genes cannot explain the transgenerational increases in mutation rates detected across a number of endpoints, including protein-coding genes, ESTR loci and chromosome aberration. Given that the mechanisms of spontaneous and induced mutation at these systems substantially differ, these observations imply that the efficiency of multiple DNA repair pathways should be simultaneously compromised in the offspring of irradiated parents. The presence of such highly coordinated changes appears to be highly unlikely. Indeed, the results of our recent study show that the efficiency of repair of some DNA lesions (detected by the alkaline Comet assay; Kassie et al., 2000) in the offspring of irradiated males is not compromised (Barber et al., 2006).

The results of our study (Barber et al., 2006) also demonstrate that the multiplicity of transgenerational changes is most likely attributed to an abnormally high level of DNA damage in F_1 offspring of irradiated parents (Fig. 2c). Given that in tissues with a high mitotic index, such as bone marrow and spleen, the life-span of cells containing deleterious lesions such as single- and double-strand breaks is restricted since these types of DNA damage are not compatible with replication, these data clearly demonstrate that transgenerational instability is an ongoing process occurring in multiple adult tissues. As unrepaired/uncorrected double- and single-strand breaks are known to be highly mutagenic and thus may result in tumour development and/or progression, our data therefore provide a plausible explanation for elevated predisposition to cancer among the offspring of irradiated parents. Given that destabilisation of genome occurs in multiple adult tissues, it is likely that transgenerational carcinogenesis may be due, in part, to this phenomenon.

The high level of DNA damage could be attributed to an oxidative stress/inflammatory response. The involvement of inflammatory-type processes in the delayed increases in mutation rates in the progeny of irradiated cells has long been suspected (Morgan, 2003a,b; Lorimore et al., 2003). Reactive oxygen species are the major source of endogenous DNA damage, including single- and double-strand breaks, abasic sites, and a variety of nucleotide modifications (Jackson and Loeb, 2001). The diversity of DNA alterations detected in the offspring of irradiated parents could therefore

be explained by this mechanism. However, according to our data, the F_1 offspring of irradiated males did not show any increases in the somatic tissue level of oxidatively damaged nucleotides. This being the case, then transgenerational instability could be attributed to replication stress. Indeed, the results of recent studies suggest that in human precancerous cells the ATR/ATM-regulated checkpoints are activated through deregulated DNA replication, which leads to the multiplicity of DNA alterations (Bartkova et al., 2005; Gorgoulis et al., 2005). It has also been shown that radiation-induced chromosome instability *in vitro* could be attributed to the long-term delay in chromosome replication (Breger et al., 2004). Given that our previous results suggest that the mechanism of ESTR mutation is most probably attributed to replication slippage (Yauk et al., 2002; Barber et al., 2004; Dubrova, 2005), delayed/stalled replication can therefore provide a plausible explanation for the transgenerational increases in mutation rate at these loci, as well as the multiplicity of DNA alterations detected in the offspring of irradiated parents.

In summary, the results of studies reviewed here provide strong evidence for long-term increases in mutation rate, observed in the offspring of irradiated parents. However, being unequivocally established in laboratory animals, the phenomenon of radiation-induced transgenerational instability in humans still awaits its final clarification. Future work should address these important issues and provide experimentally based estimates of the delayed effects of radiation in humans.

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