Paternal Exposure to Ethylnitrosourea Results in Transgenerational Genomic Instability in Mice

Running title:
ENU and Transgenerational Instability

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ABSTRACT

Recent data show that the effects of ionising radiation are not restricted to the directly exposed parental germ cells, but can also manifest in their non-exposed offspring, resulting in elevated mutation rates and cancer predisposition. The mechanisms underlying these transgenerational changes remain poorly understood. One of the most important steps in elucidating these mechanisms is to investigate the initial cellular events that trigger genomic instability. Here we have analysed the effects of paternal treatment by ethylnitrosourea, an alkylating agent which is known to form specific types of DNA adducts, on the transgenerational effects in the first-generation (F_1) offspring of exposed CBA/Ca and BALB/c male mice. Mutation rates at two expanded simple tandem repeat loci were significantly elevated in the F_1 germline of both strains. Pre- and post-meiotic exposures resulted in similar increases in mutation rate in the F_1 germline. Within each strain mutation rates were equally elevated in the germline of male and female F_1 offspring of the directly exposed males. The results of our study suggest that transgenerational instability is not attributed to a specific sub-set of DNA lesions, such as double strand breaks, and is most probably triggered by a stress-like response to a generalised DNA damage.

INTRODUCTION

The results of a number of recent studies demonstrate that mutation rates in the non-exposed progeny of irradiated cells remain highly elevated over many cell divisions following the initial exposure [Morgan, 2003]. The clinical implications of radiation-induced genomic instability, particularly its potential contribution to stepwise tumour progression, have been addressed in numerous publications [Huang et al., 2003; Goldberg, 2003]. Carcinogenesis is a multistep process in which somatic cells acquire mutations in a specific clonal lineage [Loeb et al., 2003]. How multiple mutations accumulate in the irradiated cells over a clinically relevant time period 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 [Huang et al., 2003; Goldberg, 2003]. In addition, the data on elevated mutation rates detected in the offspring of irradiated parents indicate a potential contribution of genomic instability to transgenerational carcinogenesis [Dubrova et al., 2000; Barber et al., 2002; 2006]. Taken together, these results imply that the genetic risk of ionising radiation for humans could be greater than previously predicted.

Further elucidation of the phenomenon of radiation-induced instability and proper understanding of its clinical impact is currently limited as the mechanisms underlying the long-term increases in mutation rate remain unknown. An important step to elucidate these mechanisms is to investigate the initial cellular events triggering instability signal in the exposed cells, which can subsequently lead to radiation-induced genomic instability in the progeny of irradiated cells/parents. It has been suggested that radiation-induced complex double-strand DNA breaks (DSBs) may constitute one of the signals that initiate the onset of genomic instability [Limoli et al., 1997]. If correct, then exposure to chemical mutagens that predominantly induce base damage should not result in delayed genomic instability. To test this, here we have analysed the effects of paternal exposure to the alkylating agent ethylnitrosourea (ENU) on the manifestation of genomic instability in the offspring of treated male mice. In contrast to irradiation which produces a wide spectrum of DNA lesions [Frankenberg-Schwager, 1990], exposure to ENU mainly causes alkylation of DNA at the N- and O- positions, resulting predominantly in base substitution mutations [Shibuya and Morimoto, 1993]. ENU can also induce DNA lesions attributable to either fragile alkali-labile sites [Friedberg et al., 2006] or the conversion of alkylation-induced DNA damage into double-strand breaks during DNA replication [Galli and Schiestl, 1999]. However, these lesions are far less complex and frequent than radiation-induced DSBs. This is further supported by the high prevalence of single-base substitutions in the spectrum of ENU-induced *hprt* mutations, clearly attributable to DNA alkylation [Chen et al., 2000]. In contrast, large deletions prevail in the spectrum of radiation-induced mutations at this locus, which most probably results from the mis-repair of DSBs [Nelson et al., 1994].

MATERIAL AND METHODS

Mouse Strains and Dosing

CBA/Ca and BALB/c mice were purchased from Harlan Ltd (Bicester, UK). To obtain control offspring, non-exposed CBA/Ca and BALB/c males were crossed to untreated females from the same inbred strain. CBA/Ca and BALB/c male mice, 7-8 weeks old, were given a single dose of 150 mg/kg ENU (CAS No. 759-73-9, Sigma-Aldrich, Gillingham, UK), injected into the intraperitoneal cavity immediately after dissolving in 0.9% NaCl. Exposed males were mated to control females one and ten weeks after exposure. To produce

second-generation offspring, male and female first-generation offspring were randomly taken from litters and mated with control partners from the same strain.

DNA Isolation and ESTR Typing

Genomic DNA was extracted from tails. All parents and offspring were profiled using two mouse-specific hypervariable single-locus expanded simple tandem repeat (ESTR) probes Ms6-hm and Hm-2 as described previously [Dubrova *et al.*, 1998]. DNA samples were resolved on a 40 cm long agarose gel and detected by Southern blot hybridisation. Autoradiographs were scored by two independent observers. Only bands showing a shift of at least 1 mm relative to the progenitor allele were scored as mutants.

RESULTS AND DISCUSSION

CBA/Ca and BALB/c inbred stains of mice were previously used in our studies on radiation-induced transgenerational instability in mice [Barber et al., 2002; 2006; Hatch et al., 2007]. The treated males (generation F_0) were mated to control females one and ten weeks after exposure, thus ensuring that their offspring were derived either from exposed sperm or spermatogonia stem cells, respectively [Searle, 1974]. The genotyping of animals was performed after all breeding was completed, thus ensuring that the male and female F_1 offspring were randomly taken from litters before mating with control partners (Fig. 1a). The number of mutations scored in all first- and second-generation offspring of exposed males was divided by the total number of offspring in that generation to give an estimate of germline mutation rates for the germline of F_0 and F_1 parents.

Table I presents a summary of the mutation data. Given that the paternal and maternal mutation rates did not differ for the control parents (Fisher's exact test, P=0.99 and P=0.76 for CBA/Ca and BALB/c mice, respectively), it was therefore possible to combine data across the sexes to produce single estimate of ESTR mutation rate for the control groups. Pre-meiotic exposure of stem cells (10 weeks) resulted in substantially elevated mutation rate in the germline of treated males. In contrast, the post-meiotic exposure of sperm cells (1 week) did not increase ESTR mutation rate. These data therefore confirm our previous results on the stage-specificity of mutation induction at mouse ESTR loci by ionising radiation and ENU [Dubrova et al., 1998; Vilarino-Guell et al., 2003].

We next analysed ESTR mutation rates in the germline of F_1 offspring of ENU-treated males. We first compared the transgenerational effects of exposure of post- and pre-meiotic spermatogenic cells, as well as the transmission of transgenerational instability through paternal and maternal F_1 germline. That is why within each inbred strain ESTR mutation rates were separately evaluated in the germline of F_1 male and female offspring conceived either one or ten weeks after paternal exposure to ENU (Table I). Using the two-way ANOVA, we tested the homogeneity of mutation rate (arc-transformed) for all offspring of treated males. The results of this analysis did not reveal any significant heterogeneity within strains (CBA/Ca: stage of paternal exposure, P=0.24; F_1 sex, P=0.45; BALB/c: stage of paternal exposure, P=0.83; F_1 sex, P=0.18). We therefore conclude that the F_1 ESTR mutation rates do not significantly differ within each inbred strain.

We next compared mutation rates in the germline of F_1 offspring conceived 1 and 10 weeks after paternal exposure to ENU (Table I). The magnitude of transgenerational increases for both exposures were similar. In our previous study we found that the extent of transgenerational instability in the offspring of male mice conceived 1 and 10 weeks after paternal exposure to ionising radiation did not significantly differ [Hatch *et al.*, 2007]. Within each strain, ESTR mutation rates were equally elevated in the germline of male and female F_1

offspring of exposed males (Table I), which is also in line with our previous data on radiation-induced transgenerational instability in mice [Dubrova *et al.*, 2000; Barber *et al.*, 2002]. Overall, the results of our current study show that ESTR mutation rates are highly significantly elevated in the F_1 germline of both inbred strains.

In summary, the analysis of ESTR mutation rates in the germline of ENU-treated male mice and their offspring has revealed a number of striking similarities between the mutagenic and transgenerational effects of paternal exposure to this mutagen and ionising radiation (Fig. 1b). First of all, the F_0 pre-meiotic exposure to both agents is highly mutagenic. Second, the F_1 offspring of irradiated or ENU-treated males show highly elevated ESTR mutation rates in their germline, the magnitude of which is not affected by the stage of paternal exposure and is similar for both F_1 male and female offspring. Finally, the transgenerational effects are not strain-specific and manifest in both inbred strains studied. Given the profound differences in the spectrum of ENU- and radiation-induced DNA damage, our data suggest that transgenerational instability is not attributed to a specific sub-set of DNA lesions, such as DSBs, but is most probably triggered by a stress-like response to a generalised DNA damage. Although the mechanisms underlying the long-term instability detected in the progeny of exposed cells/organisms still remain unknown, the results of a number of 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 [Morgan, 2003; Dubrova, 2003]. The results presented here raise the possibility that cellular response to DNA damage induced by high-dose exposure to ionising radiation or chemical mutagens may be compromised, which in turn, could result in the long-term epigenetic alterations in the directly affected germ cells and the F_1 offspring. Our data also suggest that exposure to a wide range of DNA-damaging mutagens could destabilise the non-exposed progeny of affected cells/parents. Indeed, it has been shown that that exposure to some chemical carcinogens and mutagens can result in a delayed increase in mutation rate in somatic cells [Limoli *et al.*, 1997; Bardelli *et al.*, 2001; Li *et al.*, 2001; Gowans *et al.*, 2006] or affect the fitness of the offspring of exposed male rats [Hales *et al*., 1992]. Given the existing high-dose human exposure to anticancer drugs, the majority of which belong to DNA-damaging agents and mutagens [Witt and Bishop, 1996], our results also raise the possibility of transgenerational effects of such exposures that may represent a potentially significant genetic hazard. Future studies should address this important issue.

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REFERENCES

Bardelli A, Cahill DP, Lederer G, Speicher MR, Kinzler KW, Vogelstein B, Lengauer C. 2001. Carcinogen-specific induction of genetic instability. Proc Natl Acad Sci USA 98:5770-2775.

Barber RC, Hickenbotham P, Hatch T, Kelly D, Topchiy N, Almeida G, Jones GGD, Johnson GE, Parry JM, Rothkamm K, Dubrova YE. 2006. Radiation-induced transgenerational alterations in genome stability and DNA damage. Oncogene 25:7336-7342.

Barber R, Plumb MA, Boulton E, Roux I, Dubrova YE. 2002. Elevated mutation rates in the germline of first- and second-generation offspring of irradiated male mice. Proc Natl Acad Sci USA 99:6877-6882.

Chen Y, Yee D, Dains K, Chatterjee A, Cavalcoli J, Schneider E, Om J, Woychik R, Magnuson T. 2000. Genotype-based screen for ENU induced mutations in mouse embryonic cells. Nat Genet 24:314-317.

Dubrova YE. 2003. Radiation-induced transgenerational instability. Oncogene 22:7087-7093.

Dubrova YE, Plumb M, Brown J, Fennelly J, Bois P, Goodhead D, Jeffreys AJ. 1998. Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation. Proc Natl Acad Sci USA 95:6251-6255.

Dubrova YE, Plumb M, Gutierrez B, Boulton E, Jeffreys AJ. 2000. Transgenerational mutation by radiation. Nature 405:37.

Frankenberg-Schwager M. 1990. Induction, repair and biological relevance of radiation-induced DNA lesions in eukaryotic cells. Radiat Environ Biophys 29:273-292.

Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T. 2006. DNA repair and mutagenesis. 2nd edition. Washington: ASM Press, 1118 p.

Galli A, Schiestl RH. 1999. Cell division transforms mutagenic lesions into deletion-recombinagenic lesions in yeast cells. Mutat Res 429:13-26.

Goldberg Z. 2003. Clinical implications of radiation-induced genomic instability. Oncogene 22:7011-7017.

Gowans ID, Lorimore SA, McIlrath JM, Wright EG. 2005. Genotype-dependent induction of transmissible chromosomal instability by gamma-radiation and the benzene metabolite hydroquinone. Cancer Res 65:3527-3530.

Hales BF, Crosman K, Robaire B. 1992. Increased postimplantation loss and malformations among the F_2 progeny of male rats chronically treated with cyclophosphamide. Teratology 45:671-678.

Hatch T, Derijck AAHA, Black PD, van der Heijden GW, De Boer P, Dubrova YE. 2007. Maternal effects of the *scid* mutation on radiation-induced transgenerational instability in mice. Oncogene 26:4720–4724.

Huang L, Snyder AR, Morgan WF. 2003. Radiation-induced genomic instability and its implications for radiation carcinogenesis. Oncogene 22:5848-5854.

Li C-Y, Little JB, Hu K, Zhang L, Dewhirst MW, Huang Q. 2001. Persistent genetic instability in cancer cells induced by non-DNA-damaging stress exposures. Cancer Res 61:428-432.

Limoli CL, Kaplan MI, Phillips JW, Adair GM, Morgan WF. 1997. Differential induction of chromosomal instability by DNA strand-breaking agents. Cancer Res 57:4048-4056.

Loeb LA, Loeb KR, Anderson JP. 2003. Multiple mutations and cancer. Proc Natl Acad Sci USA 100:776-781.

Morgan WF. 2003. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effect *in vitro*. Radiat Res 159:567-580.

Nelson SL, Giver CR, Grosovsky AJ. 1994. Spectrum of X-ray-induced mutations in the human *hprt* gene. Carcinogenesis 15:495-502.
Searle AG. 1974. Mutation induction in mice. Adv Radiat Biol 4:131-207.
Shibuya T, Morimoto K. 1993. A review of the genotoxicity of 1-ethyl-1nitrosourea. Mutat Res 297:3-38.
Vilarino-Guell C, Smith AG, Dubrova YE. 2003. Germline mutation induction at mouse repeat DNA loci by chemical mutagens. Mutat Res 526:63-73.
Witt KL, Bishop JB. 1996. Mutagenicity of anticancer drugs in mammalian germ cells. Mutat Res 355:209-234.

Table I. Summary of ESTR mutation data

Strain, group[a]	No mutations (offspring)	Mutation rate	Ratio to control	P^c
CBA/Ca				
Control (10♂, 21♀)[b]	25 (121)	0.0516	-	-
ENU F_0, 1 week (8♂)	7 (47)	0.0745	1.44	0.5042
ENU F_0, 10 weeks (10♂)	32 (66)	0.2424	4.69	2.86×10^{-9}
ENU F_1, 1 week (10♂, 8♀)	41 (114)	0.1798	3.48	2.43×10^{-7}
ENU F_1, 10 weeks (6♂, 5♀)	26 (77)	0.1688	3.27	3.18×10^{-5}
ENU F_1, males (16♂)	38 (105)	0.1810	3.50	4.01×10^{-7}
ENU F_1, females (13♀)	29 (86)	0.1686	3.26	1.27×10^{-5}
ENU F_1, total	67 (191)	0.1754	3.40	7.30×10^{-9}
BALB/c				
Control (14♂, 25♀)[b]	47 (148)	0.0794	-	-
ENU F_0, 1 week (8♂)	4 (39)	0.0513	0.65	0.5337
ENU F_0, 10 weeks (6♂)	29 (51)	0.2843	3.58	1.10×10^{-7}
ENU F_1, 1 week (10♂, 5♀)	43 (103)	0.2087	2.63	2.87×10^{-6}
ENU F_1, 10 weeks (9♂, 9♀)	34 (109)	0.1560	1.96	0.0028
ENU F_1, males (19♂)	47 (117)	0.2008	2.53	3.62×10^{-6}
ENU F_1, females (14♂)	30 (95)	0.1579	1.99	0.0037
ENU F_1, total	77 (212)	0.1816	2.29	1.78×10^{-6}

[a]The number of male and female parents is given in parentheses.
[b]Total number of paternal and maternal mutation is shown (see text).
[c]Probability of difference from the control group (Fisher's exact test, two-tailed).

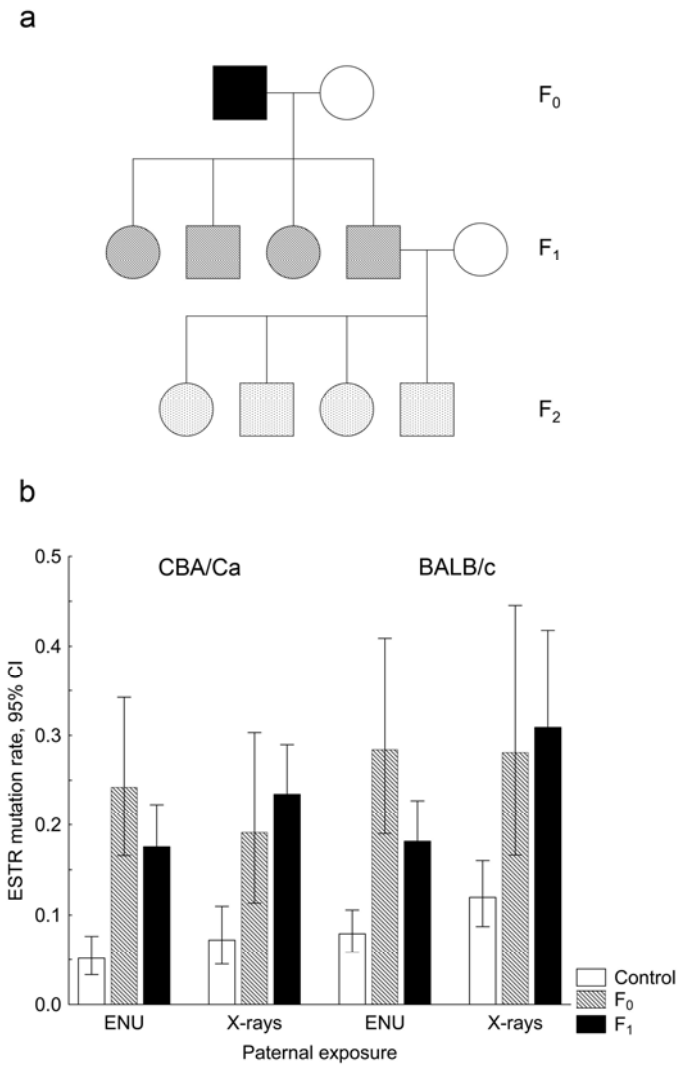

Fig. 1. The effects of paternal exposure on ESTR mutation rates in the germline of treated males and their F_1 offspring. (**a**) Design of the transgenerational study. Exposed male is in black; its F_1 and F_2 offspring are in grey; control parents with no history of exposure are in white (see text for breeding details). (**b**) ESTR mutation rates in the germline of ENU-exposed and irradiated males and their offspring. The 95% confidence intervals, CI for mutation rate, estimated from the Poisson distribution are shown. Data for irradiated mice and their F_1 offspring are taken from Barber et al. [2002]. The F_0 data are shown for male mice mated 6 (X-rays) and 10 (ENU) weeks after exposure.