Mainstream Tobacco Smoke Causes Paternal Germ-Line DNA Mutation

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Abstract

Despite the presence of known mutagens and carcinogens in cigarette smoke, there is currently no evidence to show that smoking, or exposure to cigarette smoke, can result in heritable genetic mutation. We show that male mice exposed to mainstream tobacco smoke (MTS) exhibit a significant increase in germ-line mutation frequency in spermatogonial stem cells. We exposed mature male mice to MTS for 6 or 12 weeks and investigated mutations arising in exposed spermatogonial stem cells at the expanded simple tandem repeat locus *Ms6-hm*. A generalized score test showed a significant treatment effect (P = 0.0214). *Ms6-hm* mutation frequency was 1.4 and 1.7 times higher in mice exposed to MTS for 6 and 12 weeks, respectively, compared with sham controls. The data suggest that mutations accumulate in the spermatogonial stem cells with extended exposures. Mutation spectra were identical between exposed and sham individuals, supporting the hypothesis that tandem repeat mutations arise through indirect mechanisms of mutation. Mutations in sperm that are passed on to offspring cause permanent, irreversible changes in genetic composition and can persist in future generations. Our research suggests that the consequences of smoking extend beyond the smoker to their nonsmoking descendents. [Cancer Res 2007;67(11):5103–6]

Introduction

It has been estimated that half a billion smokers will die of smoking-associated disease (1). Despite this, over a billion people worldwide continue to smoke. Unarguably, it is a major public health concern, and deterrents are greatly needed to encourage individuals to reduce or discontinue smoking. Mainstream tobacco smoke (MTS) contains more than 4,000 chemicals, many of which are genotoxic, and is considered the most extreme example of a human systemic mutagen (2). The mechanisms by which exposure to tobacco smoke causes cancers have been studied extensively over the past 20 years. One mechanism thought to result in cancer operates through the mutagenic activity of components of tobacco smoke (reviewed in ref. 2). Exposure to cigarette smoke condensate *in vitro* is mutagenic in the Ames and SOS bacterial assays and induces mutation in the $Tk^{+/-}$ locus in mouse lymphoma cells and *Hprt* mutations in Chinese hamster ovary cells (reviewed in refs. 2, 3). Many studies have shown that exposure to cigarette smoke condensate and MTS can cause DNA damage (strand breaks and micronuclei and chromosomal aberrations) in somatic cells *in vitro* and *in vivo* in animal models (4). Human smokers show increased numbers of DNA strand breaks in lymphocytes, buccal cells, and urothelial cells; higher *HPRT* mutation frequency in peripheral blood lymphocytes; and enhanced urinary mutagenicity (reviewed in ref. 2). The results of these studies show that tobacco smoke is clearly a mammalian somatic cell mutagen.

In addition to cancer and mutation, exposure to MTS is also known to cause reproductive effects such as infertility (5). DNA strand breaks, DNA adducts, chromosomal aberrations, and aneuploidy arise in human sperm as a result of exposure to MTS and provide indirect support that MTS is a human germ cell mutagen (2). However, structural and numerical anomalies, such as breaks and aneuploidies, are the result of exposure to clastogens and are likely to be lethal to the developing embryo (6). There are no data showing that exposure to MTS causes heritable DNA sequence mutation in humans or in any animal model. The implications of increased heritable DNA sequence mutation include potential changes in the incidence of genetic diseases (including cancer) among the non-exposed descendents of smokers. In this study, we investigate DNA sequence mutation at expanded simple tandem repeat (ESTR) DNA in sperm samples from mature male mice exposed to MTS for 6 or 12 weeks. ESTRs are highly unstable in the germ-line and provide the most sensitive marker available today for the measurement of induced germ-line mutation (7, 8).

Materials and Methods

Animal care and husbandry. Twenty mature (8–10 weeks old) male C57BL/6 × CBA F1 hybrid mice (The Jackson Laboratory) were used in this study. Mice were housed in a 12-h light-dark cycle with food and water *ad libitum*. Cages, food, and bedding were autoclaved. To control for handling, groups of mice were placed simultaneously in restrainers only (sham exposure). Mice were exposed to MTS in a smoke exposure system that was initially developed for guinea pigs (9) and has since been adapted for mice (10). Mice were placed in individual exposure chambers ($9 \times 3 \times 3$ cm^3) and were exposed to two cigarettes daily (1R3 reference cigarettes; Tobacco and Health Research Institute, University of Kentucky). Cigarette smoke was delivered into the exposure chambers at a rate of 0.08 L/min, 1 puff (20 mL) per 52 s. In an initial 2-week lead up period, mice were exposed to one cigarette in the 1st week and to two cigarettes in the 2nd week. Animals were then exposed 5 days/wk for a total of 6 weeks, or 12 weeks, including the 2-week lead-up period. Both exposure groups (alongside matched sham controls) were housed for an additional 6 weeks following the final cigarette to allow for the maturation of spermatogonial stem cells. Animals were euthanized, and caudal epididymus was collected and stored at −80°C. All animal procedures were carried out under the guidelines of the Canadian Council on Animal Care and procedures approved by the McMaster University Animal Research Ethics Board.

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Isolation of DNA for mutational analysis. All isolations and manipulations before gel electrophoresis were carried out in a laminar flow hood in a different laboratory room to minimize the risk of contamination of the PCR templates. The methods used were similar to those described in Yauk et al. (11). Caudal epididymus DNA was finely chopped in PBS, filtered, and centrifuged. The sperm pellet was resuspended in 1 × SSC, and the somatic cells were lysed in 0.15% SDS. The lysate was removed by centrifugation, and the resulting pellet was resuspended in 0.2 × SSC, 1% SDS, 1 mol/L 2-mercaptoethanol and digested with Proteinase K. Elimination of somatic cells was confirmed by phase-contrast microscopy. DNA was recovered by phenol/chloroform extraction. Five micrograms of DNA were digested using *Mse*I (10 units at 37°C overnight). The DNA was then diluted in buffer [5 mmol/L Tris-HCl (pH 8)] supplemented with 5 ng/μL carrier Herring Sperm DNA before PCR amplification.

Detection of mutants. Template DNA was diluted in the PCR to concentrations that allowed ~40% to 60% of amplifications to produce observable products. The *Ms6-hm* locus (accession no. S69706) was amplified with 0.5 μmol/L flanking primers HM1 forward (5′-AGAGTTTC-TAGTTGCTGTGA-3′) and HM1 reverse (5′-GAGAGTCAGTTCTAAGGC-3′) using a PTC-225 DNA Engine Tetrad Thermocycler unit (MJ Research). PCR products were visualized by gel electrophoresis, Southern blotting and hybridization with α^{32}P radiolabeled *Ms6-hm* probe. Positive PCR amplicons of the CBA 1.7-kb *Ms6-hm* allele were run on 40-cm, 0.7% agarose gels at 230 V in cooled chambers (15°C) for 20 to 24 h. Gels were Southern blotted onto nylon membranes and hybridized with *Ms6-hm* probe for visualization using a phosphoimager (Molecular Dynamics, Storm 860). The image sizes of the blots were normalized to achieve 4.5-cm spacing between the 4- and 5-kbp bands of an in-lane DNA standard (50 ng per well of 1-kbp INVITROGEN ladder). All gels were scored blindly by two individuals, without prior knowledge of sample identities. The *Ms6-hm* alleles were sized relative to the in-lane standard. The distance of each *Ms6-hm* band was measured from the internal lane standards to determine size. Bands were classified as mutants if their size differed from the progenitor allele by more than 4 repeat units. This is a conservative estimate of mutation, but allows high confidence in the measured amplicon length change. With the assumption that each mutant allele scored represents just one template molecule, the mutation frequency becomes the ratio of the number of mutant bands to template molecules. The total number of template molecules per 96-well PCR plate was estimated using a statistical approximation (see below).

Statistical analysis. The number of progenitor PCR template molecules was determined from the number of positive PCR products using the Poisson distribution, estimated within technical replicates of samples. The rate variable was estimated by taking the natural log of the observed number of PCR wells with no product to the total number of PCR wells run. The ratio of the exposed to control mutation frequencies was estimated by back-transforming results from the Wald statistic with the empirical covariance matrix using the GENMOD Procedure in the SAS/STAT software, version 8.2 of the SAS System for Windows (1999–2001 SAS Institute, Inc.). A generalized score test was used to examine treatment effect in SAS.

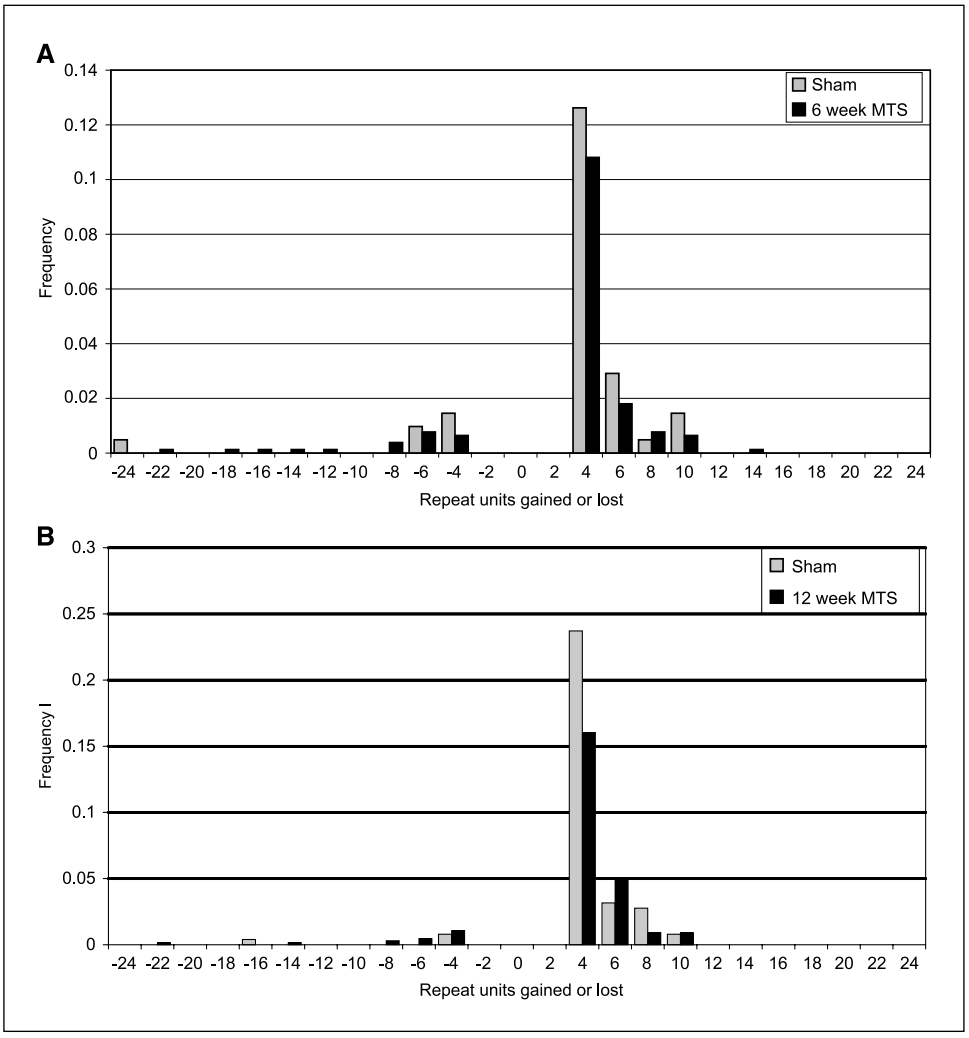

Figure 1. Frequency distribution of the size of mutants recovered from the sham- and MTS-exposed males. A significant bias towards small gains (≥4 repeat units) was observed in all groups (generalized score test, $P = 0.005$). There were no significant differences in the spectra of mutations measured between any of the groups (generalized score test, $P = 0.19$). *A,* mutation spectrum for the 6-wk group. *B,* mutation spectrum for the 12-wk group.

Table 1. Mutation data for the *Ms6-hm* locus summarized for cigarette- and sham-exposed mice

Treatment*	No. mutants	No. progenitors †	Mutation frequency ‡	Ratio relative to sham§	*P*
Sham (6 wk)	24	679 (629–731)	3.53% (3.28%, 3.81%)		
Smoke-exposed (6 wk)	39	766 (713–821)	5.09% (4.75%, 5.47%)	1.44 (0.87, 2.39)	0.1982
Sham (12 wk)	24	603 (555–652)	3.98% (3.68%, 4.32%)		
Smoke-exposed (12 wk)	42	663 (613–714)	6.33% (5.88%, 6.85%)	1.69 (1.05, 2.73)	0.0442‖
Sham (total)	48	1,281 (1,212–1,352)	3.74% (3.55%, 3.96%)		
Smoke (total)	81	1,428 (1,355–1,503)	5.67% (5.39%, 5.98)	1.56 (1.10. 2.21)	0.0214‖

NOTE: Fold-change values were calculated using the generalized linear model and can give slightly different values compared to estimates calculated by pooling samples.

*Five mice were used in each treatment. Animals were held for 6 wks following the last day of exposure to collect cells that were pre-meiotic stem cells during the exposure from the caudal epididymus. Sham and smoke refer to the pooled totals for all sham and smoke samples, respectively.

† The estimation of the number of progenitor molecules was determined from the number of positive PCR products generated using the Poisson distribution, pooling across samples. Predicted as well as the lower-upper range of the number of molecules tested, and mutation frequency.

‡ The average mutation frequency is given. Numbers in brackets indicate the uncertainty resulting from the estimate derived for the number of progenitors scored.

§The ratios of the samples versus shams are given with 95% confidence intervals (calculated using a generalized linear model).

‖$P < 0.05$, statistically significant (generalized score statistic).

Results and Discussion

Analysis of replicates of spermatogonial DNA from individual sham males yielded a mean *Ms6-hm* mutation frequency of 3.7% (range, 2.2–6.9%) and a significant bias towards gain mutations (generalized score test, $P = 0.005$; Fig. 1*A* and *B*) in keeping with published data (11, 12). There was no significant difference in mutation frequencies between 6- and 12-week sham samples. Mean *Ms6-hm* mutation frequencies were 5.1% (range, 3.9–6.9%) and 6.3% (range, 4.6–9.6%) following exposure to MTS for 6 or 12 weeks (Table 1). A significant increase in mutation frequency was found following exposure to MTS for 12 weeks ($P = 0.044$). A generalized score test showed a significant treatment effect ($P = 0.021$) and a 50% increase in overall mutation frequency for the combined exposures (Table 1).

We show that exposure to MTS for 12 weeks caused DNA sequence mutation in spermatogonial stem cells in mature mice. These data provide the first evidence that smoking causes paternal germ-line DNA sequence mutation in any mammal. Our previous work showed that *Ms6-hm* mutation frequencies measured in sperm DNA were indistinguishable from those measured in offspring using conventional pedigree analysis (11). Therefore, paternal *Ms6-hm* mutations arising in sperm may be inherited by progeny. These mutations are thought not to result from direct damage to the DNA at the site of mutation but rather result from an indirect process attributed to factors elsewhere in the paternal germ-line (8, 11). In keeping with this hypothesis, the spectra of mutations (defined in this case as the frequency distribution of size mutants) were identical between MTS- and sham-exposed mice (generalized score test, $P = 0.19$; Fig. 1*A* and *B*). One hypothesis for indirect induced mutation is DNA damage elsewhere in the genome (non-targeted DNA damage, another cellular signal, or possibly epigenetic signals) causes DNA polymerases to pause to repair damage. If a pause in a polymerase crossing an ESTR locus occurs, hairpins may result that are transformed into mutations upon replication re-initiation. It is hypothesized that the pausing of replication facilitates replication slippage events that result from the formation of secondary structures among repeat sequences in the ESTR arrays (13, 14). Our data suggest that cigarette smoke exposure interferes with normal DNA metabolism, or replication, through similar processes and results in *de novo* DNA sequence mutation. Increased germline DNA mutation may affect the incidence of genetic disease in the offspring of fathers who smoked before conception. The findings indicate that the repercussions of smoking may extend beyond cancer and mutation in exposed individuals, to their unexposed descendents, and potentially contribute to the population burden of genetic disease.

MTS exposure caused a 1.4- and 1.7-fold increase in ESTR mutation frequency over sham-exposed males after 6 and 12 weeks of exposure, respectively (Table 1). As mutation frequencies were higher in the longer-exposure (12 weeks) group, the data further indicate that mutations may accumulate in the spermatogonial stem cells. Therefore, the longer the exposure, the higher the potential risk for heritable mutation. Further work is necessary to confirm this prediction.

Previous analyses have shown that ESTR loci are sensitive to both chronic and acute high- and low-LET radiation (12), specific highly mutagenic chemicals (15), and heavily contaminated industrial air particulate pollution (16, 17). ESTR mutations arise through an indirect mechanism that results in genetic instability at repeat loci in the germ line (8, 18) but correlate with DNA sequence mutation at other endogenous protein-coding loci (19). However, a clear relationship between tandem repeat instability and mutation induction at protein coding loci is currently unknown. Nevertheless, size changes at many tandem repeats have important functional consequences, such as those at the triplet repeat disease loci (20). There may be a larger number of loci at which more subtle functional effects remain to be discovered. Therefore, the consequences of increased tandem repeat mutation should not be dismissed as unimportant (20). Accordingly, it would be prudent for men who smoke to curtail such activity before reproduction. Further studies should investigate the persistence of instability following MTS exposure to determine the minimum recommended cessation period before reproduction.

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References

1. IARC. Tobacco Smoke and Involuntary Smoking. In: IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, Vol. 83. Lyon (France): IARC, 2004.
2. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutat Res 2004; 567:447–74.
3. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate. Mutat Res 1983;114:59–89.
4. IARC. Tobacco smoking. In: IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, Vol. 38. Lyon (France): IARC, 1986.
5. Practice Committee of the American Society for Reproductive Medicine. Smoking and infertility. Fertil Steril 2004;81:1181–6.
6. Green S, Auletta A, Fabricant J, et al. Current status of bioassays in genetic toxicology: the dominant lethal assay. A report of the U S. Environmental Protection Agency Gene-Tox Program. Mutat Res 1985;154:49–67.
7. Dubrova YE, Plumb M, Brown J, et al. Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation. Proc Natl Acad Sci U S A 1998;95: 6251–5.
8. Yauk CL. Advances in the application of germline tandem repeat instability for *in situ* monitoring. Mutat Res 2004;566:169–82.
9. Simani AS, Inoue S, Hogg JC. Penetration of the respiratory epithelium of guinea pigs following exposure to cigarette smoke. Lab Invest 1974;31:75–81.
10. Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. Science 1997;277: 2002–4.
11. Yauk CL, Dubrova YE, Grant GR, Jeffreys AJ. A novel single molecule analysis of spontaneous and radiation-induced mutation at a mouse tandem repeat locus. Mutat Res 2002;500:147–56.
12. Dubrova YE. Radiation-induced mutation at tandem repeat DNA loci in the mouse germline: spectra and doubling doses. Radiat Res 2005;163:200–7.
13. Barber RC, Miccoli L, van Buul PP, et al. Germline mutation rates at tandem repeat loci in DNA-repair deficient mice. Mutat Res 2004; 554:287–5.
14. Burr KL, Velasco-Miguel S, Duvvuri VS, McDaniel LD, Friedberg EC, Dubrova YE. Elevated mutation rates in the germline of Polkappa mutant male mice. DNA Repair (Amst) 2006;5:860–2.
15. Vilarino-Guell C, Smith AG, Dubrova YE. Germline mutation induction at mouse repeat DNA loci by chemical mutagens. Mutat Res 2003;526:63–73.
16. Somers CM, McCarry BE, Malek F, Quinn JS. Reduction of particulate air pollution lowers the risk of heritable mutations in mice. Science 2004;304:1008–10.
17. Somers CM, Yauk CL, White PA, Parfett CL, Quinn JS. Air pollution induces heritable DNA mutations. Proc Natl Acad Sci U S A 2002;99:15904–7.
18. Niwa O. Indirect mechanisms of genomic instability and the biological significance of mutations at tandem repeat loci. Mutat Res 2006;598:61–72.
19. Barber RC, Hickenbotham P, Hatch T, et al. Radiation-induced transgenerational alterations in genome stability and DNA damage. Oncogene 2006;25:7336–42.
20. Armour JAL. Tandemly repeated DNA: why should anyone care? Mutat Res 2006;598:6–14.

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