Tobacco Smoke Exposure and Somatic Mutation in Newborns

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Abstract: Maternal exposure to tobacco smoke is known to have deleterious effects on the developing fetus, but it has only recently been shown that there may be life-long consequences due to genotoxic damage. Analysis of newborn cord bloods with the *GPA* somatic mutation assay demonstrates a significant effect of maternal active smoking and suggests that similar mutational induction occurs in mothers who experience only secondary exposure to environmental tobacco smoke (ETS). Moreover, in both cases, mutational induction occurs by the same molecular mechanism, likely chromosome missegregation, resulting in an effective loss of one parental chromosome 4 and duplication of the other. These data also suggest that quitting smoking during pregnancy without actively avoiding secondary ETS exposure is not effective at protecting the unborn child from the genotoxic effects of tobacco smoke.

Keywords: Maternal smoking, environmental tobacco smoke, *in utero* genotoxicity, glycophorin A assay.

INTRODUCTION

Exposure to tobacco smoke metabolites is associated with cancer in the adult and teratogenicity *in utero* [1], but it has been difficult to document a mutagenic effect in the newborn. Recently, we found that the choice of control population was critical, in that passive tobacco smoke exposure amongst our "non-smokers" induced the same level of mutation at the *HPRT* gene in newborns as did active maternal smoking, and confounded the overall analysis of smoking effects [2]. We also found a similar induction of cord blood mutations in the children of mothers who quit smoking after they became aware that they were pregnant. However, we cautioned that these results were based upon a single X-linked reporter locus. We now present results that extend these observations to a second, autosomal, reporter gene, confirming a significant effect of active maternal smoking and again suggesting that quitting smoking during pregnancy without actively avoiding secondary exposure to environmental tobacco smoke (ETS) has no protective effect on the developing fetus.

MATERIALS AND METHODOLOGY

The glycophorin A (*GPA*) human *in vivo* somatic mutation assay is based on the autosomal gene coding for the MN blood group. In $GPA^{M/N}$ heterozygotes, the standard flow cytometric *GPA* assay quantifies allele loss at the M allele and allows for the phenotypic characterization of mutant erythrocytes as arising due to either simple allele loss (N/Ø mutants) or concomitant allele loss-and-duplication (N/N mutants) [3]. For this study, we reanalyzed our own published data (N = 114) that had concluded that there was no effect of maternal tobacco smoking on their offspring, because the children of active smokers exhibited *GPA* mutation frequencies (M_f) not significantly different from those of "non-smoking" mothers (P = 0.14), a group in which more than half (59%) reported extensive long-term secondary exposure [4]. Since the reanalysis involved a unique stratification of the maternal/fetal population, we confirmed that the new groups were well matched with regard to demographics and lifestyle factors. In particular, average maternal ages ranged only from 22.8 to 24.0 years amongst the four exposure groups, and all groups were predominantly Caucasian, with successively smaller proportions of Hispanics and African-Americans. The only demographic factor previously associated with *GPA* mutation frequency, age [5], is obviously not relevant to a comparison of newborns.

RESULTS

Table **1** shows a comparison of the *GPA* M_f of children whose mothers smoked throughout pregnancy, quit during pregnancy or experienced exposure to ETS throughout their pregnancy with those of children whose mothers reported no primary or secondary exposure during their pregnancy. Both the combined exposed group and the primary smoking group (the single largest subset) had significantly higher total *GPA* M_f than the unexposed group. Although they were not individually significant, the total *GPA* M_f of the children of mothers who quit smoking during pregnancy and those who reported only passive exposures were elevated such that they were not significantly different from those of the children of active smokers (P = 0.84, 0.85, respectively).

Perhaps surprisingly, separation of the total *GPA* M_f into their mechanistic components reveals that most of the mutagenic effects of tobacco smoke in this study are manifested in the allele loss-and-duplication category, which is primarily associated with chromosomal rather than gene-specific molecular events (recombination and missegregation) [7]. Tobacco smoke exposure has usually been associated with induction of gene-specific "point" mutations [8], although these accounted for only 25% of the newborn mutations summarized in the original report [4]. While none

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Table 1. *GPA* M_f in Newborns with and without Exposure to Tobacco Smoke Metabolites *in Utero*

Maternal Exposure	N	*GPA* M_f (x 10^{-6})			P^1	P^2
		Mean ± SD	Median	Range		
***a) Total* GPA M_f[3]**						
unexposed	14	5.3 ± 2.6	4.7	0.14—1.9		
passive only	20	6.6 ± 2.7	6.2	3.2—13.6	0.086	
quit during pregnancy	11	7.0 ± 4.1[4]	6.8	3.2—16.4	0.22	
smoked throughout	48	6.8 ± 3.1[5]	6.3	2.4—18.0	0.049	0.042
***b)* GPA *N/Ø* M_f**						
unexposed	18	3.6 ± 2.6	2.9	0.8—12.2		
passive only	26	4.0 ± 2.3	3.7	1.2—10.2	0.35	
quit during pregnancy	12	4.0 ± 2.3	3.4	1.4—8.4	0.51	
smoked throughout	58	4.1 ± 2.1	4.1	0.4—10.4	0.25	0.25
***c)* GPA *N/N* M_f[3]**						
unexposed	14	1.7 ± 0.92	1.8	0.6—3.4		
passive only	20	2.6 ± 1.4	2.3	0.2—5.4	0.061	
quit during pregnancy	11	3.0 ± 2.7[4]	2.8	0.6—10.2	0.089	
smoked throughout	48	2.9 ± 2.2[5]	2.8	0.1—13.8	0.016[6]	0.014[6]

[1]Specific exposed group *vs* unexposed *GPA* M_f, Mann-Whitney *U* test
[2]Combined exposed groups *vs* unexposed *GPA* M_f, Mann-Whitney *U* test
[3]Excluding babies of mothers with NN blood type
[4]Excluding outlier with *GPA* N/N M_f of 231.5 x 10^{-6}
[5]Excluding outlier with *GPA* N/N M_f of 63.9 x 10^{-6}
[6]Statistically significant ($P < 0.05$) after adjustment for multiple comparisons [6].

of the exposed groups exhibited significantly elevated frequencies of simple allele loss mutants, for loss-and-duplication mutants the probabilities of significance of all comparisons between exposed populations and unexposed controls improved over the result with total *GPA* M_f, although, once again, only primary maternal smoking was lower than 0.05. As was observed with the total *GPA* M_f, the N/N M_f of the three tobacco exposed groups were not significantly different, and, combined, were significantly higher than the unexposed group.

DISCUSSION

Overall, our findings of significant induction of prenatal mutation at the *GPA* locus associated with maternal smoking largely reiterate our previous findings with the only other widely applied human somatic mutation assay, the *HPRT* assay [2]. Although this strengthens the evidence for the transplacental genotoxicity of maternal tobacco smoke, both active and environmental, these assays should be considered as complementary tests, in that they reflect somewhat distinct mechanisms of mutagenicity. Although loss of *HPRT* activity is not considered inviable at the cellular level, there is convincing evidence for age-related selection against HPRT-deficient cells at the organismal level [9,10], as well as clear restrictions on mechanisms, such as chromosome loss and deletion, that affect adjacent genes and genetic material [11]. On the other hand, the *HPRT* gene has cryptic VDJ recombinase recognition sequences that direct specific removal of exons 2 and 3, making this gene a reporter for mutations occurring due to "illegitimate" recombinase activity [12]. So, whereas gene-specific mutations are the major detectable lesion at the *HPRT* gene, including those occurring *via* illegitimate VDJ recombination, such "classical" mutations appear to contribute negligibly to mutation (or "loss of heterozygosity") at autosomal loci such as *GPA* [13]. Instead, the major mechanisms of "mutation" at such loci have been found to be chromosome missegregation [14], epigenetic gene inactivation [15] and homologous recombination [16].

In previous studies of *in utero* mutation at the *HPRT* locus in cord blood T-lymphocytes, we found that tobacco smoke exposure, both primary and *via* ETS, resulted in increased frequencies of both small, non-structural mutations (assumed to be point mutations) and those caused by illegitimate VDJ recombination, a mechanism that should not be possible in other cell-types, such as the erythroid lineage that uniquely expresses *GPA* [2,17,18] (or in amniocytes, despite a recent study [19]). We specifically did not observe an increase in structural mutations caused by random chromosomal deletions or rearrangements. While the increase in allele loss-and-duplication mutants observed in this study may have occurred by homologous recombination or chromosomal missegregation [7], the concurrent *HPRT* assay results [2] suggest that generalized recombination is not affected by tobacco exposure. Chromosomal missegregation, however, cannot be detected at the hemizygous X chromosome-linked *HPRT* locus, and recent studies have shown that tobacco smoke does affect chromosomal segregation [20]. Another recent study also found that maternal tobacco smoke exposure was associated

with higher *GPA* mutation frequencies [21]. In this study, however, the effect was confined to the allele loss class of mutations, which may also occur by chromosome missegregation (simple non-disjunction), or by the same type of classical point mutations observed at the *HPRT* locus. We have previously found that sorting out mechanisms in such systems depends on comparing the relative frequencies of mutations of different types [2,22], and, unfortunately, this study did not provide a detailed analysis of *GPA* M_f.

In our previous report, we suggested that the maintenance of high mutation levels in the children of women who quit smoking during pregnancy might be associated with ongoing passive exposure. Indeed, in the present study, all but one woman who quit smoking during pregnancy reported ongoing secondary exposure in the home or workplace throughout her pregnancy. Once again, we have categorized a population based on their primary smoking habits without regard for passive exposure. If the women with such ongoing passive exposure who quit actively smoking during pregnancy are added to the passive exposure only category, such passive exposure results in a statistically significant induction of *GPA* allele loss-and-duplication M_f ($P = 0.033$).

Of course, these *GPA* data were generated from a subset of the mother/daughter pairs previously analyzed for *in utero* mutation at the *HPRT* locus [2,4], with limitations on both the overall population size, and the size of the stratification classes, particularly the unexposed controls. Also, unlike the *HPRT* mutation analysis, these subjects were all derived from the same population, mothers delivering at University Hospital in Denver, Colorado, USA, and therefore represent a geographically limited population of relatively low socioeconomic status. Possible confounding factors therefore include nutrition, other exposures (including radiation due to elevation), lifestyle and workplace, although none of these factors were associated with either *HPRT* or *GPA* M_f in the original study [4].

CONCLUSION

These data confirm and support, at a second, autosomal, locus, our previous finding that both active maternal smoking and passive maternal exposure to ETS induce similar levels and types of genotoxic damage to the developing fetus. Further, it is clear that quitting smoking during pregnancy alone is not sufficient to ameliorate the effects on the baby, since maternal secondary exposure causes the same level and same type of genetic damage as active smoking.

This study also reiterates the lesson that selection of a proper control population is critical to establishing smoking effects. Indeed, we suggest that any study that failed to demonstrate such a smoking effect without controlling for the possible effects of passive exposure is irredeemably confounded and can no longer be considered valid.

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ABBREVIATIONS

ETS = Environmental tobacco smoke

GPA = Glycophorin A

HPRT = Hypoxanthine-guanine phosphoribosyl transferase

M_f = Mutation frequency (frequencies)

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