

STRAIN-DEPENDENT SUSCEPTIBILITY TO TRANSPLACENTALLY-INDUCED MURINE LUNG TUMORS

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INTRODUCTION

Lung cancer is the leading cause of cancer deaths in the US. Underlying genetic differences in the individual's response to environmental toxicants may play a critical role in determining individual susceptibility to lung cancer. While the association between exposures to environmental toxicants and lung cancer in adults is well documented, the effects of *in utero* exposures are still uncertain. In this regard, several studies have shown that the developing organism is very sensitive to chemical and physical carcinogens, suggesting that exposure of pregnant women to environmental toxicants may place the embryo and fetus at higher risk for the development of cancer because of their increased vulnerability.

Our laboratory has shown that treatment of pregnant mice with 3-methylcholanthrene (MC) resulted in the formation of lung and liver tumors in the offspring 1 year after birth. A high incidence of mutations in *Ki-ras* was induced in the lung tumors, and both strain- and organ-specific differences in the *Ki-ras* mutational spectrum were observed. The current study thus examines the biochemical and molecular mechanisms that may determine oncogenic damage and thereby modulate susceptibility to chemical carcinogens during the sensitive period of fetal development. Since the observed strain differences in the *Ki-ras* mutational spectrum may be due to differences in the metabolic activation of MC, we have determined the levels of *Cyp1a1* and *1b1* transcripts in fetal tissues and their potential association with tumor incidence and multiplicity in the C57BL/6 (B6) and Balb/c (Bc) strains of mice as well as F1 crosses between the parental strains following *in utero* exposure to MC. In addition, we have also assessed the level of DNA adducts and the rate of DNA repair in fetal lung tissue to determine their potential role in the differential susceptibility of these mice to mutations in *Ki-ras* and induction of lung tumors. Our results suggest that, similar to adult mice, susceptibility for the induction of lung cancer appears to be the dominant phenotype, as F1 offspring from crosses between C57BL/6 and Balb/c mice resemble Balb/c mice in tumor incidence and multiplicity, with only minor differences in tumor latency. Although some differences in the inducibility of *Cyp1a1* and *1b1* were observed, these did not appear to have any effect on total adducts formed or the rate of disappearance of the adducts from mouse lung tissue. Our results, combined with earlier studies examining crosses between C57BL/6 and DBA/2 mice, suggest that an underlying, dominantly acting susceptibility gene is a major determinant of individual sensitivity to lung tumor formation following *in utero* exposure to environmental carcinogens, and highlight the important interactions between genetic background and environmental exposures in determining susceptibility to lung tumorigenesis during the sensitive fetal period.

METHODS

Animals and treatment protocols

Balb/c (Bc) and C57BL/6 (B6) mice were obtained from the Charles River Laboratories (Raleigh, NC). The mice were housed in a pathogen-free environment in plastic cages with corn cob bedding and aspen pile for nesting, and allowed free access to food and water. A 12hr photoperiod light/dark cycle was maintained. The mice were treated by placing one male in a cage with one female for a 24 hr period. Pregnant mothers were treated on the 17th day of gestation (day 1 was the day after mice were placed together) by a high i.p. injection under the diaphragm with either olive oil or a 45 mg/kg dose of 3-methylcholanthrene (MC) dissolved in olive oil. For the tumor study, the fetuses were carried to term and the offspring received no further treatment following *in utero* exposure to MC. At 12-18 months of age, the mice were killed by CO₂ asphyxiation, visible lung tumors were enumerated, and lung tumors were then fixed in 10% phosphate-buffered formalin and embedded in paraffin. For the RNA study, pregnant mothers were euthanized by CO₂ asphyxiation at 1, 2, 4, 8, 12, 16, 24, and 48 hr after injection. The fetal lung and liver tissues were collected separately and flash frozen in liquid nitrogen immediately upon dissection to minimize RNA degradation. Tissue was stored at -80°C. Fetal tissues for each organ from the same litter were pooled together for RNA isolation (3 litters for each time point).

For adduct analysis, pregnant mothers were euthanized by CO₂ asphyxiation at 24 and 48 hr after injection. In addition, offspring were similarly euthanized 4, 7, 14, and 21 days after injection (1, 4, 11, and 18 days after birth). Lung tissue from fetuses and from 4 and 7 day post-injection offspring were pooled; thus, adduct values represent the means from pooled samples of individual litters. For offspring 14 and 21 days post-injection, lungs from individual mice from two different litters were assayed. Once isolated, tissues were quick frozen in liquid nitrogen and stored at -80°C until use. Adduct values represent the means \pm SD from at least 3 individual litters or mice.

RNA Isolation and cDNA Synthesis

Total RNA was isolated using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instruction. RNA samples were treated with RNase-free DNase (Promega, Madison, WI) and were desalted prior to cDNA synthesis using the Absolutely RNA RT-PCR Miniprep Kit. Synthesis of cDNA was performed using the ScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA) with the reaction conditions recommended by Bio-Rad. One μ g of RNA was used for each 20 μ l of RT-reaction. cDNA was stored at -20°C until use.

Primer Design

Primers were designed to specifically recognize target gene mRNA sequences using computer software Primer3 (<http://www.genome.gov>) and Primer Express (Applied Biosystems, Foster City, CA). The details of primers and real-time PCR products are listed in Table 1.

cDNA Standards

External controls consisted of plasmid standards for each target of interest, as well as for GAPDH and β -actin. Total cDNA was extracted from mouse tissues, and cDNA fragments were generated by RT-PCR using the same primers as given in Table 1. Each of these amplicons was purified using the DNA Extraction Kit (Millipore, Bedford, MA) and cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared by using the Plasmid Mini Kit (Qiagen, Valencia, CA). The identity of purified cDNA constructs was verified by DNA sequencing on an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). cDNA plasmid concentrations were measured by optical density spectrophotometry and the corresponding copy number was calculated using the following equation: Copy number = $6.1 \times 10^{10} \times (\mu\text{g of plasmid standard} / (\text{plasmid} + \text{water})) \times \text{Serial dilutions from the cDNA plasmids}$ were used to generate a standard curve in the range of 10^4 - 10^7 copy number.

Quantitative RT-PCR

Real-time PCR was conducted by amplifying 1-2 μ l of cDNA with the iQ SYBR Green Supermix on an iCycler iQTM Real-Time Detection System (Bio-Rad, Hercules, CA). Amplification conditions were 94°C for 10 min (hot start), followed by 40 cycles of 94°C for 30s, 57-64°C (depending on gene) for 30s, and 72°C for 30s. Melting curve analysis of amplification products were performed at the end of each PCR reaction to confirm that one single PCR product was detected by the SYBR Green dye. Quantities of specific mRNA in the samples were measured according to the corresponding gene-specific standard curve. Quantification of the samples by the software (iCycler 3.0) was calculated from the C_t by interpolation from the standard curve to yield a copy number of the target sample.

RESULTS

1. Tumor incidence in the control, olive oil treated mice was low. The resistant B6 mice had no tumors 18 months after birth; the Bc and the F1 hybrids had tumor incidences of 3-17% at 12-16 months (Table 2).
2. Bc, B6Bc, and BcB6 mice exhibited a 100% tumor incidence whereas the resistant B6 mice had an incidence of 11% (Table 2). B6 mice exhibited 4 small nodules after 18 months whereas Bc mice rarely survived beyond 14 months; BcB6 and B6Bc mice survived to approximately 16 months.
3. Bc, B6Bc, and BcB6 mice exhibited significant tumor involvement in the lungs; in many cases multiple tumors coalesced into single large masses with the majority of lesions classified as adenocarcinomas (Fig. 1). Tumor multiplicities were very similar between Bc, B6Bc, and BcB6 mice, ranging from 4.9-5.8 tumors/mouse (counting only lesions that were discrete, individual nodules) whereas B6 mice had <0.1 tumors/mouse (Table 2).
4. Basal levels of *Cyp1a1* expression in fetal lung were barely detectable; basal expression in the fetal liver was approximately 5-10 times greater than that observed in the fetal lung (Fig. 2).
5. Basal levels of expression of *Cyp1b1* in fetal lung were relatively low; however, basal expression of *Cyp1b1* in fetal lung was 5-10 times that in fetal liver. Basal expression of *Cyp1b1* in fetal lung was much higher than that of *Cyp1a1*, depending on the strain and time point (Fig. 3).
6. MC caused maximal induction of *Cyp1a1* and *Cyp1b1* RNA 2-8 hr after injection in both fetal lung and liver (Fig. 2 & 3). RNA levels for both genes then declined, but a small biphasic, secondary increase was observed in the fetal lung at 24 to 48 hr.
7. *Cyp1a1* induction by MC at 4 hr was 2-5 times greater in fetal liver (7000-16000 fold) than fetal lung (2000-6000 fold).
8. *Cyp1b1* induction in both fetal lung and liver were similar and much lower than that observed for *Cyp1a1*, with induction ratios of 8-18 fold in fetal lung and 10-20 fold in fetal liver. *Cyp1b1* appeared to be poorly induced in B6 mice.
9. MC induced covalent DNA adducts at early time points in lung DNA, reaching maximal levels 2-4 days after administration. DNA adducts were persistent to 21 days after administration and were not significantly different in B6, Bc, and F1 mice (Fig. 4).

Table 2: Tumor Incidence, Multiplicity, and Size in C57BL/6, Balb/c, and F1 Hybrid Mice Treated *in utero* with MC

	N (Number of mice)	^a Tumor Incidence	^b Tumor Multiplicity	# of coalesced tumors
B6B6	12	(0/12) 0%	0	0
Control	35	(4/35) 11%	0.06 + 0.32	2
BcBc	40	(1/40) 3%	0.03	0
Control	11	(11/11) 100%	5.82 + 3.66	18
B6Bc	23	(3/23) 13%	0.04	3
Control	19	(19/19) 100%	5.00 + 3.32	26
BcB6	30	(5/30) 17%	0.13 + 0.35	4
Control	18	(18/18) 100%	4.89 + 3.59	36

C57BL/6, Balb/c, and hybrid mice were treated *in utero* on day 17 of gestation with either olive oil vehicle or 45 mg/kg of MC. Offspring were born on the 20th day of gestation and left untreated for 12 to 18 months, at which time mice were euthanized by CO₂ asphyxiation, macroscopically visible lung tumors were counted on the surface of the lungs, and the tissue then embedded in 10% phosphate-buffered formalin for histological analysis and H&E sections.

^aTumor incidence was calculated as the number of mice with tumors/total number of mice in the group.
^bTumor multiplicity was calculated as the total number of discrete (non-coalesced) tumor masses/total number of mice in the group.

Fig. 1 Lung Morphology

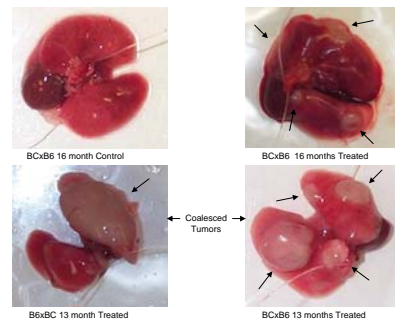
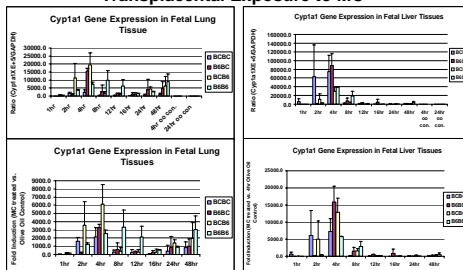


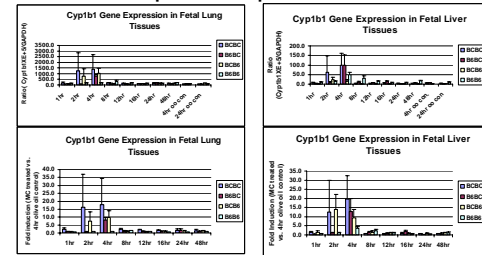
Fig. 2 *Cyp1a1* Gene Expression in Fetal Tissues Following Transplacental Exposure to MC



Cyp1a1 gene expression measured by real time PCR. Each column and bar represents the mean \pm S.D. of three individual samples. The ratio was calculated as *Cyp1a1* gene expression/*XE-5*GAPDH gene expression. Fold induction was calculated as *Cyp1a1* gene expression (normalized to GAPDH at each time point) divided by 4 hr olive oil control (mean of three individual samples).

Fig. 3

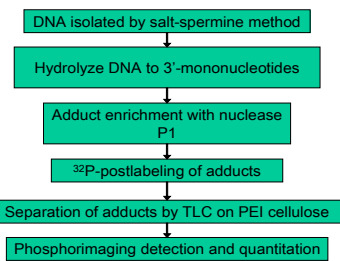
Cyp1b1 Gene Expression in Fetal Tissues Following Transplacental Exposure to MC



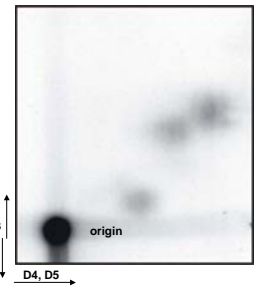
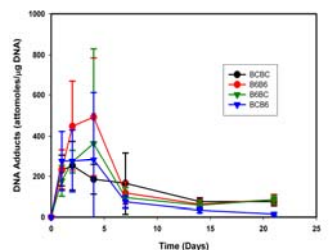
Cyp1b1 gene expression measured by real time PCR. Each column and bar represents the mean \pm S.D. of three individual samples. The ratio was calculated as *Cyp1b1* gene expression/*XE-5*GAPDH gene expression. Fold induction was calculated as *Cyp1b1* gene expression (normalized to GAPDH at each time point) divided by 4 hr olive oil control (mean of three individual samples).

Fig. 4

Flowchart of Mouse Lung DNA Adduct Analysis



3-MC DNA Adduct Levels in Mouse Lung after Single i.p. Administration



CONCLUSIONS

Susceptibility to lung cancer appeared to be the dominant phenotype, as F1 hybrid mice had similar tumor incidences and multiplicities as the parental Balb/c strain. The differences in tumor latency noted between the two strains (Table 2) were most likely due to the presence of two copies of the polymorphic *INK4* locus in Balb/c mice, which has been associated with tumor progression.

Although there were some differences in inducibility for *Cyp1a1* and *1b1* across the two parental strains, these do not appear to account for the marked differences in lung tumorigenesis, especially since adduct levels in the lung were similar in the two parental strains and F1 hybrids (Fig. 4). As previous studies from our laboratory utilizing crosses between C57BL/6 and DBA/2 mice also demonstrated a high lung tumor incidence following *in utero* exposure to MC, these results suggest the presence of an unidentified, dominantly acting gene locus in Balb/c and DBA/2 mice that confers susceptibility to lung tumorigenesis following *in utero* exposure to chemical carcinogens. Future studies will need to focus on the identification and cloning of this gene.

Table 1: Oligonucleotide Primer Sequences and Amplification Conditions

Gene	Primer Sequences	Annealing Temp (°C)	PCR Product bp	Tm (°C)
GAPDH (M32599)	(F) 5'-tctccctcacaattccatccag-3' (R) 5'-gggtgacgcaacttattgatgg-3'	63.1	100	83
β -actin ^a (X03765)	(F) 5'-atgctgacagatgcagaa-3' (R) 5'-caggagagcaatgacttctga-3'	58.7	76	81
<i>Cyp1a1</i> ^a (NM_009922)	(F) 5'-caccatccccacagac-3' (R) 5'-acaagacacagaccacctt-3'	63.1	75	81.5
<i>Cyp1b1</i> (U03283)	(F) 5'-ttgaccatcaggaactgc-3' (R) 5'-gtcgtctcttggtagggaga-3'	64.3	113	81

^aPrimers designed to span intronic sequence.