p53 Mutations in Benzo(a)Pyrene-Exposed Human p53 Knock-in Murine Fibroblasts Correlate with p53 Mutations in Human Lung Tumors

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Abstract

Human p53 mutation spectra differ significantly from one cancer type to another. One possible reason is that carcinogenic risk factors differ, and these factors elicit distinct mutation patterns. There has been no mammalian assay, however, with which to generate mutation patterns in human p53 sequences experimentally, hampering interpretation of the human tumor spectra. We have designed a new mammalian cell assay using gene targeting technology that selects and scores human p53 gene sequence mutations in human-p53 knock-in (Hupki) murine embryonic fibroblasts (HUF) that have undergone immortalization. With the Hupki assay we examined here whether benzo(a)pyrene (BaP), a major tobacco smoke carcinogen could elicit p53 mutation patterns characterizing the human lung tumor p53 mutation spectrum. We found that, in contrast to unexposed HUFs or HUFs exposed to other carcinogenic agents, HUFs exposed to BaP acquire mutations that display major features of the human lung tumor p53 mutation spectrum: (a) predominance of G-to-T mutations, (b) unequivocal strand bias of the transversions, and (c) a mutation hotspot at codons 157 to 158. These data are consistent with the hypothesis that BaP has a direct role in causing smokers’ lung tumor p53 mutations. The assay can be used to examine various hypotheses on the endogenous or exogenous factors responsible for the p53 mutations in human tumors arising in other tissues. (Cancer Res 2005; 65(7): 2583-7)

Introduction

Benzo(a)pyrene (BaP) is an environmental human carcinogen that is believed to have a major role in the induction of G-to-T mutations in the p53 gene of smoker’s lung tumors. Formal demonstration that specific mutations in human p53 gene sequences typical of human lung tumors can be induced by exposure of mammalian cells to BaP is lacking, however, leaving the issue controversial (1). One of the main hindrances to experimental testing of this and other hypotheses derived from scrutiny of human TP53 databases (~20,000 recorded mutations) is the absence of an appropriate mammalian mutagenesis assay that can select for and score mutations in HUMAN p53 sequences. Human primary cells with wild-type p53 would be the obvious choice of mutagen target in designing such an assay, but there is currently no feasible approach to recovering and characterizing a set of p53 mutant human cells harboring a variety of p53 mutations scattered over several hundreds base pairs. p53 mutant clones can be isolated readily, however, from MOUSE primary cells (2, 3). Murine embryonic fibroblasts (MEF) promptly undergo immortalization during in vitro culturing, a process that frequently involves inactivation of the p53 tumor suppressor gene (4). Thus, in principle, MEFs might be used for generating mutation spectra that could be compared against human tumor p53 mutation patterns, were it not for the fact that the p53 DNA binding domains of mouse and man, although highly conserved at the amino acid level, differ in DNA sequence. Sequence context is a fundamental determinant in shaping mutation patterns. To circumvent drawbacks of working with human primary cells, yet make use of the advantages offered by murine embryonic cells, we used gene-targeting technology to create a mouse strain that harbors human wild-type p53 DNA sequences from exons 4 to 9 in place of the homologous murine DNA sequences in both copies of the mouse p53 gene (5). The exchanged segment encodes the polyproline domain and DNA binding domain (DBD) of wild-type human p53, and the human p53 knock-in (Hupki) p53 protein retains wild-type p53 functions in this mouse (6, 7). Primary embryonic fibroblasts from Hupki mice [Hupki embryonic fibroblasts (HUF)], like their counterpart embryonic fibroblasts from murine wild-type p53 mice (MEF), readily undergo immortalization during in vitro passaging, and in the course of HUF immortalization, dysfunctional p53 point mutations typical of human tumors are selected for (8). In the present study, we explored the hypothesis that murine HUF would acquire mutations typical of human lung tumors during immortalization in vitro when exposed as primary cells to the tobacco smoke carcinogen BaP. The distinguishing features of human lung cancer p53 mutations, most of which have been found in tumors of smokers and ex-smokers, are a high proportion of G-to-T base substitutions, the striking strand bias of these transversions, and the frequent occurrence of mutations at a hotspot in exon 5 (codons 157-158; refs. 1, 9, 10). We show that the p53 point mutations in immortalized HUF cell lines derived from BaP-treated primary cultures display these features, tightening the link between tobacco smoking and tumor mutations.

Materials and Methods

Cell culture. Primary fibroblasts from Hupki embryos (Tvp53 tm1/Holl homozygous for the knock-in p53 allele harboring human p53 sequences) were distributed at 2 × 10^6 cells per well into 6-well plates and grown in DMEM supplemented with 10% FCS. Cultures were passaged at 1:2 to 1:4 dilution for at least 10 passages and then analyzed for gene mutations and gene expression alterations as described below. Two sets of 24 cultures were either exposed for 6 days to 0.1% DMSO (solvent control) at p. 2, or

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DNA adduct analysis. DNA samples were isolated from primary HUF cultures exposed in vitro to 1 μmol/L BaP for 4 days or to DMSO. Purified DNA was digested enzymatically to nucleoside 3′-monophosphates and enriched for adducts by nuclease P1 digestion as described (11). The digests were then analyzed by 32P-postlabeling.

p53 mutation analysis. Sequence changes in the human p53 gene segment of 32 HUF cell lines derived from BaP-exposed cells were examined by PCR amplification of p53 exons 4 to 9 using primers defined by Affymetrix (Santa Clara, CA) as described (8), and direct fluorescent dideoxynucleotide cycle sequencing of PCR products using protocols from Applied Biosciences International (Salt Lake City, UT). Mutations were confirmed by repeat cell harvest, DNA extraction, and sequencing of the opposite strand. The human p53 gene (exons 4-9 and splice sites) from 20 lines derived from untreated cultures (set HUF-CO-IV) also were sequenced by dideoxynucleotide sequencing; an additional 20 cell lines from untreated cultures were first screened by immunohistochemistry with p53 antiserum as described (8) and lines with a mutant p53 staining pattern then were sequenced.

DNA was also tested with Affymetrix GeneChip microarrays. Protocols and software were employed as recommended by Affymetrix. Three mutant cell lines (HUF-BaP-4, HUF-BaP-28, and HUF-BaP-116) that harbored multiple or complex mutations, were reanalyzed by ligating a PCR product spanning exons 4 to 9 into the pGEM T-Easy vector (Promega, Madison, WI), and sequencing of four or more individual clones.

Gene expression analysis. Induction of p53 downstream transcriptional targets of activated p53. Cells were exposed to 20 Gy γ-irradiation 1 hour

Table 1. p53 gene mutations in HUF cell lines derived from BaP-exposed and untreated primary cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Base change*</th>
<th>Amino acid substitution</th>
<th>Lung cancer p53 mutation† (rank)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUF-BaP-4</td>
<td>GCC to GGC (C to G)</td>
<td>A 138 G</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-13</td>
<td>GTC to GTT (C to T)</td>
<td>V 157 V</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-14</td>
<td>CGC to CCC (G to C)</td>
<td>R 158 P</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-D14B</td>
<td>GTC to TCC (G to T)</td>
<td>V 157 F</td>
<td>*(2nd)</td>
</tr>
<tr>
<td>HUF-BaP-15</td>
<td>g to t (G to T)</td>
<td>(intron 7)†</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-16</td>
<td>GAG to GAA (A to G)</td>
<td>E 224E†</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-20</td>
<td>g to c (G to C)</td>
<td>(intron 3)†</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-28</td>
<td>GTC to GTT (C to T)</td>
<td>V 157 V</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-48</td>
<td>g to t (G to T)</td>
<td>(intron 5)†</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-106</td>
<td>AGA to GGA (A to G)</td>
<td>R 280 G</td>
<td></td>
</tr>
<tr>
<td>HUF-BaP-116</td>
<td>CCT to CTC (C to T)</td>
<td>P 278 S</td>
<td></td>
</tr>
<tr>
<td>HUF-CO-15**</td>
<td>CGT to TGT (C to T)</td>
<td>R 273 C</td>
<td></td>
</tr>
<tr>
<td>HUF-CO-17**</td>
<td>CTT to TTT (C to T)</td>
<td>L 194 F</td>
<td></td>
</tr>
<tr>
<td>HUF-CO-I5</td>
<td>TGC to TGG (C to G)</td>
<td>C 135 W</td>
<td></td>
</tr>
<tr>
<td>HUF-CO-I11</td>
<td>CGG to CGC (T to C)</td>
<td>L 265 P</td>
<td></td>
</tr>
<tr>
<td>HUF-CO-I21</td>
<td>AGG to AGC (G to C)</td>
<td>R 249 S</td>
<td></td>
</tr>
<tr>
<td>HUF-CO-I21</td>
<td>AAG to AGC (A to C)</td>
<td>K 120 T</td>
<td></td>
</tr>
</tbody>
</table>

*Exons 4 to 9 and flanking splice sites were analyzed by dideoxy sequencing and with Affymetrix P53 GeneChip microarrays.

†Data from the IABC TP53 Database version R9.

‡Precise sequence change (type, location) has been detected in human lung tumors (†). The number in parenthesis gives the rank order of the most common mutations found in lung cancer.

†Exposure to 1 μmol/L BaP for 4 days (HUF-BaP-D14B) or 6 days (all other HUF-BaP cell lines).

§Splice site.

*Hotspot codon (††): >2% of all database codon mutations are at the indicated triplet.

**Previously reported (8).
before extraction of total RNA with a Qiagen RNeasy Extraction Kit. cDNA synthesis with avian myeloblastosis virus reverse transcriptase (Promega) and quantitative real-time reverse transcription-PCR (RT-PCR), done in a LightCycler (Roche Diagnostics, Nutley, NJ), using the LC-FastStart DNA Master SYBR Green I kit (Roche Diagnostics).

Results and Discussion

Our previous work suggested that primary HUFs retain various enzymatic activities for metabolizing xenobiotics to mutagenic intermediates (8). To determine whether primary HUFs (passages 1-2) are able to activate the tobacco smoke procarcinogen BaP and form DNA adducts, we did 32P-postlabeling experiments with primary HUF cells exposed to BaP. BaP can be activated in the lung by P450 enzymes to diol epoxide metabolites (anti- and syn-BaP-7,8-dihydrodiol-9,10-epoxides, BPDE). The major adduct formed by activated BaP, the (+)-anti-BaP-\(N^2\)-guanine adduct is premutagenic, mispairing with A and generating primarily G to T transversions (12–14). Loechler et al. have shown, however, that the (+)-anti-BaP-\(N^2\)-guanine adduct can induce predominantly G-to-A mutations in certain sequence contexts (15), which shows the crucial role of base sequence context in determining mutagenic properties of a given compound. To generate DNA adducts derived from BaP, we exposed primary HUFs to 1 \(\mu\)mol/L BaP for 96 hours. The adduct pattern we obtained with the nuclease P1 enrichment version of the 32P-postlabeling method consisted of one major and two minor adduct signals (Fig. 1).

Figure 2. A, comparison of the types of p53 base substitutions found in immortalized cell lines derived from BaP-treated HUF cultures with types of mutations observed in human lung and colon tumors (data from IARC TP53 Database, version R9). The mutation pattern in the BaP-treated cell lines is not significantly different from human lung tumor mutations (\(P = 0.22\)), yet is clearly unlike the mutation pattern in human colorectal tumors (\(P < 0.0001\), \(\chi^2\) test). B, DNA dideoxy sequencing electropherograms showing the G-to-T transversion at codon 157 in HUF-BaP-D14B (direct sequencing) and the double mutation in cis at codons 157 and 158 in HUF-BaP-28 subcloned into pGEM T-Easy vector (Promega). Direct sequencing of PCR product (data not shown) also detects both substitutions in HUF-BaP-28.

Figure 3. Puma expression induction 1 hour following exposure to 20 Gy \(\gamma\)-irradiation of Hupki cell lines with wild-type p53 DBD (solid columns) and Hupki cell lines harboring p53 mutations (open columns), detected by real-time RT-PCR. Primer sequences for amplification: Puma forward 5’-GGATGGCGGACGACCTC-3’ and reverse 5’-CGGGCAAGGCTGGCAGT-3’. Experiments were performed thrice, and fold change in Puma mRNA levels were calculated against GAPDH message standard. Data are displayed as in Ref. 20, showing \(\gamma\)-induced expression of p53 downstream genes in p53 wild-type but not p53 mutant cell lines of the National Cancer Institute anticancer drug screen.
total adducts), which was identified by chromatography as the (+)-anti-BaP-UN,-guanine adduct (data not shown), was present at a level of 13.9 ± 3.1 adducts per 10⁶ nucleotides (relative adduct labeling) after 4 days exposure to 1 μmol/L BaP. Our postlabeling results show that primary HUFs have adequate capacity to convert BaP to electrophilic intermediates that interact with DNA, and so we chose to perform our mutation experiments with the procarcinogen.

Immortalization of HUF cell cultures (benzo(a)pyrene-exposed and untreated control), and p53 mutation analysis of cell lines. Seventy-two primary HUF cell cultures (32 cultures exposed for 4-6 days to 1 μmol/L BaP at p. 2 and 40 unexposed/DMSO solvent-exposed cultures) survived beyond 10 passages, resumed growth after crisis, and reacquired a uniform morphology. These immortalized cultures, at this point referred to as HUF cell lines, could be maintained further in culture without signs of demise. Among 32 immortalized lines derived from BaP-treated cultures, we found a total of 16 hemizygous/homozygous or heterozygous mutations in the human-derived p53 DBD gene segment in 11 cell lines by direct dideoxy sequencing of PCR-amplified DNA and with the Affymetrix GeneChip microarray assay, whereas 4 of the 40 lines from nonexposed cultures harbored a p53 mutation (Table 1). G-to-T transversions were the most common base change in cell lines derived from BaP treatment (7 of 16, 44%), and all seven occurred at base pairs where the premutated guanine was on the nontranscribed strand (Table 1). This is in contrast to the absence of G-to-T nontranscribed strand transversions among the previously reported HUF mutations (8) from cell lines derived from cultures exposed to aristolochic acid, which targets A/T base pairs, UV light, which induces base transitions, and from cell lines established from control cultures (19 cell lines in all, 22 mutations; P < 0.0003, Fisher’s exact test). Figure 2A compares the proportion of each class of mutation in BaP-derived HUF cell lines with the human lung and colon tumor p53 mutation spectra recorded in the IARC TP53 Database (version R9; refs. 16, 17; http://www.p53.iarc.fr). For statistical comparison of base substitution categories, each base change was taken as an independent event, although some of the neighboring mutations may have been the result of a single event. The p53 mutation pattern of HUF-BaP cell lines is not significantly different from the human lung tumor spectrum, whereas it differs substantially from the human colorectal cancer mutation profile (Fig. 2B, χ² test). Most lung tumors with p53 mutations recorded to date arose in patients who are or were tobacco smokers, and tobacco smoking is considered the primary cause of human lung tumors. p53 mutations are much more common in smokers’ than in nonsmokers’ lung tumors. In addition, G-to-T mutations do not stand out among lung tumor p53 mutations of nonsmokers (10).

A parallel between HUF-BaP p53 mutations and human lung tumors can also be drawn for the distinctive hotspot sequence spanning codons 157 and 158. Six percent of human lung tumor p53 mutations occur at this site. Three of the 11 p53-mutant HUF-BaP cell lines also harbored one or more base substitution(s) at this hotspot (5 of 16 HUF-BaP base substitutions; Table 1; Fig. 2B). Notably, a guanine to thymine transversion at the second base of codon 158, and at the first base of codon 157 are the two most common and distinguishing single mutations in human lung cancers (codon 158 CGC to CTC, rank 1, 47 of 2,142 lung mutations; codon 157, GTC to TTC, rank 2, 44 of 2,142), whereas they are rare to absent among mutations in colorectal tumors and other common cancers (codon 158 CGC to CTC: 1 of 2,621 colorectal cancer mutations; codon 157 GTC to TTC: 8 of 2,621). (codon 158 CGC to CTC and codon 157 GTC to TTC, lung versus colorectal cancer, P < 0.00001, χ² test). These two most common lung mutations were found among the 11 p53-mutant HUF-BaP immortalized cell lines (Table 1).

Characterization of 21 p53 wild-type and p53-mutant cell lines derived from carcinogen-treated and untreated HUF primary cells. Human tumor p53 mutants generally are defective in transcriptional transactivation of p53 downstream target genes, and tumor cells with missense p53 mutations accumulate abnormally large amounts of the mutant protein because the wild-type p53-controlled ubiquitinylation and degradation pathways no longer function properly (18). We next asked whether, as in p53 mutant HUF cell lines isolated previously (8), the HUF-BaP p53 mutant cell lines also were defective in these wild-type properties. We measured DNA damage induced expression of three p53 target genes (p21, Mdm2, and Puma) important in cell cycle and apoptosis regulation by RT-PCR of unexposed and γ-irradiation exposed cell lines, and assessed the baseline p53 protein level in each cell line by immunoblotting. As anticipated, p53 mutant HUF-BaP cell lines that harbor p53 mutations show weak or no induction of p21, Mdm2 and Puma following γ-irradiation compared with immortalized HUFs that have retained a wild-type p53 (Fig. 3). Furthermore, the cell lines with missense p53 mutations accumulate large amounts of p53 protein (data not shown).

Because disruption of p53 function (typically by point mutation) or loss of p19ARF function by gene deletion or silencing (4) are two major and possibly mutually exclusive routes to immortalization of MEFs that have been described by other investigators (reviewed in ref. 4), it is likely that our HUF cell lines with a wild-type p53 DBD harbor defects in p19ARF. We are currently investigating genetic anomalies that could account for the consistently low or undetectable p19ARF message and protein levels we have observed in p53 wild-type HUFs. p53 mutant HUFs have consistently much higher p19ARF mRNA and protein levels. Thus far, in p53 wild-type HUFs, we have found several instances of p19ARF silencing by INK4A locus homozygous deletion, but not by promoter methylation at CpG sites nor by mutation at this locus. 4

Mutagenesis in HUFs to investigate origins of human tumor mutations. Several observations make it plausible that tobacco smoke carcinogens are a major source of G-to-T mutations in the p53 gene of smokers’ lung tumor DNA: (i) epidemiologic data on smoking and lung cancer, together with molecular data showing a general dearth of p53 mutations and the paucity of G-to-T transversions among p53 mutations in nonsmokers’ lung tumors relative to smokers’ lung tumors, and (ii) the correlation of BaP adduct patterns along the p53 gene of human DNA exposed to BaP with lung tumor p53 mutation hotspots (19). Reproduction of the human lung tumor G-to-T mutation pattern experimentally in the p53 gene has been attempted by exposure of plasmids with human p53 gene sequences to BPDE and the scoring of mutants in a yeast assay; however, although these studies corroborated observations from other mutagenesis tests in microorganisms on the predominance of G/C-to-T/A mutations induced by BaP, the data did not reveal a strand bias of this transversion, and the specific p53 mutations characteristic of lung cancer were not recovered (14).
The BaP-associated mutations we generated in the HUF immortalization assay share important features of the human lung tumor p53 mutation profile. The HUF assay can be used to investigate unusual p53 mutation patterns induced by other mutagenic human carcinogens of potential concern and to examine molecular events giving rise to mutations.

Acknowledgments

Received 10/13/2004; revised 1/12/2005; accepted 1/28/2005.

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We thank John Cairns for critical reading of the article and A. Benner for reviewing the statistics.

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