MEF immortalization to investigate the ins and outs of mutagenesis

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The importance of tumor suppressor/oncogene mutations in tumor development is clear, but the causes of the DNA sequence changes in human cancers are not. Although elegant experiments with transgenic mice harboring lacZ or cII target sequences show that exposure to mutagenic human carcinogens can cause base substitutions in vivo, it does not follow from this that the mutations found in human cancers have to be the direct result of damage by external mutagens. They could be due to endogenously generated reactive oxygen species, or polymerase infidelity, for example. Specific patterns of mutations in the defined sequence of a test system set up to address this question can provide information on the molecular events leading to DNA sequence changes in humans if the experimentally induced mutations and patient tumor mutations are compared in the same gene.

Fortuitously, inactivating point mutations in the p53 gene are driving events in the immortalization of murine embryonic fibroblasts (MEFs) in vitro. This discovery offers a natural biological strategy for selecting p53 mutants. Immortalized cell lines arising from primary MEFs harboring human p53 sequences (Hupki, human p53 knock-in) have p53 mutations that match p53 mutations in human tumors.

Endogenous and exogenous factors in carcinogenesis

Whether viruses or carcinogens are the primary cause of human cancers was a matter of discussion decades ago. Today it is obvious that both factors are important. Human papilloma viruses are key culprits in cervical cancer, and tobacco smoke causes lung cancer in smokers. Now we are in the midst of a different debate: are endogenous cellular factors, for example, spontaneous DNA replication errors or reactive oxygen species (ROS), the major generators of mutations in oncogenes and tumor suppressor genes, or are external factors more important for most human cancers (1–3)? Either source may dominate, depending on the type of cancer and patient group (4–8). In sub-Saharan Africa, dietary aflatoxin exposure has a major role in the high liver carcinoma incidence and in the induction of gene mutations these cancers display, whereas in alcoholics with liver cancer, endogenous cellular responses to tissue damage probably contributed to genetic changes in the carcinomas of these patients (9,10). While current experimental tools to examine the path leading from exposure to an exogenous carcinogen such as AFB1 to mutations in cancer genes allow us to follow the process at the molecular level (Table I), they do not show which changes were driven by endogenous processes.

Investigating exogenous exposures: the toolbox

Table I lists several chemicals and related exposures that are recognized to increase human cancer risk and therefore classified as carcinogenic to humans (Group I) by the International Agency for Research on Cancer (IARC). These chemicals require metabolic activation by various enzymes before they can form DNA adducts. The major DNA adducts in human tissues from these exposures have been identified and represent in most cases bulky modifications that induce characteristic mutations in test systems in vitro and in vivo. The molecular link between exposure to a particular carcinogen and the presence of characteristic mutations in cancer genes found in humans is best illustrated by two examples: (i) the induction of hepatocellular carcinoma by dietary aflatoxin B1 (AFB1); and (ii) the association between lung cancer and tobacco smoking. The link between urothelial tumors, mutations and the ingestion of herbs containing aristolochic acid (AAI) is a third striking instance that may emerge, as more tumor DNA from afflicted persons becomes available for mutation analysis (11–14). With respect to AFB1 and tobacco smoke, the chain of events from exposure to cancer gene mutation in human tumors has been largely elucidated and is consistent with current models of chemical carcinogenesis. The biochemical steps leading to the formation of stable pre-mutagenic N2-guanine adducts in DNA by the tobacco smoke constituent benzo(a)pyrene [B(a)P], their quantification and their mutagenic potential also have been studied in detail (Table I). In vitro methods [damage mapping along a defined DNA sequence, ³²P-post-labeling for detection of specific adducts, and the HPRT, SupF and Functional Analysis of Separated Alleles in Yeast (FASAY) mutation tests], or in vivo experiments (including transgenic rodent mutation assays, reviewed in ref. 15) corroborate data from analysis of human tissues and tumors (16). Exposure to tobacco smoke is associated with characteristic mutations along the p53 gene in lung tumors (2), and the nucleotide positions prone to mutation in this case are at sites of DNA damage caused by benzo(a)pyrene diol epoxide (BPDE) [active metabolite of B(a)P] in the human p53 sequence (17). Apart, however, from the above-mentioned examples, and the remarkable mutation signature in skin cancers of...
<table>
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<tr>
<th>Agent or relevant exposure</th>
<th>IARCevaluation group</th>
<th>Major target organ (human)</th>
<th>Target organ mutations in tumors of exposed patients (IARC database)</th>
<th>Metabolic activation</th>
<th>Major DNA adducts</th>
<th>Refs</th>
<th>Mutagenesis in mammalian-based tests</th>
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<td>In vitro</td>
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<tr>
<td>1. AFB1 Naturally occurring mixtures of aflatoxins</td>
<td>1</td>
<td>Liver (HCC)</td>
<td>(HCC) aflatoxins ( (n = 53) ) G(^6)C(\rightarrow)T:A (72%) A(^\ast):T(\rightarrow)G:C (11%)</td>
<td>Epoxidation</td>
<td>N-7-dG</td>
<td>(67, 75)</td>
<td>Hum. fibro.</td>
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<td>Mut</td>
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<td>2. B(a)P Tobacco smoking</td>
<td>2A</td>
<td>Lung (Skin, stomach)</td>
<td>Tobacco smoke ( (n = 868) ) G(^\ast)C(\rightarrow)T:A (32%) G:C(\rightarrow)A:T (15% + 11%)</td>
<td>Epoxidation</td>
<td>N(^2)-dG</td>
<td>(67)</td>
<td>CHO</td>
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<td>Mut</td>
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<tr>
<td>3. 3-NBA Diesel engine exhaust</td>
<td>n.class.</td>
<td>(n.a.)</td>
<td>(Lung)</td>
<td>n.a.</td>
<td>Nitroreduction</td>
<td>N(^2)-dG &gt; N(^6)-dA</td>
<td>(53)</td>
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<tr>
<td>4. 4-ABP</td>
<td>2A</td>
<td>Bladder</td>
<td>Aromatic amines ( (n = 46) ) G:C(\rightarrow)A:T (61% + 4%) (\ast)G:C(\rightarrow)T:A (15%) Smokers ( (n = 224) )</td>
<td>N-hydroxylation</td>
<td>C-8-dG</td>
<td>(58, 64, 67)</td>
<td>Hum. Uroep.</td>
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<td>5. Aristolochic acid Herbal remedies containing plant species of the genus Aristolochia</td>
<td>2A</td>
<td>Urothelium</td>
<td>Aristolochic acid ( (n = 1, urothelium) ) A:T(\rightarrow)T:A (100%)</td>
<td>Nitroreduction</td>
<td>N(^2)-dA &gt; N(^2)-dG</td>
<td>(13)</td>
<td>CHO</td>
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<td>6. Vinyl chloride</td>
<td>1</td>
<td>Liver</td>
<td>Vinyl chloride ( (n = 14) ) A(^\ast):T(\rightarrow)T:A (36%) G:C(\rightarrow)A:T (21% + 7%) A:T(\rightarrow)CG (14%)</td>
<td>Epoxidation</td>
<td>N-7-dG &gt; Ethenos ( (\ast)A &gt; \ast)C = \ast)G</td>
<td>(71)</td>
<td>Hum. B-lympho</td>
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<td>In vitro</td>
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For abbreviations of AFB1, B(a)P, 3-NBA, 4-ABP, see text; hum., human; fibro., fibroblast; lympho, lymphoblast; Mut., mutation; n.a., not available; uroep., uroepithelial.

\(^a\)strand bias (higher one marked), >65%.

\(^b\)insufficient data to assign strand bias.

\(^c\)IARC Monograph Series.

\(^d\)IARC p53 database (49).
sun-exposed patients, there is still scant hard evidence to suggest that bulky DNA adducts from exogenous mutagens are major sources of cancer gene mutations in patients with other major cancers that are common in Europe and North America (breast, colon and prostate cancer). Experiments using FASAY (18,19) and transgenic rodents have provided interesting new data, but mutation patterns in a number of instances have been inconsistent with expectations from human tumor mutation data. Preferentially mutated p53 sequences (hotspots) induced by BPDE in the yeast assay were completely different from p53 mutation hotspots in lung cancers of smokers (20). An in vivo mutation test with the active metabolite of 4-aminobiphenyl (4-ABP) in the cII transgene of the BigBlue™ mouse system generated a pattern that was not consistent with p53 mutation spectra in human bladder cancer (21). Do such discrepancies argue against hypotheses on the sources of mutation in lung and bladder cancers of smokers, or do they reveal limitations in the currently used test systems?

Investigating endogenous processes that elevate mutation load: quandaries

Precision in tracking DNA modifications that originated from oxidative stress rather than exogenous chemicals is not only more challenging from a technical level but is also difficult to achieve because ROS can generate various pre-mutagenic modified DNA bases, and because ROS participate in a number of cellular processes (e.g. inflammation, lipid peroxidation; Figure 1) (7,22,23). Testing the mutagenicity of individual radicals in vitro in a controlled experimental setting is informative (24,25) but does not assess the mutagenic impact of oxidative stress in mammalian cells (26,27).

One indication that oxidative stress in mammalian cells may induce mutations in cancer genes has been provided by studies on mechanisms of cell immortalization in vitro. Primary murine embryonic fibroblast (MEF) cultures senesce after a short time (10–20 population doublings) owing to the unphysiologically high oxygen levels (ca. 20%) and consequent oxidative stress when standard conditions are used (28,29). The proliferation block, however, can be bypassed by spontaneous acquisition of a p53 mutation. This allows outgrowth of the cell that lost function of the tumor suppressor, and expansion of the subpopulation into an immortalized cell line (30,31). In contrast, MEFs cultured under conditions of low oxygen (5%) do not senesce, and appear not to require any specific rare genetic event to continue replicating in vitro (32). In ROS-stressed senescent cells, the most common discrete genetic alteration known other than p53 mutation that disrupts the p53/ARF axis and releases cells from proliferation block is deletion of the p19 locus. Immortal MEF lines typically harbor either a p53 mutation or loss of p19 (30,31,33,34). Other loci that, if perturbed, may facilitate bypass of senescence include cell cycle regulators Bcl6, CDK4 and cyclinD, Dnmt3b and chromatin modulators (35–39).

With respect to in vitro immortalization, the behavior of primary human cells differs from mouse cells in at least two important ways. First, bypass of senescence in culture is a very rare and complex process, involving several pivotal genetic alterations rather than only one, before human cells can become established in vitro (30,40,41). A mutant p53 is, of itself, insufficient to permit immortalization. Second, normal human cells can proliferate in vitro for a longer period under standard culture conditions (high oxygen) than mouse cells before they enter the senescent phase, suggesting that human cells may be able to cope with oxidative stress

Fig. 1. Mutation pathways. Sources of information for oxidative DNA damage and mutations: see refs. 78–87. Abbreviations: AFB₁-Fapy, 8.9-dihydro-8(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-aflatoxin B₁.
better than mouse cells. In the light of recent experiments, it appears that p53 is in the primary line of mammalian defense against oxidative stress (42–45). This raises the conjecture that human p53 has evolved to be a more effective guardian than murine p53 against oxidative damage.

**Designing mammalian mutation tests with the human p53 DNA-binding domain as mutagen target**

The observation that immortalization occurs readily *in vitro*, and often by acquisition of p53 mutations if primary mouse cells are used, raises the possibility of developing a mutation assay with p53 as the target gene, and immortalization as a natural selection process to recover the p53 mutants. The strategy would be more appealing when mutations in the exact human p53 sequence rather than the mouse p53 sequence are scored because this allows the p53 mutations in immortalized cultures to be compared directly with human tumor p53 mutations. This refinement is desirable in principle not only because sequence context is so important in shaping mutation patterns but also because the occasional amino acid differences between wild-type mouse and human p53 protein may affect conformation or function of a particular mutant, as may be predicted from recent discovery of intragenic p53 suppressor mutations (discussed in ref. 46). For an immortalization-based mutation test with this design to work, however, it is essential that (i) the human p53 sequences replacing the mouse p53 remain functional in mouse cells and therefore able to induce the senescent phase of the cell cultures; (ii) the mutations that then arise in culture disrupt wild-type p53 function, allowing bypass of senescence; (iii) mutations can be induced when the primary cells are exposed to mutagens before senescence; and (iv) the p53 mutations recovered in the immortalized mouse fibroblasts reflect the kinds of mutations selected for in human cancer development.

In the Hupki (human p53 knock-in) mouse we have demonstrated the wild-type character of the Hupki allele, which harbors wild-type human p53 sequences from intron 3 to 9 in place of the corresponding murine sequences of the endogenous mouse p53 gene (47). In contrast to p53-deficient mice, Hupki mice homozygous for the knock-in allele are not tumor-prone. Hupki mouse p53 protein is inducible in response to various DNA damaging agents, transcriptionally active, and elicits apoptosis in gamma-irradiated thymocytes. Primary Hupki-derived MEFs, which we refer to as HUFs (for Hupki embryonic fibroblasts), senesce, immortalize readily in culture and can acquire mutations in the p53 knock-in alleles during establishment in culture, also supporting the conclusion that the pre-mutated knock-in allele is functional (48). As is true for p53 mutations found in human tumors (6,49), the mutations we have identified in Hupki immortalized cell lines are scattered widely throughout the gene, are composed primarily of missense mutations and include frameshift and non-coding sequence splice mutations. Usually the mutations are detected as homo- or hemizygous base substitutions within several passages following initial immortalization (50). When a simple subculturing protocol is followed (51), 10–15% of spontaneously immortalized HUF cultures harbor point mutations in p53. Passage I primary cells are seeded at 1–2×10^5 cells per 6 cm plate, subcultured at 1:2 to 1:4 dilution when confluent, and harvested for DNA extraction and direct p53 gene sequencing after growth has resumed and cells have been passaged for several more weeks. When the primary cell cultures are exposed to mutagen at early passage, the percent of immortalized cell lines that harbor a mutation in p53 is 2 or 3 times higher than that for spontaneously arising cell lines (i.e. from untreated cells). This increase in proportion of mutant cell lines implies that mutagen treatment induced mutations, a conclusion that is supported by the fact that exposure to different mutagens results in p53 mutant immortalized cell lines with significantly different mutation signatures (Figure 2). Almost all mutations in cell lines derived from B(a)P-exposed cells are at G:C base pairs, and the predominant base change is G to T where the guanines were on the non-transcribed strand. In contrast, exposure to AAI, the major component of aristolochic acid, generated mutant cell lines with strand-biased A to T transversions in the Hupki p53 gene (48,52). Encouraging also is the observation that the sites of mutations along the p53 gene in cell lines derived from B(a)P-treated cultures correlated with features of the p53 mutation distribution in lung tumors of smokers (50). Formation of the major pre-mutagenic adducts generated from B(a)P, AAI and 3-nitrobenzanthrone (3-NBA), a carcinogenic component of diesel exhaust, are easily detectable in Hupki primary fibroblasts after 1–2 days *in vitro* exposure to these carcinogens without addition of any metabolic activation system to the medium (48,50), and

![Fig. 2.](https://academic.oup.com/carcin/article-abstract/27/11/2141/2392173?download=true)
unpublished observations]. A further indication that the carcinoma treatment induced mutations in the exposed cell cultures is that the pattern of p53 mutations in spontaneously arising Hupki immortalized cell lines is heterogeneous (Figure 2) and distinct from that of B(a)P- or AAI-derived cell lines. Surprisingly, we have isolated only one CpG hotspot transition mutation among the 12 ‘spontaneously arising’ (untreated) cell lines. This apparently is not due to an absence of CpG methylation in HULFs because codon 248, the most common CpG site of G:C to A:T transitions in human tumors, is methylated in Hupki primary embryonic fibroblasts (E. Felley-Bosco, unpublished data) as it is in human tissues. Oxidative DNA damage during in vitro culture is a plausible cause of p53 mutations arising spontaneously during mouse embryonic fibroblast immortalization (28,29,41). It is unclear whether oxidative stress under these conditions elicits deamination of 5-methylcytosine directly.

The 50 coding sequence mutations in Hupki cell lines we have identified thus far are distributed over 35 different codons, all of which have been found mutated in at least 15 human tumor samples (IARC p53 database). Remarkably, two-thirds of the 35 codons mutated in one or more mutant Hupki cell lines are in the set of the top 40 most frequently mutated p53 codons in human cancers. The general picture emerging thus indicates that mutant p53 molecules selected for during in vitro immortalization are in large measure the same set of mutants selected for in human tumorigenesis.

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References


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