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Further studies with a cell immortalization assay to investigate the mutation signature of aristolochic acid in human *p53* sequences

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Abstract

To test hypotheses on the origins of p53 mutations in human tumors, novel strategies are needed for generating mutation spectra experimentally. To this end we developed an assay employing Hupki (Human p53 knock-in) mouse embryonic fibroblasts (HUFs). Here we examine p53 mutations induced by aristolochic acid I (AAI)), the carcinogen probably responsible for Chinese herbal nephropathy. Six immortalized cultures (cell lines) from 18 HUF primary cultures exposed at passage 1 for 48 h to 50 μM AAI harbored p53 mutations in the human DNA binding domain sequence of the Hupki p53 tumor suppressor gene. The most frequently observed mutation was A to T transversion, corroborating our previous mutation study with AAI, and consistent with the presence of persistent AAI-adenine adducts found both in DNA of exposed patients and in DNA of AAI-exposed HUF cells. One of the mutations was identical in position (codon 139) and base change (A to T on the non-transcribed strand) to the single p53 mutation that has thus far been characterized in a urothelial tumor of a nephropathy patient with documented AAI exposure. Of the seven p53 mutations identified thus far in >60 HUF cell lines that immortalized spontaneously (no carcinogen treatment), none were A:T to T:A transversions. In addition, no A to T substitutions were identified among the previously reported set of 18 mutations in HUF cell lines derived from B(*a*)P treatment in which transversions at G:C base pairs predominated.

Keywords: Mutational spectra; Aristolochic acid nephropathy; Carcinogen; Senescence

1. Introduction

Specific covalent interaction of carcinogenic substances with DNA sequences produce characteristic patterns of mutations in biological systems. Examination of mutation profiles in a defined sequence such as the *p53* tumor suppressor gene thus can provide clues to the nature of the carcinogens responsible for these genetic alterations [1,2]. This gene is particularly suited to analysis of mutation spectra because point mutations in *p53* are found frequently in human tumors, are diverse in sequence location, and are directly involved in the cancer process [3,4]. Over 20,000 occurrences of human tumor p53 mutations have been registered in the IARC p53 mutation database (http://www-p53.iarc.fr), producing a naturally arising dense spectrum of mutations for inspection; however, interpretation has been stalled by the

Abbreviations: AAI, aristolochic acid I (CAS No. 313-67-7); B(*a*)P, benzo(*a*)pyrene (CAS No. 50-32-8); CHN, Chinese herbal nephropathy; DB, database; HUF, Hupki embryonic fibroblast; Hupki, Human P53-Knock-In; IARC, International Agency for Research on Cancer; MEF, mouse embryonic fibroblast

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absence of appropriate data on experimentally induced human p53 mutations in mammalian cells with which to test inferences derived from the human tumor spectrum on the origins of somatic mutations in cancer.

Recently, we developed a mammalian cell mutation assay using murine embryonic fibroblasts from Hupki (human p53 knock-in) mice, which harbor functional, wild-type human *p53* gene sequences in place of the corresponding homologous mouse *p53* sequence at the endogenous murine *p53* locus [5–7]. With this test system, we have shown that the carcinogen benzo(*a*)pyrene [B(*a*)P], considered to be the main promutagenic component of tobacco smoke in the lung, induces strand biased G to T transversions in Hupki fibroblasts [8]. This type of mutation is the hallmark of p53 mutations in lung tumors from heavy smokers [9].

A different scenario is exemplified by the recent tragic episode involving inadvertent exposure of a group of women undergoing a slimming regimen in Belgium to a potent nephrotoxic and carcinogenic agent [10]. An alarming number of these individuals presenting with severe renal dysfunction eventually developed urothelial carcinomas [11]. The causative agent of this syndrome, the plant extract aristolochic acid AA (a mixture of AAI and AAII, both of which are mutagenic), has been clearly identified [12]. Exposure of patients to aristolochic acid was verified unequivocally by ^{32}P-postlabelling analysis of their tissue DNA [11]. Unlike the situation for smokers' lung tumors, however, a role for mutagenicity and for *p53* tumor suppressor gene mutations in particular, in development of the AA-associated carcinomas in humans has yet to be shown. With the exception of the single tumor p53 mutation we reported last year from a nephropathy patient from the United Kingdom with documented AAI exposure [13], it is unlikely that many (if indeed any) of the urothelial tumor p53 mutations already recorded in the international database of human p53 mutations arose in tumors of individuals exposed to AA. Thus, the mutation profile that AA might elicit in the human *p53* sequence remains a matter of conjecture, although the mutagenic activity of AA has been investigated previously in transgenes of rodent assays. Kohara et al. [14] reported increased mutation frequencies in the *lacZ* and *cII* genes in target organs (forestomach, kidney, bladder) of AA-treated Muta^{TM}Mouse transgenic animals. Primarily A to T transversion mutations were found by sequence analysis of *cII* mutants in the target organs. This selectivity of AAI for mutations at adenine residues is consistent with the extensive formation of dA-AAI adducts in target organs of exposed rats [15,16]. Efforts in several laboratories are underway to analyze archived tumor material from patients with documented exposure to AA in order to determine whether urothelial tumor p53 mutations do arise in patients exposed to AA and if so, do the mutations reflect the chemical reactivity of carcinogenic AA metabolites with DNA. In this study, our goals were to generate and sequence mutations in human *p53* sequences of genetically modified mouse cells that we exposed to AAI and thus to provide an initial sketch of the mutation profile that may be anticipated in tumor patients with AA-nephropathy. We found mutations in the *p53* gene of AAI-exposed cells that confirm predictions from various biochemical studies, most notably the ^{32}P-postlabelling results of DNA from aristolochic acid-exposed individuals [13,17–19]. The composite AAI mutation signature generated by this experiment and a pilot study we reported previously [6] is clearly different from the p53 mutation signature found in cells exposed to the carcinogen B(*a*)P [8].

2. Materials and methods

2.1. Cell culture

To obtain independent cultures of immortal fibroblasts, we thawed cryopreserved passage 0 embryonic fibroblasts harvested from 13.5-day-old embryos of Hupki mice (Human p53 knock-in; Jackson Laboratory Strain #004301, Trp53$^{tm1/Holl}$) and plated the cells (also referred to as primary HUFs, for Hupki Fibroblasts) into T75 flasks containing DMEM supplemented with 10% FCS. At 80% confluence the cells were trypsinized and replated in three 6-well plates per set at 2×10^5 cells per well. The following day, the medium in each well was replaced with medium containing 50 μM aristolochic acid I (as sodium salt). This concentration was chosen on the basis of prior toxicity tests in primary HUFs, which showed a sharp decrease in cell survival after 48 h from 80% at 20 μM, to less than 20% at 100 μM concentrations of AAI. After 48 h, the mutagen-containing medium was removed, cells were washed briefly with PBS and fresh medium without mutagen was added to each well. Each of the 18 independent cultures was passaged at 1:2–1:4 dilution as the culture approached confluency. During crisis, which typically became manifest by passage 5, up to 15–20 days were required before cells were again ready for passaging. Cultures that resumed growth thereafter and regained a homogenous morphology with increasing passage number were cultivated to passages 12–14 before harvesting for DNA extraction and mutation analysis (described below). The AAI-treated cultures that reached this stage of immortalization could be passaged for many months subsequently, without signs of deterioration or growth arrest. These immortalized cultures are referred to here as HUF cell lines and are coded with the prefix NF-AAI. Control cultures of HUFs subjected to the same protocol as outlined above were not exposed to carcinogen-containing medium; immortal cell lines established from these cultures are designated by the prefix CO [8].