p53 Gene Mutation Pattern in Rat Liver Tumors Induced by Vinyl Chloride

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ABSTRACT

Vinyl chloride (VC) induces angiosarcoma of the liver (ASL) and hepatocellular carcinomas (HCCs) in humans and rodents. We examined the presence of p53 gene mutations in ASL and HCC induced by VC in Sprague Dawley rats; 25 ASL and eight HCCs were analyzed for point mutations in exons 5–8, using PCR amplification, single-strand conformation polymorphism analysis, and direct DNA sequencing. Mutations were found in 11 (44%) of the ASL and in 1 HCC. A 12-base pair deletion was found in one tumor; all others were base pair substitutions. Nine of the point mutations were observed at A:T base pairs (5 A:T $\rightarrow$ T:A; 2 A:T $\rightarrow$ G:C; and 2 A:T $\rightarrow$ C:G), and of three G:C $\rightarrow$ A:T transitions, only one was at a CpG site. In ASL, four mutations were found in exon 5, two in exon 6, and six in exon 7; the base pair substitution found in one HCC was in exon 8. One ASL exhibited two point mutations, including a silent one. Two ASL exhibited the same mutation in codon 203 and two other samples in codon 235. Codon 235 was found to be mutated in three ASL.

These data show that p53 is often mutated in ASL induced by VC in rats and, as observed in ASL in humans exposed to VC, the majority of the missense mutations involved A:T base pairs. The characteristic patterns of mutations found suggest that a common mechanism operates in VC-induced p53 mutagenesis in both species, and these mutations are consistent with the formation of DNA etheno adducts by VC in the liver. The A:T $\rightarrow$ T:A transversion observed in the first nucleotide of codon 235 in two rat ASL is equivalent to the A:T $\rightarrow$ T:A transversion characterized previously in codon 255 in one human ASL associated with VC exposure.

INTRODUCTION

Sporadic ASL is a rare disease in the human population, whereas in individuals exposed to VC, thorium dioxide (Thorotrast), or arsenic compounds, the risk of developing this type of cancer greatly is increased (for review, see Refs. 1 and 2). VC also induces ASL and HCCs in rodents as well as other cancers (3, 4).

Experimental studies have shown that VC is activated into chloroethylene oxide by cytochrome P450-dependent microsomal monoxygenases (5, 6). This epoxide is thought to be the reactive intermediate involved in the formation of VC-DNA adducts and three cyclic, promutagenic adducts with DNA bases have been identified in vivo following exposure of rats to VC, namely, eA, N$^2$-ethenocytosine, and N$^2$-3,ethenoguanine (reviewed in Ref. 7). Their promutagenic properties have been characterized extensively in vitro and in vivo and involve mainly base pair substitution mutations (reviewed in Ref. 8).

Site-specific mutagenesis studies in Escherichia coli and in mammalian cells have shown that both N$^2$-3,ethenoguanine and 3,N$^2$-etheno adenine can induce G:C $\rightarrow$ A:T transitions, and the latter can, in addition, lead to C:G $\rightarrow$ A:T transversions (9, 10); eA can direct misincorporation of G, C, or A during replication, thus inducing A:T $\rightarrow$ C:G, A:T $\rightarrow$ G:C, or A:T $\rightarrow$ T:A base pair substitutions, respectively (11, 12).

Previous studies have shown that in human ASL associated with VC, mutations in the p53 tumor suppressor gene occurred in three of six tumors and the mutations were all A:T-to-T:A transversions (13–15). Such mutations are uncommon in human cancers (2.7% of a total of 5085 cancers and 8% of a total of 290 primary liver cancers; Ref. 16). Moreover, in 30 ASL not associated with VC exposure, only two p53 mutations were found, and both were G:C-to-A:T transitions (17, 18). These data suggest that the pattern of p53 mutations detected in these tumors is associated specifically with a past exposure to the carcinogen VC. Specific p53 mutation patterns have also been observed in certain other human tumors directly attributable to exposure to a given carcinogen. Distinct changes in the p53 coding sequence have been found in HCCs associated with aflatoxin exposure, lung cancers associated with tobacco smoke, or skin cancers associated with solar UV radiation (19, 20).

The present studies were undertaken to examine whether in ASL induced in rats by VC in a well-characterized experimental system, the types of p53 mutations were consistent with the DNA adduct(s) induced by this carcinogen, and the findings are compared with the previous observations in humans.

MATERIALS AND METHODS

Tumor Induction. ASL and HCCs were obtained from Sprague Dawley rats following exposure to VC by inhalation or by gavage. Twenty-one samples were from the inhalation exposure experiment described by Froment et al. (21). The other 12 samples were from the following carcinogenicity bioassays carried out by Maltoni et al. (Ref. 3; number of samples examined in parentheses): inhalation experiments, BT4001 (n = 5), BT6 (n = 1), BT10 (n = 1), and BT14 (n = 1); gavage experiments, BT11 (n = 3) and BT27 (n = 1). Liver tissue samples had been either frozen in liquid nitrogen and stored at −80°C or fixed in alcohol and embedded in paraffin.

DNA Isolation. Tumor areas in the liver samples were localized by histological examination of tissue sections stained with H&E. Several consecutive micromtome unstained sections were then prepared. Paraffin sections were dewaxed and rehydrated before further processing. Tumor areas from several (frozen or paraffin) sections were microdissected with a scalpel blade, pooled, and soaked overnight at 4°C in 200 μl of buffer containing 50 mm Tris-HCl (pH 7.5), 150 mm NaCl, 2 mm EDTA, and 1% (v/v) SDS. DNA extraction was performed essentially as described by Shafritz et al. (22).

PCR. The analyses of p53 gene mutations in liver tumors induced by VC in rats were confined to exons 5–9, because in human cancers the majority of p53 point mutations are localized within these evolutionarily conserved regions that code for the DNA-binding domain of the protein (19). The DNA sequence of the rat p53 gene was taken from Soussi et al. (23), Hulla and Schneider (24), and Vancutsem et al. (25). The first nucleotide of the initiation codon was designated nucleotide 1 in the cDNA sequence (26, 27). Two ASL exhibited the same mutation in codon 203 and two other samples in codon 235. Codon 235 was found to be mutated in three ASL.

Received 11/26/96; accepted 3/10/97.

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1 This work was supported in part by National Institute of Environmental Health Sciences Grant ES 05948 (to A. B.) and by Grant EVSV-CT920199 (to R. M.) from the European Commission.

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4 The abbreviations used are: ASL, angiosarcoma(s) of the liver; VC, vinyl chloride; HCC, hepatocellular carcinoma; SSCP, single-strand conformation polymorphism; eA, 1,N$^2$-etheno adenine.
City, CA) using the solid-phase phosphoramidite method. Pairs of intronic primers or a combination of an intronic primer and an exonic primer was used to exclude the possible coamplification of rat p53 pseudogenes. The location and sequence of each primer is shown in Fig. 1. Amplified fragments were between 125 and 273 bp, which provides optimal sensitivity for SSCP analysis. PCR amplification was performed under standard conditions. The reaction mixture (50 μl) contained 1 μg or less DNA, 1 μM of each primer, 0.4 mM of each deoxyribonucleoside 5’-triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2—4 mM MgCl₂, and 2 units of Taq DNA polymerase (Boehringer Mannheim France, Meylan, France). Thirty-five cycles of amplification were carried out in a DNA Thermal Cycler (Perkin-Elmer, Saint-Quentin, France). Each cycle consisted of 10 s at 94°C, 10 s at 55°C, and 30 s at 72°C, with a final extension step of 5 min at 72°C.

SSCP Analysis. The amplified DNA was electrophoresed with molecular weight markers on a 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME). The band corresponding to the expected fragment was excised under UV light, and the DNA was eluted in 50 μl of water. Ten μl of eluted DNA were submitted to a second round of PCR, as described above, but in the presence of 10 μCi of [α-32P]dTTP (Amersham France S. A., Les Ulis, France). The 32P-labeled sample was diluted 10 times in a loading buffer, denatured at 100°C for 4 min, and chilled on ice, and an aliquot (with a radioactivity of about 20,000 cpm) was loaded onto a Mutation Detection Enhancement Hydrolink gel (Bioprobe Systems, Montreuil-sous-Bois, France) prepared with or without 5% glycerol. Electrophoresis was carried out at a constant power of 8 W for 15 h at room temperature, in 0.6X Tnt-borate EDTA buffer. The gel was dried on filter paper and exposed to autoradiography. Arrows, mutant DNA bands; others were G:C → A:T transitions, one occurring at a CpG site and others were G:C → A:T transitions, one occurring at a CpG site and another at a CpNpG site. Three different mutations were recovered in codon 235 (Table 1; Fig. 3), and mutations in codons 203 and 253 were observed twice (Table 1). One sample exhibited a 12-base pair deletion in exon 5, between nucleotide positions 531 and 542. Within this sequence, a direct repeat of three base pairs (TGA) flanked both sides of the deleted segment, with only one being included in the 12-bp deletion; this segment was enclosed in an inverted repeat (CACCAT).

Detection of p53 Gene Mutations by SSCP. Mutations of the p53 gene were investigated by SSCP analysis in 25 ASL and eight HCCs induced by VC in rats. Exons 5—9 were sequenced. The cDNA sequence published by Soussi et al. (23) was used as a reference. RESULTS

Detection of p53 Gene Mutations by SSCP. Mutations of the p53 gene were investigated by SSCP analysis in 25 ASL and eight HCCs induced by VC in rats. Exons 5—9 were sequenced. The cDNA sequence published by Soussi et al. (23) was used as a reference.

Mutation Spectra. The shifted SSCP fragments were collected and submitted to a standard PCR followed by an asymmetrical PCR, to amplify either the sense or the antisense strand. For both the asymmetrical PCR and DNA sequencing, the same primers were used as for amplification of genomic DNA (see Fig. 1). Sequencing data, shown in Table 1, confirmed that the shifted SSCP fragments, observed in 12 cases, were mutated. Positive samples were confirmed further by sequencing from a second independent PCR from tumor DNA. Eleven of these mutations were base pair substitutions resulting in an amino acid change. One tumor sample (sample 2) yielded two shifted SSCP bands with different point mutations, one of them being silent. A total of nine base pair substitutions arose at A:T base pairs (five A:T → T:A, two A:T → G:C, and two A:T → C:G). The three others were G:C → A:T transitions, one occurring at a CpG site and another at a CpNpG site. Three different mutations were recovered in codon 235 (Table 1; Fig. 3), and mutations in codons 203 and 253 were observed twice (Table 1). One sample exhibited a 12-base pair deletion in exon 5, between nucleotide positions 531 and 542. Within this sequence, a direct repeat of three base pairs (TGA) flanked both sides of the deleted segment, with only one being included in the 12-bp deletion; this segment was enclosed in an inverted repeat (CACCAT).
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Table 1  p53 mutations in rat liver tumors induced by VC

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA sequence change</th>
<th>Codon (exon)</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12-bp deletion</td>
<td>177–181 (5)</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>TTC → TCT</td>
<td>147 (5)</td>
<td>Gly → Ser</td>
</tr>
<tr>
<td>3</td>
<td>GGT → AGT</td>
<td>152 (5)</td>
<td>Lys → Thr</td>
</tr>
<tr>
<td>4</td>
<td>AGT → AAG</td>
<td>160 (5)</td>
<td>Thr → Val</td>
</tr>
<tr>
<td>5</td>
<td>ATG → TGG</td>
<td>235 (7)</td>
<td>Met → Arg</td>
</tr>
<tr>
<td>6</td>
<td>ATG → CTG</td>
<td>235 (7)</td>
<td>Met → Leu</td>
</tr>
<tr>
<td>7</td>
<td>TAT → GTT</td>
<td>235 (7)</td>
<td>Thr → Cys</td>
</tr>
<tr>
<td>8</td>
<td>TAT → GTT</td>
<td>235 (7)</td>
<td>Thr → Cys</td>
</tr>
<tr>
<td>9</td>
<td>ATC → TTC</td>
<td>253 (7)</td>
<td>Ile → Phe</td>
</tr>
<tr>
<td>10</td>
<td>ATC → TTC</td>
<td>253 (7)</td>
<td>Ile → Phe</td>
</tr>
<tr>
<td>11</td>
<td>CGG → CAC</td>
<td>246 (7)</td>
<td>Arg → His</td>
</tr>
<tr>
<td>12</td>
<td>GAG → GTG</td>
<td>283 (8)</td>
<td>Glu → Val</td>
</tr>
</tbody>
</table>

a Tumors were obtained from Sprague Dawley rats exposed to VC either by gavage (animals 1–3) or by inhalation (animals 4–12). Of 25 ASL analyzed, 11 (samples 1–11) exhibited p53 gene mutations, and out of 8 HCCs, 1 (sample 12) was found with a p53 gene mutation.

b Codons and exons were numbered as described in "Materials and Methods."

DISCUSSION

The findings of this study show that p53 mutations are a frequent event (11 of 25 cases; 44% prevalence) in ASL, but are less frequent (1 of 8 cases) in HCCs induced in rats by VC. In general, p53 mutations are not observed in HCCs induced in rats or mice by a variety of carcinogens (33), with the exception of the mutations observed in HCCs induced in rats by tamoxifen, in which 50% of the tumors showed p53 mutations (25). The second major finding of this study is that the majority of mutations (in ASL) were missense mutations (10 of 12; 83%), 8 of which arose at A:T base pairs, and 4 of which were A:T-to-T:A transversions (Table 1). These data in rats clearly indicate a specific mutation spectrum in ASL associated with exposure to VC. The only mutation detected in HCCs was also an A:T-to-T:A transversion.

The rat p53 mutations found at A:T base pairs clustered at a few sites in exons 6 and 7; codon 235 was mutated in three tumors, and codons 203 and 253 were each mutated in two tumors. It should be noted that the three p53 mutations observed in ASL from VC-exposed workers were all A:T-to-T:A transversions (Refs. 13–15; Table 2). The A:T-to-T:A transversion at the first nucleotide of codon 235 (exon 7) in one of these human tumors is equivalent to the A:T-to-T:A transversion found at codon 253 in two rat ASL (Table 1). The two other A:T-to-T:A transversions occurred at codons 179 (exon 5) and 249 (exon 7) of the human p53 gene. These data indicate that similar molecular genetic changes occur at the level of the p53 gene in human and rat ASL associated with exposure to VC. This is also supported by the observations of Soini et al. (17) and Andersson et al. (18) who examined p53 mutations in human ASL not associated with VC exposure. In 13 cases of human ASL associated with Thorotrast (thorium dioxide) exposure, no p53 mutations were detected, and in 17 sporadic ASL (not associated with VC, Thorotrast, or arsenic exposure), only two contained p53 mutations, consisting of G:C → A:T transitions at codons 136 and 141. Thus, p53 mutations are as uncommon in sporadic ASL as in other human cancers, and their profile is different from that of VC-associated ASL (see "Introduction" and Table 2).

VC after metabolic activation (6) induces several DNA adducts, and various studies have shown that these DNA adducts can be responsible for specific mutations. eA gives rise to A:T → T:A, A:T → C:G, and A:T → G:C base pair substitutions in E. coli and simian kidney cells (Refs. 11 and 12; Table 2). In Sprague Dawley rats, eA was shown to accumulate preferentially in hepatic DNA during prolonged exposure to VC; it accumulated to a much lower extent in extrahepatic tissues (34). These data suggest that the missense mutations observed at A:T base pairs in the p53 gene could arise from eA. The formation of this ethenobase has not yet been demonstrated in hepatic endothelial cells following exposure to VC; however, there is evidence for other types of DNA adducts of their preferential accumulation in hepatic endothelial cells (35, 36).

Other mutations observed in this study, the partial deletion and the G:C → A:T transitions, may result from endogenous processes and/or from alkylation of DNA by reactive metabolites of VC yielding N2,3-ethenoguanine and 3,N′-ethenocytosine (see Table 2).

Specific mutation patterns in the p53 gene have been observed in some other cancers. For example, human and murine UV-induced skin cancers often exhibit p53 mutations, such as C → T substitutions and CC → TT double-base changes at dipyrимidine sites, which are specifically in-

Table 2 Comparison of mutation spectra in the p53 gene in ASL in humans and rats and promutagenic properties of ethenobasesa

<table>
<thead>
<tr>
<th>Species</th>
<th>Tumor origin</th>
<th>No. mutations/no. of cases</th>
<th>No. and types of mutations</th>
<th>Ethenobaseb</th>
<th>Base pair substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>VC associated (13–15)</td>
<td>3/6</td>
<td>3 A:T → T:A</td>
<td>eA (11, 12)</td>
<td>eA → G</td>
</tr>
<tr>
<td></td>
<td>Thorotrast associated (17, 18)</td>
<td>0/13</td>
<td>2 G:C → A:T</td>
<td>eA → T</td>
<td>eA → C</td>
</tr>
<tr>
<td>Rats</td>
<td>VC associated (this study)</td>
<td>13/33c</td>
<td>5 A:T → T:A, 2 A:T → G:C, 2 A:T → C:G, 3 G:C → A:T</td>
<td>eC (10, 44)</td>
<td>eC → A</td>
</tr>
<tr>
<td></td>
<td>GAG → GTG</td>
<td>2/17</td>
<td>1 deletion</td>
<td>eG (9, 45)</td>
<td>eG → A</td>
</tr>
</tbody>
</table>

a Reference numbers in parentheses.
b eC, 3,N′-ethenocytosine; eG, N2,3-ethenoguanine.
c Twenty-five ASL and eight HCCs. One tumor sample exhibited two mutations.
d One A:T-to-T:A transversion was present in a HCC.
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duced by UVB radiation (37–39). More recently, other p53 signature mutations have been reported in murine skin cancers induced by PUVA therapy (a combination of psoralen and UVA radiation; Ref. 40). Another well-known example is the AGG-to-AGT base pair substitution at codon 249 of the p53 gene that occurs in more than half of the human HCCs associated with high aflatoxin B1 dietary intake and in nonmalignant liver tissue from HCC patients (e.g., Refs. 41 and 42). The data presented here in rats strongly support the finding in humans that A:T base pair mutations induced by UVB represent a specific mutational “signature” of the environmental carcinoigen VC. This observation supports the notion that p53 mutation spectra can be used as fingerprints of past carcinogen exposure in molecular epidemiological studies (20, 43).

ACKNOWLEDGMENTS

We thank Dr. B. Bancel (Laboratory of Pathological Anatomy, Hôpital de la Croix-Rousse, Lyon, France) for her assistance in examining the rat liver tumor samples obtained at IARC and Drs. C. Wild, J. Hall, and P. Hainaut for helpful discussions.

REFERENCES


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6. REFERENCES


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