Mutagenesis of ras Proto-Oncogenes in Rat Liver Tumors Induced by Vinyl Chloride

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ABSTRACT

Vinyl chloride is a DNA-damaging carcinogen which induces liver angiosarcomas in humans and animals. Activation of the Ki-ras 2 gene by a GC→AT transition at the second base of codon 13 in liver angiosarcomas associated with occupational exposure to vinyl chloride has been reported recently. In order to compare the molecular pathways of carcinogenesis in humans and animals, Sprague-Dawley rats were exposed to vinyl chloride and hepatic tumors, including two hepatocellular carcinomas and five liver angiosarcomas, were investigated for mutations at codons 12, 13 and 61 of the Ha-ras, Ki-ras and N-ras genes. High molecular weight DNA was amplified by the polymerase chain reaction and point mutations were analyzed by allele specific oligonucleotide hybridization, direct sequencing of polymerase chain reaction products and sequencing after cloning. None of the tumors exhibited a mutation in codons 12, 13 and 61 of the Ki-ras gene, nor in codons 12 and 61 of the N-ras gene. However, an activating AT→TA transversion at base 2 of codon 61 of the Ha-ras gene was detected in two liver angiosarcomas, suggesting that the nature of the ras gene affected by a given carcinogen depends on host factors specific to cell types. Several additional base pair substitutions were found in exon 1 of the N-ras B and C sequences. NIH 3T3 transfection assays and Southern blot analysis of DNA from transformed NIH 3T3 cells confirmed the presence of a dominant activated N-ras gene. These results emphasize the differences in the molecular pathways leading to tumors in humans and rats and within a given species between different cell types.

INTRODUCTION

Chemically induced tumorigenesis in rats is widely used to assess the carcinogenic risk of chemicals for humans and to study the molecular mechanisms of carcinogenesis in order to improve cancer prevention and therapy in humans. Whether the molecular pathways leading to a given tumor are similar in both species and whether the results obtained for animals can be extrapolated to humans remain speculative in most instances. A first clue has been provided by molecular studies of tumors induced by AFB11. AFB1, a toxic substance produced by Aspergillus flavus, is one of the most potent hepatocarcinogenic agents inducing HCCs in humans and animals (1, 2, 3). It is found as a grain contaminant in countries with a warm and moist climate, especially Southeast Asia and Southern Africa. In hepatocellular carcinomas from patients of such countries, mutations at hot spots, particularly at codon 249 of the p53 tumor suppressor gene, have been attributed to the genotoxic effect of AFB1 (2, 3).

However, this mutation has not been found in HCC induced by AFB1 in nonhuman primates and rats (4, 5). Also, in these countries hepatitis B virus is endemic and is often associated with the hepatocarcinomas studied, blurring the etiology of the tumor (2, 3).

Liver tumors induced by VC provide a unique opportunity to compare the molecular pathways of carcinogenesis in humans and animals. VC is a gas industrially used to synthesize polyvinyl chloride. Its carcinogenic properties were fortuitously discovered in rats in 1971 by Viola et al. (6) and confirmed by Maltoni et al. (7), who demonstrated, using Sprague-Dawley rats in a dose-response study, that VC induced, among various types of tumors, angiosarcomas of the liver. In 1974, Creech and Johnson (8) reported three deaths by liver angiosarcoma in the same polymerization plant.

A multitude of studies have established the metabolic pathways of VC, its mutagenic properties, and allowed the identification of VC-DNA adducts (9, 10, 11). It is generally admitted that the adducts are responsible for point mutations in genes implicated in the control of cell proliferation and differentiation. Among these genes, members of the ras gene family, Ha-ras, Ki-ras, and N-ras, are frequently activated by point mutations in codons 12, 13, and 61. These activated genes seem to play a key role in the development of spontaneous or carcinogen-induced animal tumors (12).

We have previously reported that the c-Ki-ras 2 gene, activated by a GC→AT transition at base 2 of codon 13, was found in liver angiosarcomas of humans who had been highly exposed to VC (13). Here, we report the results of an investigation into Ha-ras, Ki-ras, and N-ras gene activation, by point mutations, in liver tumors induced by VC in Sprague-Dawley rats.

MATERIALS AND METHODS

Tumor Induction. Six pregnant Sprague-Dawley (OFA) rats were obtained from Iffa-Credo (L’Arbresle, France). They were housed in a temperature and humidity controlled room with a 12-h light/dark cycle and fed with Biscuits Extra Labo rodent chow (Piétremont, Provins, France) and water ad libitum. Using an inhalation chamber, described previously (11), four female rats with their pups (both sexes) were exposed 8 h per day, 6 days per week to 500 ppm of VC from days 3 through 28 postpartum. At day 28 post partum, young animals were weaned, and the males (22) and females (22) were separated, redistributed into four groups, and further exposed for 2 weeks (total exposure, 33 days). During exposure, rats were housed in four plastic and brass cages placed in the same housing conditions as described above, and the adults had free access to food and water. The control group comprised two females with their pups (14 males, 14 females) of the same age not exposed to VC. Following exposure, rats were observed daily until death or appearance of tumors. Rats in poor condition or with obvious tumors were killed by inhalation of diethyl ether. Some animals died spontaneously. The surviving rats were sacrificed at 19 months. All animals underwent total necropsy. Several organs (lung, liver, kidney, brain, spleen, and lymphocyes) were removed, and gross tumors were excised from unaltered tissue. All the tissues and tumors were frozen in liquid nitrogen and stored at −80°C. A part of each tissue was also fixed in alcohol, embedded in paraffin, and stored for histological analyses.

DNA Preparation. For DNA preparation, only tumor samples exhibiting a well-conserved structure, in which the different lesions could be identified histologically and further easily isolated, were chosen. A 3-μm section was first cut from a frozen liver sample using a cryostat microtome and stained with
eicos and hematoxylin. After histological examination, about 100 sections of 12 µm were cut out from the tumoral part of the frozen block and pooled. A last 3-µm section was stained as a control. The high-molecular-weight DNA was prepared essentially as described previously (13).

**PCR.** DNA was amplified by PCR for c-Ha-ras, c-Ki-ras, and c-N-ras sequences around codons 12–13 and 61 as described (13). On the basis of sequences previously published, the exonic primers used for amplification of Ha-ras codons 12 and 61 were, respectively, 5'-GAGACAACTCCAGGCTTT-3' and 5'-TGGTGGTGAGCGGACCTT-3', and 5'-AGACGTGGTT-TACTGGGATCT-3' and 5'-CGATGTACGGTGCTCCGCAT-3', yielding PCR fragments of 63 or 73 base pairs (14). In addition, amplification of exon 2 of the Ha-ras gene was also performed with the intronic primers described by Fujimoto et al. (15). The primers used for amplification of Ki-ras codons 12 and 61 were, respectively, 5'-GAGCTGATAATTACGG-3' and 5'-CATCTGTAAGCAGCTTATC-3', and 5'-CTCTCTAAGGACAGTAG-3' and 5'-CAACAAAGAGCCTCTCCA-3', yielding PCR fragments of 108 or 128 base pairs (16). N-ras gene amplification was performed either with the primers designed for the human N-ras gene (17) or with the intronic primers of the N-ras A gene exon 1 (15), followed by a second PCR with the corresponding to the expected fragment was excised under UV light, and the DNA was eluted. Ten µl of eluted DNA were submitted to a second round of PCR with a primer ratio of 50:1 to obtain single-stranded DNA fragments. The single-stranded DNA was purified on a Sephacryl S300 column (Pharmacia, Saint Quentin, France) and directly sequenced (18) by the dideoxynucleotide chain termination method using DNA Sequenase Version 2.0 (United States Biochemical Corporation, Cleveland, OH) and primers 5' end-labeled with [γ-32P]ATP (specific activity, >185 TBeq/mmol; Amersham) or primers 5' end-labeled with [α-32P]dATP (specific activity, >185 TBeq/mmol; Amersham, Les Ulis, France).

**Cloning and Sequencing.** Exon 1 of the N-ras gene was amplified from cH ras exon 1, and was designed according to sequences described previously (14). Oligonucleotides were synthesized using an A391 automatic synthesizer (Applied Biosystems, Paris Nord II, Rissoy, France).

**Direct Sequencing Analysis.** DNA from tumor samples was amplified, and 10 µl of the PCR products were subjected to electrophoresis in 4% NuSieve agarose gels (FMC BioProducts, Rockland, ME). The band corresponding to the expected fragment was excised under UV light, and the DNA was eluted. Ten µl of eluted DNA were submitted to a second round of PCR with a primer ratio of 50:1 to obtain single-stranded DNA fragments. The single-stranded DNA was purified on a Sephacryl S300 column (Pharmacia, Saint Quentin, France) and directly sequenced (18) by the dideoxynucleotide chain termination method using DNA Sequenase Version 2.0 (United States Biochemical Corporation, Cleveland, OH) and primers 5' end-labeled with [γ-32P]ATP (specific activity, >185 TBeq/mmol; Amersham, Les Ulis, France).

**Southern Blot Analysis.** Ten µg of DNA extracted from individual foci of NIH 3T3 transformants were digested with 50 units of the restriction endonuclease BamHI, using the conditions recommended by the supplier (Boehringer, Meylan, France), and subjected to electrophoresis in 0.7% agarose gels. DNA was then transferred to nylon membranes (Gene-Screen Plus; NEN DuPont de Nemours, Boston, MA) and hybridized in 50% (v/v) formamide, 10% (w/v) dextran sulfate, 1% (w/v) sodium dodecyl sulfate, 1 M NaCl, and 1.5 mg/ml sonicated and denatured herring sperm DNA at 42°C for 48 h with probes labeled with [α-32P]dCTP (specific activity, >110 TBeq/mmol; Amersham) by random priming. The filters were washed twice with 2X standard saline citrate (1X standard saline citrate: 0.15 M sodium chloride and 0.015 M sodium citrate) at room temperature, once with 0.5X standard saline citrate and 1% sodium dodecyl sulfate at 65°C, and exposed to X-ray film (Hyperfilm MP; Amer sham) at −70°C, with intensifying screens. The probe used in this study was a 0.9-kilobase PvuII fragment of the human N-ras gene isolated from the plasmid pKN-9K. This plasmid was kindly provided by the Japanese Cancer Research Bank.

**RESULTS**

**Tumor Histology.** The liver lesions were histologically classified according to the recommendations of the Institute of Laboratory Animal Resources (22). For all of the VC-treated rats, livers which appeared macroscopically normal at the necropsy were found by histological examination to contain multiple nodular hyperplastic foci of hepatocytes. A H&E paraffin section of a VC-exposed rat liver, exhibiting such a hyperplastic nodule, is shown in Fig. 1A. Liver tumors including 8 HCCs, 15 angiosarcomas, and 2 benign cholangiomas were found in the VC-treated rats. It should be noted that several livers contained two or more different lesions. Moreover, some hepatocarcinomas contained hyperplastic endothelial cells, and some liver angiosarcomas were found to exhibit hepatocyte dysplasia.

In addition to liver tumors, other tumors were detected, including three lung angiosarcomas, probably metastatic, three nephroblastomas, one abdominal angiomylema, one leukemia, one Zymbal gland carcinoma, one pituitary gland adenoma, four mammary fibromas, and three mammary carcinomas.

Our study included two HCC (samples 1 and 2), and five ASL (samples 3 to 7). In both HCCs, nonatypic endothelial cell hyperplasia was observed on the H&E sections of paraffin-embedded tissues (Fig. 1B); the sections from the frozen tissues used for the DNA preparation exhibited the same histological pattern. Sample 3 was diagnosed as an angiosarcoma of sinusoidal pattern with neighboring areas of more anaplastic pattern (Fig. 1C). The frozen section also exhibited an anaplastic pattern with areas of necrosis. Three different lesions were observed in sample 4: an hepatocellular carcinoma of trabecular and papillary pattern, a sinusoidal angiosarcoma, and areas of strongly atypical endothelial cell hyperplasia. The DNA was extracted from a fragment exhibiting mainly sinusoidal angiosarcoma. Samples 5, 6, and 7 were more typical of pure angiosarcomas, ranging from sinusoidal to cavernous pattern, with the endothelial cells surrounding degenerative and sometimes dysplastic hepatocytes (Fig. 1D). For these tumors, the frozen sections exhibited the same histological pattern as the paraffin sections.

**Analysis of Ki-ras and Ha-ras Gene Mutations.** For analysis of mutations at codons 12, 13, and 61 of the Ki-ras gene, and at codon

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12 of the Ha-ras gene, the DNA extracted from frozen tumor fragments was amplified by PCR and analyzed by ASO hybridization. The amplified DNA efficiently hybridized with the wild-type probes but not with the mutant sequence probes. These results were confirmed by direct sequencing analysis of the Ki-ras gene (data not shown). Therefore, none of the seven tumors analyzed exhibited any mutations in codons 12, 13, or 61 of the Ki-ras gene, or in codon 12 of the Ha-ras gene.

When analyzed by ASO hybridization, amplified DNA from samples 1 and 2 exhibited a positive signal with the wild-type Ha-ras codon 61 probe and with the mutated probe for an A→T mutation at the second base (data not shown). This mutation was confirmed by direct sequencing of DNA amplified by PCR using exonic or intronic primers to both strands (Fig. 2). Thus, tumor samples 1 and 2 contained an AT→TA transversion at the second base of codon 61 (Table 1), corresponding to a Gln-Leu amino acid substitution.

Analysis of N-ras Gene Mutations. Because a polymorphism of exon 1 of the N-ras gene was reported for Fisher, Long-Evans, and Wistar rats (23, 24, 25), a cloning strategy was used to investigate the exon 1 sequence of this gene. PCR amplification products of DNA extracted from liver, lung, and spleen from control Sprague-Dawley rats were cloned, and 75 clones were individually sequenced. The three N-ras sequences described previously by McMahon et al. (23) were found. N-ras A, homologous to the human N-ras sequence, was found in 26% of the clones analyzed. The sequence N-ras B, differing from the N-ras A sequence by a base pair substitution at the first base of codon 14 (GTT→ATT), was also found in 26% of the clones. The sequence N-ras C, homologous to the N-ras A, but with substitutions at codon 8 (GGC→GGT), codon 13 (GCC→GTC), and codon 18 (GGT→GTG), was found in 14% of the sequenced clones (data not shown). A fourth N-ras sequence, classified as N-ras B because of the presence of the substitution at the first base of codon 14 but containing, in addition, two substitutions at codons 15 (GGG→AGG) and 24 (ATC→ACC), was also found in 13% of the clones and in all the samples tested.

Sequences differing from these four main sequences were also observed in a few clones (12/75). N-ras A sequences with base pair substitutions at codons 8, 18, or 24 and N-ras B sequences with substitutions at codons 15 or 24 were identified. The N-ras C sequence also exhibited modifications at codon 8 or at codons 8 and 18 (data not shown). Remarkably, all these minor modifications were
confined to codons 8, 15, 18, and 24, which thus appear to be hyper-mutable codons.

Initially, assuming that N-ras B and C were pseudogenes (26), the DNA extracted from the VC-induced liver tumors was amplified using intronic primers and sequenced directly. No mutation was detected by this method in any of the seven DNA analyzed.

However, a mutation could have been missed due to the dilution of the signal arising from the mutated DNA by the signal from the nonmutated DNA. Moreover, McMahon et al. (23) have found that the N-ras C sequence transformed cells in an NIH 3T3 transfection assay. Therefore, both to detect infrequent mutations and to analyze the pseudogenes, the investigation of the N-ras gene mutations was also performed by cloning the PCR products produced with exonic primers, followed by the sequencing of individual clones. The three N-ras sequences, N-ras A, B, and C, were found in all samples, together with some of the base substitutions at codons 8, 15, 18, and 24 found in untreated rats. However, we found additional base pair substitutions in some tumoral DNAs. These base pair substitutions included a GC→AT transition at codon 13 (GGC→GAC) and an AT→CG transition at codon 36 (ATA→CTA) in the N-ras A gene, inducing Gly-Asp and Ile-Leu amino acid substitutions; they were observed in the DNA extracted from samples 5 and 7, respectively (Fig. 3). Other nucleotide substitutions, confined to the hyper-mutable codons (15 and 18) and observed in N-ras B and C genes, were also apparently associated with VC (Table 1).

**ras Gene Analysis Using the DNA Transfection Assay.** All the tumor DNA samples, except sample 2, for which a sufficient amount of DNA was not available, were subjected to transfection assays, and the resulting transfected cells were subjected to nude mice tumorigenicity tests and focus-formation assays. DNA from tumors 1 and 3 led to few foci of transformed cells as compared with DNA from tumors 4, 5, 6, and 7; the delay of appearance of the mice tumors was in accordance with these results (Table 2).

The transfected NIH 3T3 cells were selected using G418, and individual foci of transformed cells were isolated and expanded. DNA samples from foci of transformed cells were screened for N-ras sequences, by Southern blot analysis, after the first round of transfection. As shown in Fig. 4, hybridization of nontransformed NIH 3T3 cell DNA detected two bands of 12.8 and 10 kilobases for the cellular N-ras gene of mice. Clearly, additional bands were present in transformants corresponding to samples 5 and 7, indicating the presence of a dominant transforming N-ras gene. Direct sequencing of the DNA from isolated foci showed both rat and mouse N-ras sequences. However, the mutations initially found in the tumors could not be confirmed in the transformed NIH 3T3 cells.

**DISCUSSION**

In order to compare the molecular mechanisms of carcinogenesis in humans and animals, Sprague-Dawley rats were exposed to gaseous vinyl chloride, a carcinogen known to induce liver tumors, and particularly angiosarcomas, in both species (7, 8, 27, 28). Point mutations at hot spots in ras genes were investigated in VC-induced HCCs and ASLS.

In our study, ASLS, a tumor otherwise rare in humans and rodents (29, 30, 31), have been obtained with an incidence of 34%, confirming the relationship between VC and the occurrence of ASL established previously (6, 7). HCCs (18%) and benign cholangiomas (4%) have also been obtained after VC exposure, with incidences similar to those observed by Maltoni et al. (7) for liver tumors.

In untreated Sprague-Dawley rats, the three sequences, N-ras A, B, and C, described previously by McMahon et al. (23) for the Fisher
The adduct 1,6-ethenoadenine, which has been identified in the livers of N-methylurea treatment (36), were either spontaneous or due to endogenously produced carcino urethan-treated mice (42, 43) and in VC-treated rats (11), could be the oxirane intermediates and react with nucleic acids to form ethenobases.

Adding genetic modifications, termed I-compounds and resembling polycyclic aromatic hydrocarbons, might occur during normal metabolism, oxidative stress, or chronic inflammation may accumulate in the DNA of healthy humans (35). It has also been shown recently that mutations due to the Taq DNA polymerase errors can be identified and are, therefore, somatic mutations. Due to the short length of the PCR products (109 and 160 bases for the N-ras exon 1) and the number of PCR cycles compared with the number of the mutated clones, artifacts due to endogenously produced carcinogens. Indeed, adducts produced during normal metabolism, oxidative stress, or chronic inflammation may accumulate in the DNA of untreated rats (33). In addition, a series of unidentified nucleotide substitutions were observed in a few clones, in the four different N-ras sequences, and from the controls and VC-treated rats. Their relative scarcity suggests that they are present only in a few cells and that they are, therefore, somatic mutations. Due to the short length of the PCR products and the number of PCR cycles compared with the number of the mutated clones, artifacts due to Taq DNA polymerase errors can be reasonably excluded. Therefore, we assumed that these mutations were either spontaneous or due to endogenously produced carcinogens. Indeed, adducts produced during normal metabolism, oxidative stress, or chronic inflammation may accumulate in the DNA of untreated rats (33). In addition, a series of unidentified nucleotide modifications, termed I-compounds and resembling polycyclic aromatic DNA adducts, have been described in rats (34). Such genetic lesions may very possibly be able to induce mutations. Using a clonal assay, random somatic mutations have been described in the hprt gene of healthy humans (35). It has also been shown recently that mutations found in N-nitroso-N-methylurea-induced mammary tumors in Fisher rats were, in fact, present in the mammary tissue before the N-nitroso-N-methylurea treatment (36).

Samples 1 and 2, both identified as HCCs, exhibited an AT→TA transversion at the second base of codon 61 of the Ha-ras gene. AsL gene activation is usually a rare event in chemically induced HCC in rats (37, 38). However, this same AT→TA transversion in the Ha-ras gene is frequently observed in urethan-induced liver tumors in different mouse strains (39, 40, 41). Both urethan and vinyl chloride are activated into oxirane intermediates and react with nucleic acids to form ethenobases. The adduct 1,6-ethenoadenine, which has been identified in the livers of urethan-treated mice (42, 43) and in VC-treated rats (11), could be the premutagenic lesion of the Ha-ras gene; it directs the misincorporation of thymine (44). This mutation has not been detected in the angiosarcomas from this study, indicating that there might be cell type-specific factors affecting the accessibility of the ras genes.

Two of the five ASLS studied contained, respectively, a GC→AT transition at codon 13, the same transition as found in human ASL, and an AT→CG transversion at codon 36 in the N-ras A. The GC→AT transition is consistent with the miscoding properties of the VC adducts. N2,3-ethenodeoxyguanosine or 3,N6-ethenodeoxycytidine, and the AT→CG transversion with the formation of 1,N6-ethenodeoxycytidine (43, 44). Whether these mutations are directly responsible for the development of the ASL remains to be determined since they were found in only a few clones. It is possible that the signal from the tumoral cells lining the sinusoids has been diluted by the signal from the residual hepatocytes and other cells. Another hypothesis is that only a few tumoral cells have acquired the mutation. An uneven distribution of ras-mutated cells within a tumor has been reported in rat colon tumors and in mouse liver tumors (45, 46). This scarcity of the ras-mutated cells within a tumor has been attributed to ras mutations being a late event or due to tumor heterogeneity. Also, these mutations could be induced by endogenous adducts and be uninvolved in the tumorigenesis. Yet, Rearranged and amplified copies of N-ras sequences have been identified in the transfectants, confirming the involvement of an activated N-ras gene in the tumors.

We have reported earlier that the c-Ki-ras 2 gene was activated, with a relatively high incidence, by a GC→AT transition at the second base of codon 13 in ASLS of VC-exposed workers (13). In contrast, none of the five ASLS induced by VC in Sprague-Dawley rats exhibited a point mutation at codons 12, 13, or 61 of the Ki-ras gene. AsLS are located on different chromosomes in rats and humans (12) and are probably surrounded by different genes, which could modify the overall accessibility in both species. A different response of mammalian species toward the same chemical carcinogen and the resulting mutation patterns have also been observed in AFB1-induced HCC in humans and nonhuman primates (2, 3, 4).

The relative scarcity of relevant ras gene mutations in rat VC-induced ASL, in contrast to the human tumors in which the activation of the Ki-ras gene seems to be a key event (13, 47), points to a different carcinogenic mechanism in rats, which does not implicate a direct genotoxic effect of VC on the ras genes. Moreover, in our study, the highest tumorigenic effect observed in nude mice inoculated with transfected NIH 3T3 cells was obtained for the tumors in which the presence of an activated ras gene could not be demonstrated. This result, taken together with the few mutations apparently observed in the rat tumors, suggest strongly that genetic events inducing either the inactivation of a tumor suppressor gene, as in the human tumors (48), or the activation of an unidentified oncogene, are involved and remain to be determined.

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