Int Arch Occup Environ Health (1996) 68 : 394–398

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M.-J. Marion · I. DeVivo · S. Smith · J.-C. Luo
P. W. Brandt-Rauf

The molecular epidemiology of occupational carcinogenesis in vinyl chloride exposed workers

75-01-4

Introduction

The production of specific mutations in cellular oncogenes and tumor suppressor genes is thought to be involved in the development of certain cancers related to exposures to chemical carcinogens. The occurrence of certain point mutations in oncogenes and tumor suppressor genes results in the expression of mutant forms of their encoded protein products, which is believed to contribute to the process of cellular transformation and thus cancer. The detection of the expression of such mutant protein products *in vivo* could therefore serve as potential biomarkers for the molecular epidemiologic study of chemical carcinogenesis in human populations exposed to carcinogens [1]. A model system for such study is provided by workers who have been occupationally exposed to high levels of vinyl chloride (VC) and are at risk for the development of a sentinel neoplasm, angiosarcoma of the liver (ASL) [2].

VC is a known animal and human carcinogen which is rapidly absorbed following respiratory exposure and is primarily metabolized in the liver by the cytochrome P450 2El system to the electrophilic metabolites, chloroethylene oxide (CEO) and chloroacetaldehyde (CAA). CEO and CAA react with DNA bases to form adducts that are mutagenic in bacterial systems and mammalian cells, including: 7-(2′-oxoethyl) guanine; 1, N^6-ethenoadenine; 3, N^4-ethenocytosine; and N^2-3-ethenoguanine [3]. In animal experiments, the oxoethyl adduct is the major liver DNA adduct formed, representing 98% of all adducts, but it is the least persistent with a half-life of about 62 h [4] On the other hand, the less common etheno adducts are highly persistent with half-lives of more than 30 days, suggesting that they are poorly recognized by the liver DNA repair system [5]. Thus, the production of such etheno adducts could account for the occurrence of specific point mutations (including G.C→A.T transitions and A. T→T.A transversions) identified in the cellular oncogenes and tumor suppressor genes of VC-associated ASLs (Fig. 1).

For example, the cellular *ras* oncogenes are frequently found to contain specific point mutations in many human malignancies [6]. In a study of *ras* oncogene mutations in tumors of VC-exposed workers, 15 of 18 (83%) ASLs examined to date have been found to contain a G→A transition at the second base of codon 13 of the c-Ki-*ras*-2 gene [7,8]. This *ras* mutation at codon 13 (GGC→GAC) is known to be capable of converting the gene into an actively tranforming oncogene in model systems, and, thus, could be causally related to the carcinogenic process [9]. This *ras* mutation results in the substitution of an aspartic acid for the normal glycine at amino acid residue 13 in the encoded p21 protein product. This amino acid substitution is believed to produce a conformational change in p21 which may be responsible for altering its intrinsic GTPase activity, affecting signal transduction within the cell contributing to cellular transformation [10]. This altered Asp 13 p21 protein can be distinguished from normal p21 and other mutant p21s immunologically with a mouse monoclonal antibody that is specific for this mutant protein [11].

In addition, the p53 tumor suppressor gene is also frequently found to contain point mutations in a significant proportion of human malignancies [12]. In a study of p53 gene mutations in tumors of VC-exposed workers, 2 of 4 (50%) ASLs examined to date have been found to contain

Work presented at the "23rd Congress on Occupational and Environmental Health in the Chemical Industry" (Medichem 1995) "The Chemical Industry as a Global Citizen – Balancing Risks and Benefits", 19–22 September 1995, Massachusetts Institute of Technology, Cambridge, Massachusetts

M.-J. Marion
Unit for Research on Hepatitis and Human Retroviruses, INSERM, Lyon, France

I. DeVivo · S. Smith · J.-C. Luo · P. W. Brandt-Rauf (✉)
Division of Environmental Health Sciences, Columbia University, School of Public Health, 60 Haven Avenue, B-1, New York, NY, 10032, USA
FAX: 212-305-4012

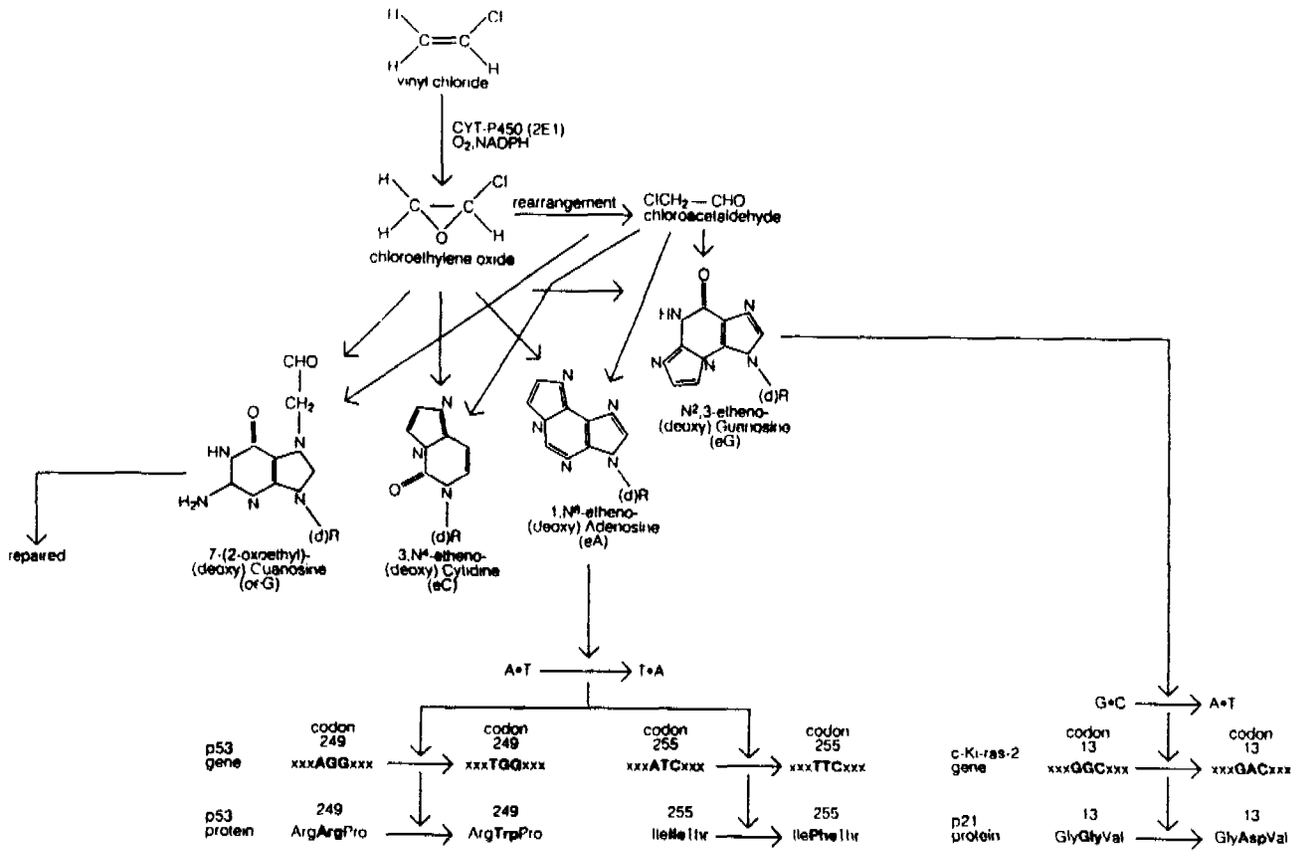

Fig. 1 Proposed carcinogenic mechanism of vinyl chloride in the production of angiosarcomas of the liver via the induction of mutations in p21 and p53

an A→T transversion at the first base of codon 249 or 255 of p53 [13]. These mutations result in the substitution of a tryptophan for the normal arginine at amino acid residue 249 or of a phenylalanine for the normal leucine at amino acid residue 255 in the encoded p53 protein product; these amino acid substitutions occur in a domain of the p53 protein which has been highly conserved throughout evolution, suggesting that this domain is critical for determination of the structure and hence function of the protein [12]. Other amino acid substitutions in this conserved region of p53 have in fact been demonstrated to alter the protein's normal structure and function and to result in the stabilization of the mutant protein, significantly prolonging its half-life in comparison to the wild-type protein and thus causing its accumulation in transformed cells [12]. The resultant increased amounts of mutant p53 can be detected by a mouse monoclonal antibody that is specific for mutant conformations of the protein [14]. Thus, similar VC-induced amino acid substitutions would also be expected to alter p53's normal ability to act as a negative regulator of cell division by causing a conformational change in the protein that would result in its accumulation which could be detected immunologically in the same fashion.

For cells in culture that contain a mutant *ras* gene and express mutant p21 or contain a mutant p53 gene and express mutant p53 protein, it is possible to use monoclonal antibodies to detect the mutant p21 or mutant p53, respectively, in the extracellular supernatant [15,16]. In an analogous situation *in vivo* where individuals may contain cells that have mutations in *ras* or p53 due to exposure to chemical mutagens, one might expect to be able to detect the presence of mutant p21 or mutant p53 in their extracellular fluids, including serum. We have therefore used immunological techniques, including immunoblotting for mutant p21 and an enzyme linked immunosorbent assay (ELISA) for mutant p53, to examine the expression of these proteins in the serum of individuals with VC exposure.

Methods

From a cohort of more than 400 workers employed in VC-polymerization plants in France since 1950, a sample of 27 of the most heavily exposed individuals have been studied for their serum expression of both mutant p21 and mutant p53 proteins [15,16]. For these individuals, information was available on age, gender, race, smoking status, alcohol consumption, occupational exposure history, and current medical status, including the presence of hepatic or other neoplasia. Estimates of VC exposure were based on years worked with VC as well as on estimated ppm-years of VC exposure based on the exposure matrix of Heldaas et al. (for production, autoclave cleaning, maintenance and packing/drying job categories exposures averaged 2000 ppm from 1950 through 1954,

1000 ppm from 1955 through 1959, 500 ppm from 1960 through 1967, 100 ppm from 1968 through 1974, and 1 ppm thereafter) [17]. The 27 individuals studied were white males with an average age of 58 (range = 37–74) and with an average exposure to VC of 21 years (range = 2–37) or 7,107 ppm-years (range = 9–26,708). These individuals included five cases of ASL (four of which were known to contain codon 13 c-Ki-*ras* gene mutations and two of which were known to contain codon 249 or 255 p53 gene mutations), one case of hepatocellular carcinoma (HCC) without either the *ras* or p53 gene mutations, and 21 VC-exposed workers without hepatic malignancies. For all workers, serum samples had been collected between 1987 and 1992 by routine venipuncture techniques and stored frozen at −20°C until the time of analysis.

A sample of 18 control patients – group-matched for age, gender and race – was selected from a previously described cohort of surgical patients hospitalized with non-cancer diagnoses [15, 16]. These controls were white males with an average age of 65 (range = 54–81) and with no known exposure to VC, and they were also comparable to the exposed cohort in terms of cigarette smoking and alcohol consumption. For all controls, serum samples had been collected between 1987 and 1989 by routine venipuncture techniques and stored frozen at −80°C until the time of analysis.

Serum analysis for mutant p21 was performed by immunoblotting as described previously using the primary mouse monoclonal antibody, D146, raised against a synthetic peptide corresponding to amino acid residues 5–16 of the p21 protein with aspartic acid at position 13 [15]. Briefly, 2 µl of serum was diluted with 4 ml of sample buffer (0.125 M trizma base, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), placed in a boiling water bath for 2 min, and then loaded into individual wells of a 5%–17% gradient polyacrylamide gel with a 5% stacking gel and electrophoretically separated at constant current at room temperature. The samples were then electrophoretically transferred onto nitrocellulose membranes at constant current at 4°C. The nitrocellulose blots were blocked with 3% BSA in PBS for 2 h at room temperature and incubated with the primary antibody D146 (1:250) overnight at 4°C. After washing, the blots were developed with a secondary antibody (horse anti-mouse)-avidin-biotin-peroxidase detection system. Serum 21 kDa bands were scored in comparison with the 21 kDa bands of cell lysate from the positive control human cancer cell line, HCT 116, known to contain the c-Ki-*ras* codon 13 aspartic acid mutation, by laser densitometric analysis of the volumetric integration of the optical density of the bands. Positive mutant p21 serum bands were confirmed as c-Ki-*ras* p21 by immunoblotting with another primary mouse monoclonal antibody, 147–67C6, raised against a synthetic peptide corresponding to amino acid residues 157–180 of the c-Ki-*ras* p21 protein.

Serum analysis for mutant p53 was performed as described previously using an ELISA based on the mouse monoclonal antibody PAb 240 which identifies an epitope between amino acids 210 and 214 that is normally concealed in the wild-type p53 protein but which is revealed in many mutant p53 proteins due to the aforementioned conformational changes produced by the mutations [18]. Briefly, microtiter wells were precoated with PAb 240 and then 100 µl of serum was added to each well and incubated overnight at 4°C. After washing, 100 µl of a rabbit polyclonal reporter antibody for p53 was added to each well and allowed to incubate at room temperature for 2 h. After washing again, the remaining reporter antibody was bound to a horseradish peroxidase-conjugated goat anti-rabbit IgG, and color was developed by incubation with the chromogenic substrate 2, 2′-azino-di-[3-ethylbenzthiazoline sulfate]. Absorbance of each well was read on a spectrophotometric plate reader at 405 nm, and the concentration of mutant p53 was determined by comparison to the absorbance of standard solutions of purified, recombinant, human mutant p53.

Results and Discussion

The four of five cases of ASL (80%) among the VC-exposed workers that were known to contain c-Ki-*ras* gene mutations in their tumor DNA were found to have detectable amounts of the corresponding mutant p21 protein in their serum, whereas the one case of ASL and the one case of HCC that were known not to contain such a mutation in their tumor DNA were found not to have mutant p21 in their serum. Similarly, the two of five cases of ASL (40%) that were known to contain p53 gene mutations in their tumor DNA were found to have detectable amounts of mutant p53 protein in their serum, whereas the three cases of ASL and one case of HCC that were known not to contain p53 mutations in their tumor DNA were found not to have mutant p53 in their serum. These results suggest that detection of serum mutant p21 and serum mutant p53 can be valid surrogates for mutant *ras* and mutant p53 gene expression at the tissue level in vivo.

The results among the other VC-exposed workers without malignancies and the unexposed controls showed considerable differences between these two groups in terms of their serum expression of these mutant proteins. None of the 18 unexposed controls (0%) were found to have detectable levels of the mutant p21 in their serum, whereas 9 of 21 VC-exposed workers (43%) were serum-positive for mutant p21. Similarly, only 1 of 18 unexposed controls (6%) were serum-positive for mutant p53 compared to 3 of the 21 VC-exposed workers (14%). These results suggest that mutational activation of the *ras* and p53 genes may be an early event in VC-induced carcinogenesis and that identification of the mutant p21 and mutant p53 proteins may be potential biomarkers of cancer risk in these individuals. For example, one can make an estimate of the predictive value of the biomarkers based on the current data. At this time, each biomarker by itself has a relatively low estimated positive predictive value (0.29 for mutant p21 and 0.40 for mutant p53). However, with the results of the two tests combined, the positive predictive value for both tests positive at the same time increases to 0.67 (with a negative predictive value if both tests are negative or only one test is positive of 0.88). Furthermore, over time one would anticipate an increase in the predictive value of the tests if they accurately represent critical events in the causal pathway for carcinogenesis, as presumed. Follow-up of the serum-positive and serum-negative workers in this cohort should provide confirmation of this. For example, to date, all serum-negative individuals remain healthy, but one of the serum-positive individuals has developed a lesion consistent with ASL. In addition, the current results with the higher percentage of p21 positives among the exposed compared to p53 positives may indicate that *ras* gene mutation is a very early event in VC-induced carcinogenesis but that p53 gene mutation may occur at a somewhat later stage. Such a step-wise model of VC-induced carcinogenesis would thus be similar to other models of carcinogenesis such as that proposed for colon cancer in which *ras* gene mutation is an early event and p53 gene mutation occurs later [19].

The serum results for all workers and controls were also stratified by degree of VC exposure in terms of years worked and ppm-years exposed (Table 1 A and B). With

Table 1 Dose-response relationship between serum biomarkers, mutant p21 and mutant p53, and VC exposure in years and PPM-Years. Maximum likelihood logistic analysis statistically significant for increasing biomarker positivity with increasing exposure (for A, $P = 0.0021$; for B, $P = 0.0019$)

	Both biomarkers	One biomarker	Both biomarkers
	Negative	Positive	Positive
A. Years of exposure			
0	17	1	0
< 15	3	4	0
15–25	4	5	1
> 25	4	4	2
B. PPM-Years of exposure			
0	17	1	0
< 1,800	4	4	0
1,800–18,000	6	7	2
> 18,000	1	2	1

increasing exposure by years or ppm-years, there was a statistically significant increasing likelihood of positivity for one or both serum biomarkers (maximum likelihood logistic analysis, $P < 0.01$). For example, among the individuals without either serum biomarker, the percentage of individuals in the strata defined by years fell from 94% (17/18) among the unexposed to 40% (4/10) among those with more than 25 years of exposure, whereas among the individuals with both serum biomarkers, the percentage rose from 0% (0/18) among the unexposed to 20% (2/10) among those with more than 25 years of exposure. Similarly, among the individuals without either serum biomarker, the percentage of individuals in the strata defined by ppm-years fell from 94% (17/18) among the unexposed to 25% (1/4) among those with more than 18,000 ppm-years of exposure, whereas among the individuals with both serum biomarkers, the percentage rose from 0% (0/18) among the unexposed to 25% (1/4) among those with more than 18,000 ppm-years of exposure. Likewise, the average exposure can be seen to be increasing with the presence of the biomarkers from 7.8 years (2,421 ppm-years) among those negative for both biomarkers to 19.3 years (6,571 ppm-years) among those positive for one biomarker to 26.3 years (10,708 ppm-years) among those positive for both biomarkers. Once again assuming that the presence of the biomarkers represent the occurrence of critical events in the causal pathway for carcinogenesis, these results would be consistent with epidemiologic studies that have suggested an increased risk of ASL with increasing VC exposure [17].

An important goal of molecular epidemiology studies such as this is to contribute to improved disease prevention and control. For example, as noted, biomarkers of presumed critical events in the disease pathway may be better able to identify individuals at risk before clinical onset and thus allow more effective intervention. At the present, there is no specific intervention that could be offered to individuals with the presence of these biomarkers that is known to mitigate the effects of VC-induced cancer-related events. However, progress is being made in developing such interventions. For example, we have already demonstrated that the compound L-β-(5-hydroxy-2-pyridyl)-alanine (azatyrosine) is capable of causing permanent reversion of the human cancer cell line HCT116 (which is known to contain the Asp 13 c-Ki-*ras* VC-induced cancer-related mutation) to a normal cellular phenotype that will no longer grow in soft agar and will no longer produce tumors in nude mice [1]. It is hoped that this type of compound will serve as a prototype chemoprophylactic agent that can eventually be employed in human studies for the prevention of ASLs in VC-exposed cohorts.

Furthermore, it is anticipated that studies of biomarkers such as these may have broader implications for carcinogen risk assessment and regulation. For example, the presence or absence of biomarkers among subgroups of workers with varying levels of exposure could provide intermediary evidence for a potentially protective exposure level. Thus, in most Western countries, the workplace exposure limit for VC has been 1 ppm since 1974. However, if some individuals with such low exposure levels (e.g., below 40 ppm-years or 1 ppm for 40 working years) tested positive for these biomarkers, this could suggest that current permissible exposure limits are not adequately protective. Conversely, if it is only individuals with much higher exposures who test positive for these biomarkers, this would support the level of protection provided by current exposure limits. As noted above, most of the individuals in the present cohort had extremely high levels of exposure. However, the range of exposure was quite broad and included a few individuals with low levels of exposure. Thus, it is interesting to note that one of these workers with an estimated exposure of only 9 ppm-years tested positive for mutant p21 (although negative for mutant p53). Expansion of studies such as these to include greater numbers of workers with low levels of exposure will be necessary to adequately address these regulatory concerns, as well as related issues of cancer prevention and control.

Acknowledgements We thank Dr. C. Trepo, D. Tang, Y. Zhang and S. Kahn for their assistance with this research. This work was supported in part by grants from the U.S. Environmental Protection Agency (R818624), the National Institutes of Health (CA69243), the Association pour la Recherche sur le Cancer and le Groupment des Enterprises Francaises pour la Lutte contre le Cancer.

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