Molecular mechanisms of carcinogenesis by vinyl chloride

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Summary. In 1974 vinyl chloride (VC), a gas used in the plastics industry, was shown to be a human carcinogen, inducing a very rare type of tumor, angiosarcoma of the liver. The same type of tumor was induced in rodents exposed to VC thus providing an excellent model for mechanistic studies. Here, we review the numerous studies on the mechanism of action of VC with particular emphasis on the DNA products induced by this strong alkylating agent. In particular, the genotoxicity, repair mechanisms, *in vivo* formation and tumor mutation spectra by etheno-adducts will be analysed and possible approaches for future research suggested.

Key words: vinyl chloride, mutagenesis, carcinogenesis, DNA repair, DNA adducts.

Riassunto *(Meccanismi molecolari di cancerogenesi del cloruro di vinile)*. Nel 1974 il cloruro di vinile (CV), un gas usato nell'industria plastica, è stato riconosciuto come cancerogeno nell'uomo inducendo un tumore molto raro, l'angiosarcoma del fegato. Il CV induce lo stesso tipo di tumore nei roditori che rappresentano pertanto un eccellente modello per studi meccanicistici. In questo studio presentiamo una breve rassegna dei numerosi studi sul meccanismo d'azione del CV con particolare enfasi sui prodotti di modificazione del DNA indotti da questo potente agente alchilante. In particolare, verranno analizzati la genotossicità, i meccanismi di riparazione, la formazione *in vivo* degli eteno-addotti e gli spettri di mutazione nei tumori associati ad esposizione a CV. Verranno inoltre presentate proposte per possibili sviluppi di ricerca futura in questo settore.

Parole chiave: cloruro di vinile, mutagenesi, carcinogenesi, riparazione del DNA, addotti del DNA.

INTRODUCTION

Vinyl chloride (VC) is a gas used in the plastics industry to produce PVC. In 1974 the carcinogenicity of VC has been recognized on the basis of the experimental evidence in rodents and on the unfortunate high number of case reports of liver hemangiosarcomas (ASL) in occupationally exposed workers. In light of the discovery of the carcinogenicity of VC the exposure levels were drastically reduced arriving at the current levels of ≤1 ppm. Decades after the discovery of the VC's carcinogenicity an important issue is the protection of those subjects who worked in VC polymerization plants in the 1960s, when VC exposure levels were still high. An example is the polymerization plant of Porto Marghera, near Venice. A follow-up study conducted by Pirastu *et al.* [1] on a total of 1658 workers employed in this plant from start of production (1950), reported a mortality from all malignant neoplasms similar to expected but a significantly increased risk for primary liver cancer. This study confirmed the causal relationship between Vinyl Chloride Monomer (VCM) exposure and liver angiosarcoma but also indicated an excess risk for hepatocellular carcinoma (HCC) and liver cirrhosis as well as lung cancer among specific categories of workers.

In 2000 the author of this paper was asked by Dr. Casson to provide scientific advice on the molecular mechanisms of carcinogenesis of VC within the legal action against Montedison-Enichem, the owner of the VC plant in Porto Marghera. In that occasion she had the great pleasure of working with Prof. Romano Zito, to whom this issue of Annali dell'Istituto Superiore di Sanità is dedicated. He provided his expertise and knowledge on the metabolism and toxicokinetics of VC as well as on experimental carcinogenesis studies. In this review the current knowledge of the mechanisms of carcinogenesis of VC will be presented. The major issues have been discussed at great length with Romano in the course of frequent and unforgettable meetings when preparing the final report.

METABOLISM OF VC

VC is known to be rapidly metabolized to generate reactive metabolites that react with protein, DNA and RNA. The most reactive metabolites are chloroethylene oxide (CEO) and its derivative chloroacetaldeyde (CAA) [2]. The reaction products of these intermediates with nucleic acids are the same [3] and, as expected, the mutagenic activity is similar [4]. However, CEO reacts with DNA much more rapidly than CAA and thus it is considered the relevant ultimate electrophile of VC. CEO is formed mainly by the cytochrome P-

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450 (CYP) isoenzyme CYP2E1 [5, 6], while epoxide hydrolase and glutathione-S-transferase are involved in its detoxification [7]. Human genetic polymorphisms in these genes might affect the genotoxicity of VC and therefore cancer risk in VC exposed workers. What are the reaction products with DNA? Targets for modification are guanine, adenine and cytosine moieties *(Figure 1)*. 7-(2-oxoethyl)-guanine is the predominant DNA product [8, 9], although of minor biological relevance [10]. Etheno(ε)-adducts such as 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εC), N^2,3-ethenoguanine (N^2,3-εG), and 1,N^2-ethenoguanine (1,N^2-εG) are also produced in cellular DNA by reaction with oxidised metabolites of VC [8, 9]. These adducts are promutagenic (reviewed in [11]). By comparison of the adduct profile of VC and 2,2'-dichloroethyl ether, that produces only CAA as metabolite, it has been concluded that the biologically relevant metabolite of VC is indeed CEO [12, 13]. Epoxides are decisive for DNA binding but they are very unstable. It is likely that VC biotransformation into CEO occurs in the hepatocytes thus allowing the epoxide to reach and hit the adjacent sinusoidal lining cells that are the target for angiosarcomas.

DNA ADDUCTS AND REPAIR

7-(2-oxoethyl)-guanine constitutes 98% of total adducts but it is not mutagenic. By contrast, ε-adducts are clearly miscoding. ε-adducts are also produced by endogenous processes through the interaction of lipid peroxidation (LPO)-derived aldehydes and hydroxyalkenals. They have been found in DNA isolated from tissues of nonexposed humans and rodents [14, 15]. In particular, in human tissues the levels of εA reported range between 0.011-0.85/10^6 nucleotides (depending on the technique used), the levels of εC 0.012/10^6 nucleotides and the levels of N^2,3-εG between 0.02-0.033/10^6 nucleotides. However, the levels of these adducts were significantly increased by agents contributing to lipid peroxidation and oxidative stress such as VC. This has been clearly shown by Morinello *et al.* [16, 17] who analysed N^2,3-εG levels over a long period of time and a broad range of VC doses in different organs of exposed rats. For instance increases of 10 to 100 times over background in the levels of N^2,3-εG were detected in hepatocytes of treated mice depending on the exposure regimen. There was no statistically significant difference in the adduct concentration between hepatocytes and non-parenchymal cells (the target cells for angiosarcomas) indicating that besides adduct concentration specific factors (for instance differential cell proliferation) are responsible for cell specificity in susceptibility to VC-induced carcinogenesis.

The base excision repair (BER) pathway is involved in the removal of ε-adducts, with DNA glycosylases being the key enzymes of this pathway (reviewed in [18]). They remove ε-adducts from DNA by hydrolysing the N-glycosidic bond between the damaged base and deoxyribose, leaving an abasic site in DNA. AlkA protein of *Escherichia coli* catalyses the excision of N^2,3-εG and εA [19], although its main substrate are alkylated bases. The alkyl-N-purine-DNA glycosylase (ANPG) is the human counterpart of *E. coli* AlkA and releases alkylated bases as well as hypoxanthyne and εA [20]. In contrast with AlkA, ANPG is also able to excise from DNA 1,N^2-εG [21]. The *E. coli* MUG protein removes various ε–bases: εC and 1,N^2-εG [22]. MUG was initially characterised as

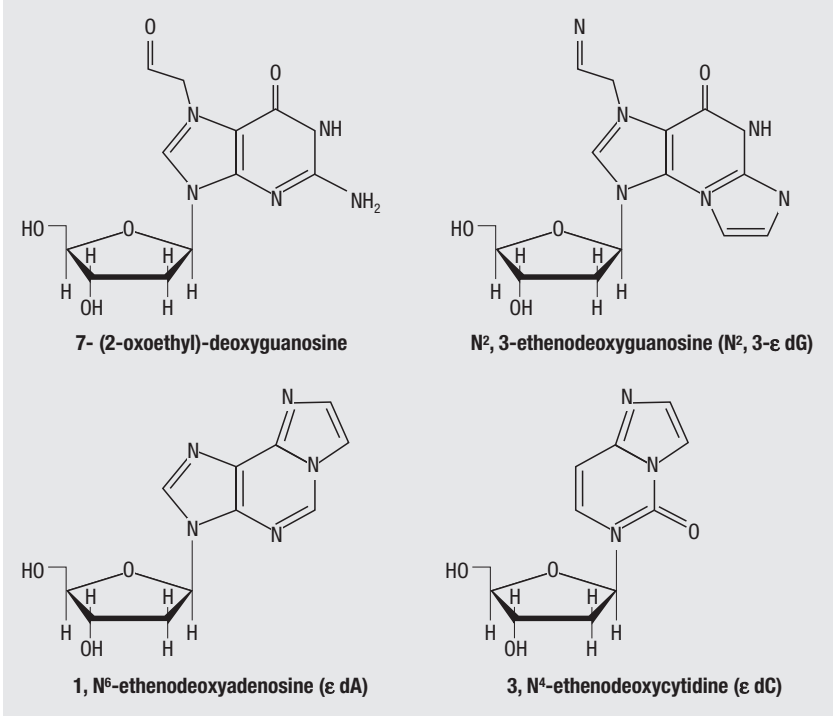

Fig. 1 | *Chemical structures of exocyclic DNA adducts.*

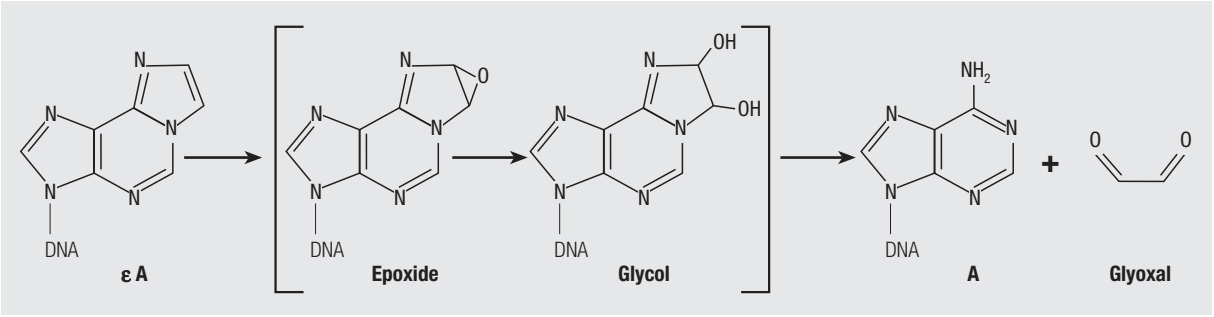

Fig. 2 | *Proposed mechanism for the repair of etheno-adducts by AlkB (modified from [28]).*

an enzyme specific for removal of uracil from G:U mismatches but later it was shown that its primary function is the repair of εC. The human thymine DNA glycosylase (hTDG), the homologue of the *E. coli* MUG, exhibits wide substrate specificity including, besides T mispaired with G, also εC [22, 23] and mismatched uracil. Recently other two human enzymes able to excise εC have been identified: methyl-CpG binding domain protein (MBD4/MED1) [24] and single-stranded monofunctional uracil DNA glycosylase (SMUG1) [25], but their εC- DNA glycosylase activity is weak as compared with hTDG. Mammalian cells can repair ε–adducts by BER initiated by the abovementioned DNA glycosylase *in vivo*, but recently an additional pathway has been involved in the protection against ε-lesions. The discovery has been stimulated by the observation that *E. coli* induced for the adaptive response are resistant to induction of mutation by the VC metabolite CAA. One of the genes involved in adaptive response is alkB whose gene product AlkB couples the oxidative decarboxylation of α-ketoglutarate with hydroxylation of 1-alkylpurines and 3-alkylpyrimidines to yield lesion-free DNA [26, 27]. Essigmann's group discovered that etheno DNA base lesions, that are produced by CAA, are indeed repaired by the AlkB protein of *E. coli* [28]. As mentioned above AlkA can also repair εA and it is likely that AlkA and AlkB have specialized niches (for istance AlkB but not AlkA repairs damaged single-stranded DNA at a physiologically relevant rate). The mechanism that has been proposed for reversal of εA and εC by AlkB *(Figure 2)* involves epoxidation of the exocyclic double bond with subsequent epoxide ring opening and, ultimately, liberation of the dialdehyde glyoxal. In mammals, the corresponding AlkB homologs may defend the genome against the deleterious effects of oxidative stress.

The fact that etheno-adducts are recognised and excised with high efficiency by various DNA repair proteins suggests that these enzymes may constitute important contributors to genetic stability

GENOTOXICITY OF VINYL CHLORIDE

Studies conducted in *Salmonella typhimurium* revealed that VC and its metabolites, CEO and CAA are mutagenic, and in particular induce base pair substitutions mainly G>A transitions and G>T transversions [29, 30]. In *E. coli* CEO induced various types of base pair substitutions, mainly G>A transitions and to a lesser extent A>T transversions [31].

Gene mutations by base pair substitution induced by VC, urethane and their reactive metabolites were also described in a variety of test systems including *Drosophila*, yeast, mammalian cells and rodents. In addition to mutations other genotoxic effects were reported such gene conversion, sister chromatid exchanges, micronuclei, mitotic recombination, chromosomal aberrations and cell transformation [32]. Few molecular analysis of VC induced mutations have been done. The mutagenic potential of CAA, the ultimate carcinogenic form of VC, was analyzed in human cells using a shuttle vector containing the *sup*F gene [33]. More than half of the single base pair substitutions were G>A transitions. The majority of the mutations involved G:C bp in 5'AAGG3' or 5'CCTT3' sequences suggesting sequence specificity in VC-induced mutagenesis. A report on *hprt* mutational spectrum of VC, CEO and CAA in human B-lymphoblastoid cells [34] indicated that VC and CEO displayed similar toxicity/mutation profiles and a similar frequency of large deletions, whereas CAA displayed greater toxicity and a larger frequency of deletion mutations. These results suggest that the majority of mutations induced by VC occur through its metabolite, CEO.

MUTAGENIC PROPERTIES OF ETHENO-BASES

The highly mutagenic and genotoxic properties of ε-adducts have been established *in vitro* by analysing steady-state kinetics of primer extension assays and, more recently, *in vivo* by site-specific mutagenesis in mammalian cells. In these last experiments a single etheno-adduct is introduced into a plasmid at a defined site by *in vitro* DNA sinthesis and then transfected into a bacterial or mammalian cell system. After replication in the host cell system, the plasmid progeny is recovered and sequence changes are revealed by DNA sequencing [35]. The main findings are summarized in *Table 1*. In *E. coli* and simian kidney cells, εA induced mainly A>G followed by A>T and A>C base changes

Table 1 | *Base pair substitutions induced by etheno-bases*

DNA lesion	Base changes *In vitro*	*E. coli*	Mammalian cells
εA	A>G, A>T, A>C[a]	A>G, A>C, A>T[b, c]	A>G, A>T, A>C[c]
εC	C>A, C>T, C>G[d]	C>T, C>A[e, f]	C>A, C>T, C>G[g]
N^2,3εG	G>A[h]	G>A[i]	
1,N^2-εG	G>T, G>C[l]	G>T, G>C, G>A[m]	G>A, G>T [n]

[a][58]; [b][36]; [c][37]; [d][38]; [e][39]; [f][40]; [g][41]; [h][42]; [i][43]; [l][44]; [m][45]; [n][46].

[36, 37]. εC generated C>A and C>T changes and less frequently C>G *in vitro* [38], in *E. coli* [39, 40] and in monkey cells [41]. Induction of 1- and 2-base deletions was also reported [36-40]. N^2,3εG generated G>A transitions *in vitro* [42] and in *E. coli* [43]. 1,N^2-εG induced a variety of base pair substitutions [44] as well as frameshifts [45]. In hamster cells, besides base pair substitutions, deletions, rearrangements and single- and double- base pair substitutions were described to occur at sites near but not at the lesion site [46]. The mutation frequency of these ε-lesions varied depending on several factors including the type of lesion, the DNA polymerase involved and the sequence context.

TUMOR MUTATION SPECTRA

The events leading to cancer are complex and interactive. Analysis of the frequency, type and site of mutations in important cancer-related genes may provide clues to the identification of etiological factors and sources of exposure (reviewed in [47, 48]). In 1991 Marion *et al.* [49] described the activation of the Ki-*ras* gene in human ASL associated with VC exposure. In particular, G>A transitions were observed at codon 13 in 5/6 tumor samples analysed.This finding was later confirmed by others *(Table 2)*. Interestingly, ASL of different etiology contained lower frequency of Ki-ras mutations and they were localized at codon 12 instead of codon 13, suggesting that mutations found in VC-associated ASL are specific.

Mutations of *p53* are apparently rare in ASL but there is preliminary evidence of a specific mutation spectrum in these patients associated with occupational exposure to VC. In 21 ASL patients with ASL not associated to exposure to VC, Soini *et al.* [50] have reported only 2 mutations. In 6 patients with occupational exposure to VC, Hollstein *et al.* [51] have found 3 mutations, and all of them are A>T transversions, a type of mutation which is very rare in all other types of cancer (they represent 2.8% of all mutations, with a maximum of 8.9% in squamous cell carcinoma of the esophagus). In contrast, the two mutations found in patients with other exposures (including thorotrast) were both G>A transitions [50, 52]. Although the tumor sample size is too small to draw any definitive conclusion, it should be noted that the type of mutation found in the *p53* gene of VC-associated ASL *(Table 2)* are reminiscent of the mutation specificity described for etheno-adducts *(Table 1)*.

Specific mutational patterns were also found in the Ha-*ras* gene in HCC and in the *p53* gene of ASL in rats. In particular, VC-induced HCC (7/8) contained an A>T transversion at codon 61 in the Ha-*ras* gene [53, 54]. This type of mutation is rarely detected in chemically induced HCC in rats. When *p53* mutations were analysed in liver tumors (25 ASL and 8 HCC) in VC-treated rats again a very rare type of mutation was detected. They were mostly localized at AT base pairs and the majority were transversions [55]. It is also interesting to mention that the A>T transversion observed at codon 253 in two ASL of rats is equivalent to the A>T transversion detected at codon 255 in one human VC-associated ASL. In conclusion, mutations detected in cancer-related genes in the rat model, following exposure to VC, are specific molecular events that involve mainly A:T base pairs *(Table 2)*. This type of mutation is reminiscent of the mutational signature

Table 2 | *Gene mutations found in tumors associated with exposure to vinyl chloride*

Species	Tumor	Gene	Mutation (frequency)	Reference
Humans	ASL	Ki-*ras*	G>A (5/6)	[49]
Humans	ASL	*p53*	A>T (3/6)	[51]
Humans	ASL	Ki-*ras*	G>A (8/15)	[59]
Humans	HCC	Ki-*ras*	G>A (5/12)	[60]
Rats	ASL	*p53*	Various base-pair substitutions (10/25) deletions (1/25)	[55]
Rats	HCC	Ha-*ras*	A>T (7/8)	[53, 54]

of εA *(Table 1)* that is known to accumulate in hepatic DNA following exposure to VC.

What is the knowledge on mutational spectra induced by other carcinogens that induce etheno-adducts? In tumors induced by urethane and its electrophilic metabolites in mice, codon 61 of the Ha-*ras* gene (liver, skin) and of the Ki-*ras* gene (lung) seems to be a characteristic target. These tumors frequently contain A>T transversions that are compatible with the pro-mutagenic properties of etheno-adducts and with their formation in target tissues [11].

These findings all together suggest that etheno-adducts might be initiating lesions in tumor formation. However, it should be taken into account that several factors might affect a mutational spectrum. The mutation spectrum is the product of the probability patterns of several sequential steps of mutagenesis: damage, repair, polymerase misreading and biological selection. A correct interpretation of tumor-specific mutation spectra requires a good and detailed understanding of the molecular mechanisms that are responsible for the formation, escape from repair mechanisms and biological selection of these mutations.

Finally, serum anti-*p53* antibodies have been found in ASL patients and in some workers with occupational exposure to VC [56]. A recent study conducted on VC workers in Italy [57] demonstrated a trend for increasing likelihood of *p53* over-expression with increasing exposure to VC and supports the usefulness of detection of this marker in identifying individuals with a history of VC exposure and thus at high risk of ASL.

CONCLUDING REMARKS

Cancer is a complex process involving interactions between multiple factors of endogenous and exogenous origin. As a result of this complexity, the contribution of a single risk factor to the generation of a tumor-specific mutation spectrum is difficult to assess in many situations. In the case of VC several events that connect the exposure to the final outcome (*i.e.* tumor formation) have been clarified: 1) the metabolic activation pathway; 2) DNA adduct formation; 3) the mutagenic potential of the DNA base modifications induced; 4) coherent mutation spectrum detected in cancer-related genes. Less is known on the mechanisms behind the cell and organ specificity. VC induces specifically hepatic angiosarcomas that originate from hepatic sinusoidal cells. The understanding of the specific susceptibility of these cells requires further studies. Moreover, the mechanisms of extra-hepatic tumor formation by VC should be elucidated. From the point of view of adduct formation the aspect that needs further discussion is that VC produces DNA adducts that also occur physiologically in the context of cellular aerobic metabolism. This involves that in a low dose range the VC-related response cannot be distinguished from endogenous background. Finally, the due relevance in the process of risk assessment must be given to the potential chronic exposure of humans and inter-individual variability in VC metabolism and etheno-adduct repair.

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