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Minireview

Formation of DNA etheno adducts in rodents and humans and their role in carcinogenesis*✪

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Ethenobases are exocyclic adducts formed with DNA by some environmental carcinogens such as vinyl chloride or urethane. In the last few years, they have received a renewed interest due to the development of sensitive techniques of analysis that made it possible to measure their formation *in vivo*. This minireview summarizes the information gained recently from the work of several laboratories, including ours. Increased levels of DNA etheno adducts have been measured in target tissues from rodents exposed to vinyl chloride or urethane. Hepatic tumours caused by exposure to vinyl chloride in humans and in rats and lung tumours induced by urethane in mice exhibit base pair substitution mutations in the *ras* and *p53* genes which seem to be exposure-specific and consistent with the promutagenic properties of ethenobases. Background levels of etheno adducts have been detected in DNA from non-exposed humans or animals, pointing to an alternative, endogenous pathway of formation. This background may be affected by dietary factors. It could arise from the reaction of *trans*-4-hydroxy-2-nonenal (or its epoxide 2,3-epoxy-4-hydroxynonanal), a lipid peroxidation product, with nucleic acid bases. Elevated levels of etheno adducts are found in hepatic DNA from humans and rodents with genetic predisposition to oxidative stress and lipid peroxidation in the liver, and with an associated increased risk of liver cancer. These data suggest that DNA ethenobases could serve as new biomarkers of oxidative stress/lipid peroxidation.

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Abbreviations: CAA, 2-chloroacetaldehyde; εA, 1,N^6-ethenoadenine; VC, vinyl chloride; CEO, chloroethylene oxide; εC, 3,N^4-ethenocytosine; 7OEG, 7-(2-oxoethyl)guanine; εG, ethenoguanine; EC, urethane (ethyl carbamate); LEC, Long-Evans with cinnamon-like colour; LEA, Long-Evans with agouti colour; LPO, lipid peroxidation; ASL, angiosarcomas of the liver; HCC, hepatocellular carcinomas; VCA, vinyl carbamate; VCO, vinyl carbamate epoxide; HNE, *trans*-4-hydroxy-2-nonenal; HE, 2,3-epoxy-4-hydroxynonanal.

For the last 25 years, ethenobases have been the most extensively studied of the exocyclic DNA adducts, a new class of DNA lesions that is being paid an increasing attention [1, 2]. Ethenobases were first described by Kochetkov *et al.* [3] who identified them as reaction products of 2-chloroacetaldehyde (CAA) with adenine and cytosine. Initially, they were used as fluorescent analogues (1,N^6-ethenoadenine (εA) nucleotides) in biochemical or enzymatic studies or as probes for nucleic acids structures (reviewed in [4]). The interest in these lesions was renewed begining from 1975 when it was discovered that they could be generated in the presence of microsomal fraction by vinyl chloride (VC) [5–7], previously identified as a human [8] and rodent [9, 10] carcinogen. In the following years, using replication and transcription fidelity assays and oligo- or polynucleotides modified with CAA or chloroethylene oxide (CEO), two VC metabolites (Fig. 1), it was established that εA and 3,N^4-ethenocytosine (εC) had miscoding or ambiguous base pairing properties [11–13] and thus could be involved in the mutagenic and carcinogenic effects of VC [14, 15]. In contrast, 7-(2-oxoethyl)guanine (7OEG), the major VC-DNA adduct (Fig. 1), did not appear to be a miscoding lesion [16]. The promutagenic properties of ethenobases were subsequently confirmed by a series of studies, based on steady-state kinetics or primer extension assays *in vitro* and site-specific mutagenesis experiments in *Escherichia coli* and in mammalian cells (reviewed in [2]). The four ethenobases produce mainly base pair substitution mutations. εA can lead to AT → GC transitions and to AT → TA and AT → CG transversions [17, 18]; εC generates CG → AT transversions and CG → TA transitions [19, 20]; N^2,3-ethenoguanine (N^2,3-εG) induces GC → AT transitions [21] and 1,N^2-ethenoguanine (1,N^2-εG) GC → TA and GC → CG transversions, in addition to -1 and -2 base frameshifts [22]. The mutagenic potencies of εA and εC have been shown to be higher in mammalian (simian kidney) cells than in *E. coli*, possibly reflecting a different fidelity of the DNA replication complexes [18, 23]. The discovery of the promutagenic properties of ethenobases led to the hypothesis that they could be implicated in the initiation of carcinogenesis by VC [11] or urethane (ethyl carbamate, EC), another etheno adduct-forming carcinogen

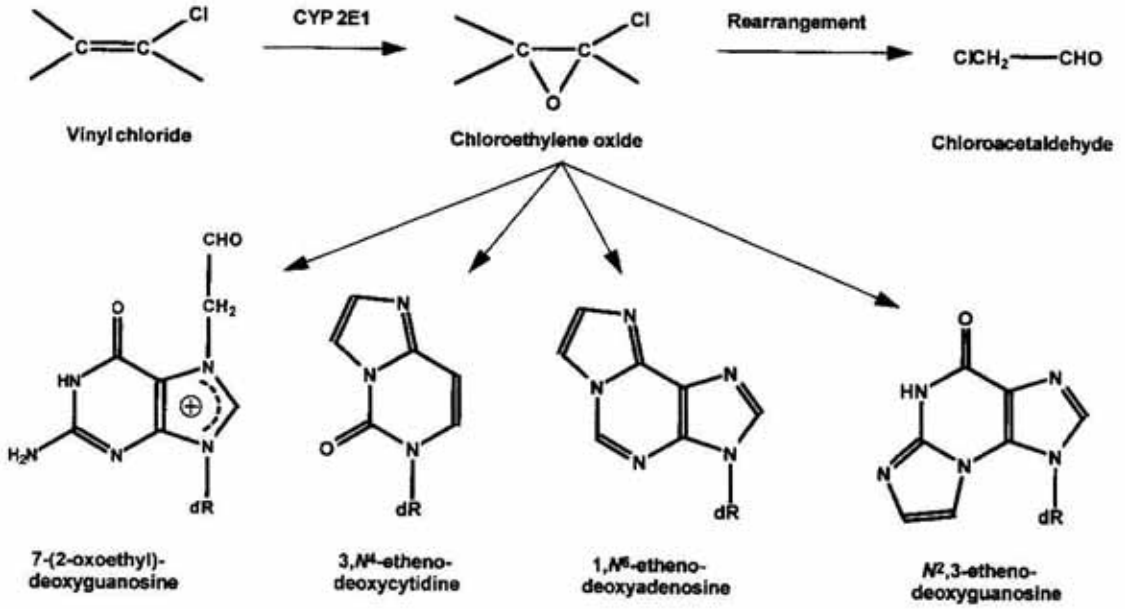

Figure 1. Metabolic activation of vinyl chloride and formation of DNA adducts *in vivo*.

[24]. Until recently, this hypothesis could not be tested *in vivo* due to the low levels of etheno adducts formed. However, with the advent of sensitive techniques, based on GC-MS [25], HPLC/RIA [26] or on immunoaffinity/^{32}P-postlabelling [27, 28], it became possible to study the formation and repair of etheno adducts in experimental animals and humans. In parallel, progress has been made in the knowledge of molecular mechanisms operating in VC- and EC-induced carcinogenesis. Finally, background levels of etheno adducts have been detected in tissues from non-exposed humans and rodents, probably arising from lipid peroxidation (LPO) products. In the light of these recent advances, the role of etheno adducts in carcinogenesis will be discussed in this short review.

ROLE OF ETHENO ADDUCTS IN VINYL CHLORIDE-INDUCED CARCINOGENESIS

Metabolic activation of vinyl chloride and formation of DNA ethenobases (Fig. 1)

VC, a gas industrially used for the production of polyvinyl chloride (and of copolymers), is carcinogenic to humans and rodents, inducing mainly angiosarcomas of the liver (ASL) and hepatocellular carcinomas (HCC) [29, 30].

VC is mutagenic in bacteria, yeasts, Drosophila and mammalian cells in culture (reviewed in [31]). It has been demonstrated that the biological effects of VC are dependent on its metabolic conversion, by microsomal monooxygenases, into CEO which can rearrange spontaneously into CAA. In humans and rats, oxidation of VC is catalyzed by cytochrome P450 2E1 (CYP 2E1) [32, 33] and this reaction occurs essentially in the liver [33]. Although both compounds, CEO and CAA, can react with DNA bases and are mutagenic, qualitative and quantitative differences in their biological and chemical activities have been observed. CEO is more mutagenic than CAA in bacteria [34], yeasts [35] and mammalian cells [36]. CEO is a potent tumour inducer in mice [37] whereas the carcinogenicity of CAA is much weaker [38], pointing to CEO as the ultimate carcinogenic metabolite of VC.

In vitro, both CEO and CAA can form etheno adducts with nucleic acid bases, however the former exhibits a greater reactivity [39]. In addition, 7OEG has been characterized as a major reaction product of CEO with guanine [40] whereas CAA does not yield this adduct [41]. 1,N^6-Ethenoadenosine (εAdo) and 3,N^4-ethenocytidine (εCyd) were characterized as reaction products of VC with ribonucleosides, in the presence of a microsomal activation system [5, 7]. Analysis of DNA incubated *in vitro* with rat liver microsomes, an NADPH-regenerating system and [^{14}C]VC revealed the formation of 7OEG, the major DNA adduct, and of 1,N^6-etheno-2′-deoxyadenosine (εdAdo) and 3,N^4-etheno-2′-deoxycytidine (εdCyd) [42]. More recently, Müller *et al.* [43] quantitated six adducts in DNA treated with CEO, including 7OEG, the four ethenobases and 5,6,7,9-tetrahydro-7-hydroxy-9-oxoimidazo[1,2-α]purine. The reactivity of CAA towards double-stranded B-DNA is very low [4, 39]. CAA reacts with unpaired A and C bases to yield εA and εC, respectively. Treatment of DNA with CAA has also been reported to result in the formation of N^2,3-εG and 1,N^2-εG moieties [39, 41, 44].

Following exposure of rodents to VC, 7OEG was found to be the major DNA adduct [42, 45, 46]. εAdo and εCyd were identified in hydrolysates from liver RNA of rats exposed to [^{14}C]VC [6, 7]. The occurrence of etheno lesions in DNA after exposure of animals to VC *in vivo* was more difficult to demonstrate. In 1978, Green & Hathway [47] presented mass-spectral evidence, but no quantitative data, for the formation of εdCyd, and tentatively of εdAdo, in liver DNA from rats exposed to VC (250 p.p.m. in drinking water) for two years. In contrast, N^2,3-εG could be detected in he-

patic DNA after short exposure of 12-day-old rats to [^{14}C]VC [48, 49]. Under similar experimental conditions, 5-fold lower levels of 7OEG were found in adult rats, whereas N^2,3-εG was not detectable [49]. The relative yields of ethenobases in RNA *versus* DNA, or in adult rats as compared to pre-weanling rats (which have a greater DNA replication rate), are probably determined by the hydrogen bonding and accessibility of nucleic acid bases.

Monoclonal antibodies against εdAdo and εdCyd were developed in 1989 by Eberle *et al.* [26] and used successfully in competitive RIA for the detection of these adducts, following their pre-separation from DNA hydrolysates by HPLC. The levels of εdAdo and εdCyd were quantitated in the DNA of lung and liver tissue of preweanling Sprague-Dawley rats after short exposure to VC *in vivo* (2000 p.p.m., 10 days). In parallel, a sensitive method, based on electrophore labelling followed by negative ion chemical ionization MS was developed by Fedtke *et al.* [25] to measure N^2,3-εG. Using these new methods, the three ethenobases were subsequently analyzed, together with 7OEG, in DNA from preweanling rats exposed to 600 p.p.m. VC for 5 days (4 h/day). The four VC adducts were quantitated in liver, lung and kidney. 7OEG was the major DNA adduct detected, representing about 98% of all adducts. N^2,3-εG and εdCyd were present at about 1% of 7OEG content while εdAdo was present in even lower amounts. The persistence of the adducts in hepatic DNA after exposure was also investigated in this study. Whereas 7OEG had a $t_{1/2}$ of 62 h, the three etheno adducts were apparently highly persistent [50]. In another study, the formation of εdAdo and εdCyd was measured in several tissues from BD VI rats (7-days or 13-weeks old) exposed to 500 p.p.m. VC for 14 consecutive days (7 h/day) [51]. Both etheno nucleosides were detected in liver, lungs and brain (with higher levels found in liver) but not in kidneys of preweanling rats. In adult rats, the levels of each adduct in liver DNA were six times lower than in young rats.

This first series of studies revealed that etheno adducts are formed at low levels in young rats exposed to VC (molar ratios of ethenobase to parent base in DNA varied from 4×10^{-8} to 2×10^{-6}), and at still lower levels in adult animals. In order to facilitate dosimetry and repair studies on etheno adducts in adult rodents and in humans, a new assay based on immunopurification and ^{32}P-postlabelling of the etheno deoxyribonucleosides 3′-monophosphates was developed [27, 28]. In this assay, DNA is hydrolyzed enzymatically into 3′-monophosphates. Etheno nucleotides are separated on immunogels prepared with monoclonal antibodies EM-A-1 (against εdAdo) and EM-C-1 (against εdCyd) coupled to Sepharose CL 4B. After ^{32}P-postlabelling on the 5′-position with [γ-^{32}P]ATP and T_4 polynucleotide kinase, the 5′-[^{32}P]monophosphates are separated by two-dimensional thin-layer chromatography on polyethyleneimine-cellulose and quantitated by autoradiography and liquid scintillation counting or using a Phosphorimager. Normal nucleotides are quantitated by reverse-phase HPLC and UV detection. The detection limit of this method is about 5 adducts/10^{10} parent bases, using 50 μg DNA. This method was applied to investigate the formation and accumulation of εdAdo and εdCyd in adult Sprague-Dawley rats exposed to 500 p.p.m. VC for 1, 2, 4 or 8 weeks (4 h/day, 5 days/week) [52]. Background levels of ethenobases (range, 0.04 to 11×10^{-8}) were found in DNA from the liver, lungs, kidneys and circulating lymphocytes of unexposed, control rats. A dose-dependent increase of both etheno adducts was observed in liver DNA from VC-treated rats. In contrast, there was an accumulation of εC but not of εA in lungs and kidneys of these animals; no significant increase of ethenobase levels above control values was observed in circulating lymphocytes. In agreement with the previous observation made with preweanling rats [50], both etheno adducts were found to be persistent in liver DNA, after two months following the termination of VC exposure. These data,

together with previous data, do not show a correlation between total etheno adduct levels and organospecificity of VC-induced carcinogenesis: the liver, the main target organ in Sprague-Dawley rats, did not exhibit higher levels of etheno adducts after VC exposure than the other tissues examined. However, the accumulation of εA in liver DNA could be involved in VC-induced hepatocarcinogenesis (see below). This accumulation was not observed in organs less susceptible to tumourigenesis such as lungs and kidneys.

Mutation spectra in tumours induced by exposure to vinyl chloride

Mutagenesis of *ras* proto-oncogenes and of the *p53* tumour suppressor gene has been investigated in liver tumours associated with VC-exposure in humans and rats, thereby providing the opportunity to examine whether the mutations observed are compatible with the formation of promutagenic DNA etheno-bases.

Human liver angiosarcomas

Marion *et al.* [53] reported the activation of the Ki-*ras* gene by point mutation in human liver ASL associated with VC exposure. A GC → AT transition was detected at the 2nd nucleotide in codon 13 of the Ki-*ras* gene, in 5 out of 6 tumours analyzed. This mutation results in the substitution of a Gly residue by an Asp residue in the p21 protein product. This Ki-*ras* mutation profile differs from that observed in ASL of other etiologies: 5/19 sporadic and 2/5 Thorotrast-induced ASL exhibited a GC →AT transition at base 2 of codon 12 in the Ki-*ras* gene [54]. In addition, a subset of these tumours (4) contained a 2nd mutation (double mutants) in the Ki-*ras* gene: a GC → TA transversion at the first base of codon 12. These data support the hypothesis linking VC exposure to ASL with a specific mutation pattern of the Ki-*ras* gene, i.e. a GC → AT transition at the 2nd nucleotide of codon 13.

p53 Gene mutations were found in 3 out of 6 human ASL associated with VC exposure [55, 56]: all were AT → TA transversions and they involved codons 179, 249 and 255. In contrast, in 13 cases of human ASL associated with Thorotrast exposure, no *p53* mutations were detected [57, 58], and in 17 sporadic ASL (not associated with VC, Thorotrast or arsenic exposure), only two contained *p53* mutations, consisting of GC → AT transitions at codons 136 and 141 [57]. Thus, *p53* mutations are uncommon in sporadic ASL and their profile is different from that of VC-associated ASL.

From the above studies, we can conclude that human ASL associated with VC exposure shows a characteristic mutational spectrum in both the Ki-*ras* and *p53* genes. The substitutions found at G : C or A : T base pairs in these tumours are compatible with the hypothesis implicating promutagenic DNA etheno adducts (N^2,3-εG, εC or εA) as the initiating lesions.

Rat liver tumours

In order to compare the molecular pathways of carcinogenesis in animals and humans, hepatic tumours (10 ASL and 8 HCC) obtained from Sprague-Dawley rats exposed to VC, were investigated for mutations of the Ha-*ras*, Ki-*ras* and N-*ras* genes ([59]; Boivin, S. *et al.*, unpublished observations).

In ASL, no mutations of the Ki-*ras* and Ha-*ras* genes were found. Mutations involving codon 13 (GGC → GAC) and codon 36 (ATA → CTA) of the N-*ras* A gene were detected in two ASL. Several additional base pair substitutions were found in exon 1 of the N-*ras* B and C sequences (pseudogenes) [59].

In HCC, a CAA → CTA mutation was detected in codon 61 of the Ha-*ras* gene [59]. This transversion was detected in all HCCs of grades II/III tested (5/5) but not in HCCs of grade I (0/3) (Boivin, S. *et al.*, unpublished observations).

The presence of gene mutations in exons 5–8 of the *p53* gene was examined in 25 ASL

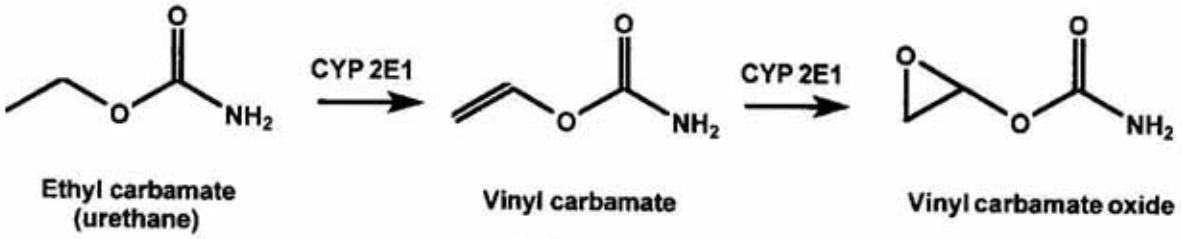

Figure 2. Metabolic activation of urethane.

and 8 HCC induced by VC in Sprague-Dawley rats [60]. Mutations were found in 11 (44%) of the ASL and in 1 HCC. A 12-base pair deletion was found in one tumour; all others were base pair substitutions. Nine of the point mutations were observed at A : T base pairs (5 AT → TA; 2 AT → GC; and 2 AT → CG), and of three GC → AT 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 253. Codon 235 was found to be mutated in three ASL.

Therefore, two molecular events have been shown to occur frequently in VC-induced hepatocarcinogenesis in rats: activation of the Ha-*ras* gene in HCC and mutation of the *p53* gene in ASL, both involving A : T base pairs. This suggest that εA, which accumulates preferentially in hepatic DNA during exposure to VC, could be involved in the initiation of these tumours.

ROLE OF ETHENO ADDUCTS IN URETHANE-INDUCED CARCINOGENESIS

Metabolic activation of urethane and formation of DNA ethenobases (Fig. 2)

Urethane (ethyl carbamate, EC) is found in fermented food products and alcoholic beverages [61]. It has been used formerly in human medicine as an anaesthetic and as a co-solvent in analgesic preparations. EC is a pluripotent carcinogen in rodents, inducing, among others, tumours of the liver, lung, skin (reviewed in [61, 62]). Under conditions of appropriate metabolic activation, it is mutagenic in bacteria and in eukaryotic cells. The pathway of activation of EC into electrophilic metabolites that bind to cellular macromolecules involves two steps: desaturation to vinyl carbamate (VCA) followed by oxidation to vinyl carbamate epoxide (VCO) [24, 63]. The two steps are catalyzed by CYP 2E1 [32]. VCA, in the presence of a hepatic activation system, is more mutagenic than EC to *Salmonella typhimurium* [63], as well as more active in inducing micronuclei [64] and sister chromatid exchanges [65] in mice. EC and VCA give rise to similar spectra of tumours in mouse and rat tissues, but the potency of the latter is much greater [63]. VCO is a strong electrophile and direct-acting mutagen; it is a stronger initiator of carcinogenesis than its precursors EC and VCA and, unlike these two compounds, is a complete carcinogen in the skin of mice [66].

EC yields the same adducts with nucleic acid bases as does VC. The first evidence that EC could yield etheno adducts was obtained by Ribovich *et al.* [67] who detected εAdo and εCyd in hepatic RNA of mice given labelled EC. Like for VC, 7OEG is the major adduct formed by EC or VCA in hepatic DNA of rats and mice [24, 68, 69]. Following repeated applications of VCO, 7OEG and N^2,3-εG have been characterized in hepatic DNA of Sprague-Dawley rats and in skin DNA of CD-1 mice [66]. The formation of these adducts has been

confirmed *in vitro*. εAdo has been detected in microsomal reaction mixtures as a product of adenosine with EC or VCA [69, 70]. Reactions of VCO with DNA yielded 7OEG and N^2,3-εG [65]. More recently, Fernando *et al.* [71] used the immunoaffinity/^{32}P-postlabelling method to measure εdAdo and εdCyd in DNA from mice treated with EC, VCA or VCO. Following five daily i.p. injections of 250 or 280 nmol/g b.w. VCA to adult mice (strains CD-1, C3H/HeJ and C57BL/6J), increased levels of εA and εC were detected in liver and lung DNA, as compared to levels measured in control DNA samples. Under similar dose regimens, lower levels of etheno adducts were detected in strain B6C3F1. DNA etheno adducts were also formed in liver and lung after treatment with EC in adult mice, but at a 3-fold lower levels as compared with VCA. These results further support the hypothesis that metabolic activation of EC to VCA to VCO is involved. In 12-day old C3H/HeJ and C57BL/6J mice, 2- to 3-fold higher etheno adduct levels were detected in liver DNA, when compared with adults, after a single treatment with 250 nmol/g b.w. VCA, suggesting that young animals are more susceptible to adduct formation.

Mutation spectra in murine tumours induced by urethane

A number of studies have shown that liver and lung tumours induced by EC or its metabolites in mice often contain an activated *ras* gene. Since the formation of DNA etheno adducts has been demonstrated in these target tissues in mice [71], it is worth to examine whether the mutation patterns found are specific to EC-treatment and consistent with the known promutagenic properties of DNA etheno adducts.

The Ha-*ras* gene is often activated at codon 61 in spontaneous and chemically-induced liver tumours (hepatocellular adenomas and carcinomas) in mice (reviewed in [72, 73]). *Ras* mutations have been analyzed in EC- and VCA-induced liver tumours in strains of low, intermediate or high susceptibility to hepatocarcinogenesis. The frequency of Ha-*ras* activation in these tumours is not higher than in spontaneous tumours and is also independent of the susceptibility of the strain to hepatocarcinogenesis. However, both EC and VCA treatment leads to a higher frequency of CAA → CTA codon 61 mutations in liver tumours, in mouse strains from high and intermediate susceptibilities. This effect is not observed (or not significant) in strains of low susceptibility. Whatever the strain, the frequency of CAA → AAA codon changes decreases in EC- or VCA-induced liver tumours. There is no obvious effect of EC or VCA treatments on the frequency of CAA → CGA codon changes. In spontaneous tumours, the frequencies of CAA → AAA and CAA → CGA activating mutations seem to increase with susceptibility of the mouse strains. The elevated frequency of AT to TA transversions at the second base of codon 61 in liver tumours from EC- and VCA-treated mice would be consistent with the formation of the initiating lesion εA in DNA by the electrophilic metabolite VCO. Moreover, a high proportion of hepatocellular adenomas from VCA-treated B6C3F1 mice (susceptible strain) exhibit this mutation, suggesting that it appears relatively early in the pathogenesis of liver neoplasia [74]. Inconsistent with the above hypothesis is the fact that liver tumours induced by a number of other genotoxic chemicals in the sensitive B6C3F1 mouse also exhibit a high frequency of CAA → CTA codon 61 mutations [73].

Ki-*ras* gene activation is a frequent event in spontaneous and chemically-induced lung tumours in mice (reviewed in [73, 75]). In most of the strains used in studies on EC-induced carcinogenesis, a high proportion (> 70 %) of spontaneous lung tumours exhibit an activated Ki-*ras* proto-oncogene, most activating mutations being localized in codons 12 and 61. Following treatments with EC or VCA, the frequency of Ki-*ras* activation in lung tumours still increases (in sensitive as well as in resis-

tant strains) and practically all Ki-*ras* mutations in these tumours arise in codon 61. Comparison of the mutation spectra of the Ki-*ras* gene in spontaneous and in EC- or VCA-induced lung tumours shows a significantly higher frequency of CAA → CTA codon changes in tumour samples from treated animals [73, 75–78]. With the exception of 2-acetylaminofluorene, this effect has not been observed with other genotoxic carcinogens. The codon 61 A to T transversion is present at all stages of EC-induced lung tumorigenesis (hyperplastic areas and small adenomas) and thus arises early in tumour development [76, 79]. Following a single i.p. injection of EC, this mutation has been detected in lungs of mice at day 14 but not at day 7 after the treatment [80]. Therefore, the A to T transversion at the second base of codon 61 seems to be EC- or VCA-specific and could result from the formation of an εA adduct.

Alterations of the *p53* gene in EC-induced tumours in mice are less well documented than *ras* mutations. Only one study reports the identification of missense *p53* mutations involving codons 164, 241, 247 and 253 in 4/11 EC-induced, late stage lung adenomas [79]. A silent base pair substitution at codon 264 was observed in one small adenoma out of 22 examined. No *p53* mutation was found in hyperplastic lesions. The point mutations consisted of 3 GC → AT transitions, 1 GC → CG transversion and 1 AT → GC transition. Therefore, *p53* mutations occur relatively late during the benign stages of EC-induced lung carcinogenesis in mice and they involve mainly G : C base pairs. As data on spontaneous lung tumours are lacking, it is presently not possible to conclude that these *p53* mutations are EC-specific. It should be noted, however, that other studies on murine lung tumours induced by different chemical carcinogens failed to identify *p53* gene mutations.

Altogether, these data suggest that the Ki-*ras* and *p53* gene mutations found in lung tumours from mice exposed to EC or VCA could arise from the formation of ethenobases.

ENDOGENOUS FORMATION OF DNA ETHENO ADDUCTS

Background levels in unexposed rodents and humans and diet-related effects

The use of the ultra-sensitive immunoaffinity/^{32}P-postlabelling method revealed the existence of background levels of εdAdo and εdCyd in tissues from unexposed rodents and humans. Analysis of nine liver DNA samples from humans with unknown exposure showed the presence of εdAdo and εdCyd residues in the range of < 0.05 to 2.7 adducts per 10^8 parent bases [28]. In adult Sprague-Dawley rats (7- or 14-week old) fed a standard pelleted diet, background levels were found in all the tissues examined. They were relatively low in liver, with molar ratios of ethenobase/parent base at around 0.04 to 0.07 × 10^{-8}, and higher in lungs, kidneys and circulating lymphocytes, with molar ratios between 1.7 and 11.2 × 10^{-8} [52]. It should be noted that these background levels seemed to be affected by diet, particularly in the liver (unpublished).

εdAdo and εdCyd were also detected in DNA from control mice of several strains (CD-1, B6C3F1, C3H/HeJ and C57BL/6J) fed a high fat diet (Wayne's Breeder Blocks) [71]. In adult and 12-day-old mice, levels of both adducts ranged from 0.3 to 1.4 × 10^{-8} in liver and lungs. Some variations with strain were noted. Preliminary data obtained in this study also suggested that background levels of εA and εC in DNA were affected by the type of diet given to the animals. εdAdo and εdCyd were analyzed in adult B6C3F1 mice which had been fed either a high fat diet (Wayne's Breeder Blocks), a purified diet (AIN-76) or a non-purified natural ingredient diet (NIH-07). In liver and lung DNA, higher levels of etheno adducts were found in mice fed the AIN-76 diet as compared to mice fed the NIH-07 and Wayne's Breeder Blocks diets. This diet-related effect was also reproducibly seen in mice treated with EC or VCA, in that animals given AIN-76 diet exhibited higher etheno ad-

duct levels than mice fed the two other diets [71].

The effects of diet on DNA etheno adduct levels were more specifically adressed by Nair *et al.* [81] in a pilot study in humans. These authors analyzed εdAdo, εdCyd and a malondialdehyde-deoxyguanosine adduct in white blood cells from individuals who participated in a carefully controlled dietary study. Effects of a diet rich in polyunsaturated fatty acids (sunflower oil-based diet) were compared with those of a diet rich in monounsaturated fatty acids (rapeseed oil-based diet). The malondialdehyde-deoxyguanosine adduct, quantified by ^{32}P-postlabelling/reverse phase HPLC, was at a higher level in male and female subjects on the former diet than in individuals on the latter diet. In female subjects on sunflower oil diet, εA/A and εC/C molar ratios ranged from 1.3 to 901 × 10^{-8} and from 0.6 to 716 × 10^{-8}, respectively; mean levels of both etheno adducts were about 40-fold higher than those found in subjects on the rapeseed oil diet. In contrast, such dietary effects on etheno adduct levels were not observed in males. These results show that dietary sunflower oil can increase LPO-derived DNA adducts *in vivo* (see below).

Formation of etheno adducts by lipid peroxidation products *in vitro*

The first evidence that LPO products could form etheno adducts with nucleic acid bases was obtained in 1988 by Sodum & Chung [82]. These investigators identified 1,N^2-εdGuo as a reaction product of dGuo with *trans*-4-hydroxy-2-nonenal (HNE). This etheno adduct was only formed when a hydroperoxide was added to the reaction mixture, suggesting that the reactive intermediate was an epoxy aldehyde, 2,3-epoxy-4-hydroxynonanal (HE). Direct Michael addition of HNE to nucleic acid bases yields exocyclic, substituted propano adducts. Subsequently, several types of adducts, including substituted etheno and ethano derivatives, were characterized as reaction products of 2,3-epoxy-4-hydroxynonanal with nucleosides (reviewed in [83]). The major adduct formed by this compound with native DNA is 1,N^2-εdGuo. In addition, the minor substituted 1,N^2-ethano-2′-deoxyguanosine derivatives are easily converted into 1,N^2-εdGuo under mild alkaline conditions [84]. Etheno adducts can also been formed in reactions of enals (α,β-unsaturated aldehydes such as HNE, crotonaldehyde) with nucleosides (dGuo, dAdo) in the presence of oxygen, suggesting a pathway that involves autooxidation of the enals [85].

Another approach using *in vitro* model systems of LPO yielded further evidence that LPO products can form etheno adducts [86]. Using several methods of analysis, etheno adducts were detected in rat liver microsomal suspensions in the presence of inducers of LPO (Fe(II) or cumene hydroperoxide) and adenine or cytosine nucleotides or nucleosides. Incubation of arachidonic acid (presumably the exclusive source of HNE during microsomal LPO) supplemented with Fe(II) and cAMP led to the formation of 1,N^6-etheno-cAMP. These data showed that etheno derivatives could also be formed in a complex subcellular mixture.

Genetic diseases with increased liver peroxidation

The above evidence that LPO products react with nucleic acid bases to form ethenobase adducts, including εA and εC, provides an explanation for the existence of a low background level of these etheno residues in tissue DNA of unexposed animals and humans. To examine whether the stimulation of LPO *in vivo* could lead to the formation of ethenobases in DNA, an animal model of LPO was investigated [87]. εdAdo and εdCyd were quantitated in hepatic DNA from LEC (Long-Evans with cinnamon-like colour) and LEA (Long-Evans with agouti colour) rats of different age. LEC rats have been established as a mutant inbred strain displaying copper accumulation in the

liver and hereditary hepatitis. The onset of hepatitis (at about 4 months after birth) is accompanied by an elevation of the level of LPO in the liver. Animals that survive for more than one year develop HCC in the chronic phase. LEA rats are a sibling line of LEC rats which do not suffer from liver disease. The data show an accumulation with age of etheno adducts in hepatic DNA from LEC rats, which parallels both the copper accumulation and the progression of hepatic disease. No such accumulation was observed in the LEA strain. Etheno adducts in the liver of LEC rats could arise from oxygen radicals generated by copper-catalyzed Fenton-type reactions; reactive oxygen species can induce LPO by abstraction of allylic hydrogens from unsaturated fatty acid residues of the cell membranes.

LEC rat is an animal model for human Wilson's disease. Patients with Wilson's disease also accumulate copper in the liver, due to a mutation in a gene that codes for a copper-binding P-type ATPase protein. Elevated LPO has been demonstrated in liver tissue of these patients. Nair *et al.* [88] examined the etheno adduct levels in hepatic DNA from patients with Wilson's disease, as well as in patients with primary hemochromatosis, another genetic disease causing iron accumulation in the liver and associated with an increased risk of liver cancer. Mean εA/A values in Wilson's disease patients and primary hemochromatosis patients were 6.6 and 7.1×10^{-8}, respectively, which is about four times the levels detected in normal liver DNA. Mean εC/C values were 6.7 and 7.0×10^{-8}, respectively, i.e. three times higher than in normal liver. These data show that elevated levels of DNA etheno adducts are formed in Wilson's disease and primary hemochromatosis patients due to oxidative stress and LPO.

Etheno DNA adducts along with other oxidative DNA base damages may thus be involved in the initiation of liver cancer in LEC rats and in patients with Wilson's disease or primary hemochromatosis.

CONCLUSIONS

Ethenobases in DNA are promutagenic lesions leading to base pair substitution mutations. They can arise from endogenous sources or from exposure to xenobiotics such as VC or EC. Up to now, three of them, i.e. εA, εC and N^2,3-εG, have been detected *in vivo*. However, due to the availability of an ultrasensitive method for detection of εA and εC, based on immunoaffinity purification and ^{32}P-postlabelling, most data reported so far deal with the formation of these adducts.

Increased levels of ethenobases are formed in target tissues for carcinogenesis in rodents exposed to VC or to EC. Following exposure of rats to VC, persistence of these adducts has been observed in hepatic DNA. During prolonged exposure, εA and εC accumulate in the liver, the major target organ for VC-induced tumourigenesis, whereas only εC accumulates in kidneys and lungs. The accumulation/persistence of ethenobases could result from a poor repair *in vivo*. However, this is unlikely because it has been shown that ethenobases are repaired efficiently by mammalian DNA glycosylases *in vitro* [89–91] and by crude extracts from rat [92] or human [93] cells. An alternative explanation, put forward by Guichard *et al.* [52], could be the concurrent formation of ethenobases through LPO products during and after exposure to VC. Whatever the origin of these etheno adducts, the *ras* and *p53* gene base pair substitutions observed in liver tumours induced by VC in rats are consistent with the promutagenic properties of ethenobases. Human ASL associated with VC exposure exhibit mutation spectra in the Ki-*ras* and *p53* genes that differ from those found in ASL of different etiology and that are also consistent with the formation of ethenobases as initiating lesions.

The formation of ethenobases has been demonstrated in target tissues from rodents exposed to EC or to its metabolites. Despite a substantial accumulation of data showing activation of Ha-*ras* in liver tumours and of Ki-*ras*

in lung tumours, a specific effect of exposure remains difficult to establish, because the same *ras* mutations are also observed in spontaneous tumours and in tumours induced by other chemicals in mice. However, the elevated frequency of the A : T to T : A transversion detected at codon 61 in the Ki-*ras* gene in lung tumours induced by EC and VCA seems to be specific to these chemicals. One study suggests that ethenobases could also be involved in the induction of base pair substitutions of the *p53* gene in these tumours [79].

There is now strong evidence that the endogenous background of DNA etheno adducts detected in tissues from humans and rodents arises from LPO products, more specifically enals such as HNE. Background levels of etheno adducts are highly variable. Part of this variability may be associated with dietary factors, in particular with the dietary intake of ω-6 polyunsaturated fatty acids which oxidize readily to form enals. The measurement of etheno and other exocyclic DNA adducts could thus be a useful tool in molecular epidemiological studies to elucidate the role of dietary fat intake on endogenous DNA damage as well as the protective role of antioxidants [81].

Finally, background levels of εA and εC are elevated in the liver from humans and rodents suffering from hereditary metal storage diseases, these diseases being associated with an increased oxidative stress/LPO in hepatic tissue and with a high risk of liver cancer. DNA etheno adducts along with other oxidative DNA lesions may thus be involved in the initiation of experimental and human liver cancers.

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