Copper-dependent Formation of Miscloding Etheno-DNA Adducts in the Liver of Long Evans Cinnamon (LEC) Rats Developing Hereditary Hepatitis and Hepatocellular Carcinoma

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ABSTRACT

Formation of etheno-DNA adducts in the liver was investigated in Long Evans cinnamon (LEC) rats, a Long Evans strain with hereditary abnormal copper metabolism, which develop spontaneous hepatitis and hepatocellular carcinoma. Using an ultrasensitive immunofluorometric/32P-postlabeling assay (J. Nair et al., Carcinogenesis, 16: 613–617, 1995), the etheno adducts 1,N2-ethenoxyadenosine (edA) and 3,N2-ethenoxyctydine (edC) were measured in the liver of 7-, 18-, 30-, and 87-week-old LEC rats. Levels were highest in the liver of 18-week-old rats 85 ± 17 (edA) and 85 ± 30 (edC) adducts per 107 parent nucleotides, and the increase in the levels of both etheno adducts was age dependent. Age-matched Long Evans agouti rats, a tumor-free sibling line of LEC rats, had much lower levels of etheno adducts. Etheno adduct levels in LEC rats were well correlated with the hepatic copper levels, and peak adduct levels coincided with the age of commencement of fulminant hepatitis. Our results demonstrate for the first time a copper- and age-dependent formation of highly miscoding etheno-DNA adducts in the liver of LEC rats. These adducts are formed from lipid peroxidation products (F. El-Ghissassi et al., Chem. Res. Toxicol., 8: 273–283, 1995) and thus could arise in the liver of LEC rats from oxygen radicals generated by copper-catalyzed Fenton-type reactions. Etheno-DNA adducts along with other oxidative DNA base damages may thus be involved in liver carcinogenesis in LEC rats.

INTRODUCTION

LEC (Long Evans Cinnamon) rats, an inbred strain, accumulate copper in the liver and have low plasma levels of copper and ceruloplasmin (ferroxidase) activity. They are thought to be an appropriate animal model for studying the role of hepatitis in liver cancer development (1, 2) and for human Wilson’s disease (3), a copper storage disorder (4). About 4 months after birth there is a sudden onset of spontaneous hepatitis in LEC rats. Animals that survive for more than 1 year develop hepatocellular carcinoma during the chronic phase. Recently, the WD gene has been cloned and its product characterized as a copper-binding P-type ATPase (4, 5). A linkage analysis revealed that copper accumulation is linked with the hts gene in LEC rats (6). Furthermore, hts was found to be linked with the Atp7b gene (7), and a deletion at the 3′ region was detected in the LEC rat Atp7b gene (8). Although the pathogenesis of hepatitis in LEC rats is quite similar to that of Wilson’s disease (2), the molecular mechanisms that lead to these pathological changes and finally to hepatocellular carcinoma are not known.

MATERIALS AND METHODS

Chemicals and Reagents. All of the common laboratory reagents used were of highest purity. The normal deoxyribonucleoside 3′-monophosphates, deoxyuridine 3′-monophosphate (internal standard, 3′-dUMP), micrococcal endonuclease (grade VI), and spleen phosphodiesterase (from calf spleen) were obtained from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). Proteinase K and RNase were purchased from Boehringer Mannheim (Mannheim, Germany). T4 polynuclease kinase was purchased from Pharmacia (Uppsala, Sweden), and [γ-32P]ATP from Amersham (Les Ulis, France). 3′-edAMP and 3′-edCMP and immunofluorometric purification, 32P postlabeling, and subsequent separation of 5′-monophosphates of edA and edC has been developed (14). The method has a detection limit of four etheno adducts in 1010 normal bases requiring 50-μg DNA samples. This article reports the age-dependent accumulation of etheno adducts in LEC rats using the method described above, thus providing for the first time evidence that DNA damage associated with LPO occurs in the livers of LEC rats.

Reactive oxygen species generated from free copper ion and hydrogen peroxide (9) or ascorbic acid can induce LPO by abstraction of allylic hydrogens from unsaturated fatty acid residues of the cell membrane (10). By measuring mitochondrial- and microsorne-derived, conjugated dienes, and thiobarbituric acid-reactive substances, elevated LPO has been demonstrated in liver tissue of the patients with Wilson’s disease (11). The levels of the LPO in symptomatic LEC rats at 4 months of age were also significantly higher than those in asymptomatic and normal rats (12).

We recently presented evidence (13) that LPO products react with nucleic acid bases to form etheno base adducts, including edA and edC, providing an explanation for the existence of a low background level of these etheno residues in tissue DNA of unexposed animals and humans (14). edA and edC are highly miscoding exocyclic DNA adducts that are also formed in the liver of vinyl chloride-exposed animals and also implicated in vinyl chloride-induced liver angiosarcomas in humans (15, 16). An ultrasensitive detection method based on immunofluorometric purification, 32P postlabeling, and subsequent separation of 5′-monophosphates of edA and edC has been developed (14). The method has a detection limit of four etheno adducts in 1010 normal bases requiring 50-μg DNA samples. This article reports the age-dependent accumulation of etheno adducts in LEC rats using the method described above, thus providing for the first time evidence that DNA damage associated with LPO occurs in the livers of LEC rats.

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3 The abbreviations used are: LEC, Long Evans cinnamon; edA, 1,N2-ethenoxyadenosine; edC, 3,N2-ethenoxyctydine; LEA, Long Evans agouti; edAMP, edA monophosphate; edCMP, edC monophosphate; LPO, lipid peroxidation; HPLC, high-performance liquid chromatography; GOT, serum glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase.
Analysis of Etheno Adducts. DNA was prepared from liver samples by hydroxyapatite purification (22) with the modification of RNase treatment as described (14). edA and edC in DNA were determined using an ultra-sensitive procedure developed recently by us (14), based on immunoaffinity purification of the etheno adducts and subsequent $^{32}$P postlabeling followed by separation as 5'-monophosphates on polyethyleneimine-cellulose-coated thin layer plates. In brief, 50-μg aliquots of DNA were hydrolyzed to 2'-deoxyribonucleoside 3'-monophosphates. Normal nucleotides in the enzymatic hydrolysate were quantitated using HPLC and UV detection at 254 nm and by comparison to authentic standards. Purification of etheno adducts from DNA hydrolysates was carried out on immunoaffinity columns prepared with antibodies against edA or against edC. The bulk of the normal nucleotides was removed by washing with PBS in water, and then the etheno adducts were eluted with methanol/water. $^{32}$P postlabeling of the etheno adducts was done according to the procedure by Hollstein et al. (23), with the modification as described by Nair et al. (14). One hundred 3'-dUMP were added as internal standard to the dried samples amol obtained from the immunoaffinity columns and labeled with [γ-$^{32}$P]ATP (10 μCi; specific activity, >5000 Ci/mmol) and T₄ polynucleotide kinase. After incubation and centrifugation, the mixture was spotted on the left side of prewashed polyethyleneimine cellulose plates and developed on D₁ direction with 1 M acetic acid (pH 3.5). The plates were dried, cut 2 cm above from the origin, and developed on D₂ direction with saturated ammonium sulfate (pH 3.5). The plates were dried, clipped 1.5 cm from the right margin, and exposed to X-ray films in cassettes supported with intensifying screens. Sections of the TLC plates corresponding to the spots of 5'-edAMP, 5'-edCMP, and 5'-dUMP (spots 1, 2, and 3, respectively, in Fig. 1) were cut out, and radioactivity was determined by liquid scintillation counting. Background counts were determined from appropriate blank areas on TLC plates and subtracted. Parallel samples containing standard 3'-edAMP, 3'-edCMP, and 3'-dUMP were also labeled and separated in a similar way. The number of adducts per parent nucleotide was obtained from the ratio of the quantity of etheno adduct measured on TLC to the quantity of parent nucleotide in the sample derived from HPLC analysis as described by Nair et al. (14), taking into account the labeling efficiency as

![Fig. 1. Autoradiograms of $^{32}$P-labeled liver DNA samples. A, standard compounds; B, from 7-week-old LEC rats; C, 18-week-old LEC rats; and D, and 18-week-old LEA rats. D₁, 1 M acetic acid (pH 3.5); D₂, saturated ammonium sulfate (pH 3.5). Spot identification: 1, 5'-edAMP; 2, 5'-edCMP; and 3, 5'-dUMP (internal standard).]
measured with 5'-dUMP. The detection limit of this method is four etheno adducts per 10^10 unmodified nucleotides.

**Copper and GOT and GPT Determination.** The copper levels in liver tissues and GOT and GPT activities were determined as previously described (6).

**Statistical Analysis.** The results obtained in three to four rats are expressed as means ± SD. The differences in adduct levels were analyzed for statistical significance using Student's t test; the correlation of adduct levels to copper contents was obtained by linear regression analysis.

**RESULTS**

Using our recently developed ultrasensitive immunoaffinity/32P-postlabeling technique, edA and edC levels were measured as their nucleoside 5'-monophosphates in liver DNA from 7-, 18-, 30-, and 87-week-old LEC rats and from 18- and 87-week-old LEA rats, a noncopper accumulating, tumor-free sibling line of LEC rats that does not develop hereditary hepatitis or hepatocellular carcinoma. Typical autoradiograms obtained with the labeled nucleoside 5'-monophosphates from liver DNA samples of LEC and LEA rats are shown in Fig. 1. The nonassigned spots are the unremoved normal nucleotides from the DNA samples and from [γ-^32P]ATP.

Both etheno adducts were found to be highest in liver DNA of 18-week-old LEC rats, reaching an edA level of 85 ± 17 and an edC level of 85 ± 30 per 10^9 parent nucleotides (Fig. 2). These values were about 5-fold higher than those found in 7-week-old LEC rats. In age-matched LEA rats at 18 weeks, the edA and edC levels were 28-fold and 5-fold lower, respectively. The etheno adduct levels in liver DNA of LEC rats were highest at the age when the hepatic copper level was highest (Fig. 2). The peak adduct levels also coincided with the commencement of hepatitis, which was at around 18 weeks. In older LEC rats, the levels of edA and edC declined and were only about one third of the peak level. The concentration of hepatic copper also decreased in older animals.

Table 1 shows the statistical analysis of the adduct levels in the various age group samples from LEC and LEA rats and the relationship between adduct levels and copper content. The age-dependent increase of both etheno adducts in the liver of 7- versus 18- or 30-week-old rats was highly significant. Hepatic edA and edC levels in LEC rats at 18 weeks were significantly higher than those in the LEA rats of the same age group, and the edA level in 87-week-old LEC rats was significantly higher than that in 87-week-old LEA rats. Both edA and edC levels in liver DNA of LEC rats were significantly correlated with the copper content in the liver (r = 0.77 and 0.67;
enzymes decreased in the serum of older rats. Again, LEA rats did not show those increases and correlations.

**DISCUSSION**

Hepatitis affects more than 200 million people worldwide, and liver cancer often develops in association with chronic hepatitis caused by hepatitis B or C infections. However, the underlying mechanisms of how these chronic inflammatory conditions lead to hepatocellular carcinoma are poorly understood. The LEC rat is considered to be a good model for studying the human Wilson’s disease and the role of copper in liver cancer development, since the pathogenesis of the liver in LEC rats shows a remarkable similarity to the clinical course of human liver cancer development (3).

Using an ultrasensitive detection assay, we have previously shown background levels of edA and edC in liver DNA of untreated rodents and humans (14). In *vitro* experiments have demonstrated that these etheno adducts can readily be formed from LPO products (13). Reaction of *trans*-4-hydroxy-2-nonenal, a major LPO product, with deoxyguanosine is known to form the 3,4′-etheno derivative in *vitro* (24). Because of the availability of the method, we investigated the formation of edA and edC to assess whether LPO induced by copper accumulation could cause these specific DNA damages in LEC rats. Our results demonstrate for the first time a copper- and age-dependent formation of etheno-DNA adducts in the liver of LEC rats. These appear to originate from LPO products such as *trans*-4-hydroxy-2-nonenal that are produced due to accumulation of copper and generation of reactive oxygen species in the liver. Whether the accumulation of etheno adducts in LEC rats is further accelerated by the inhibition of DNA repair pathways due to hepatitis that removes these lesions in the liver needs to be investigated. Recently, a human 3-methyladenine-DNA glycosylase has been shown to release all four known etheno-base adducts from DNA in *vitro* (25). Because etheno adducts are highly miscoding DNA lesions which are implicated in urethane-induced tumors and in vinyl chloride-induced liver carcinogenesis in animals and humans (16), they are possibly also involved in the genesis of hepatocellular carcinomas in LEC rats.

Our data also suggest that the analysis of etheno-DNA adducts appears to be a useful biomarker for LPO- and oxidative stress-induced DNA damage. Studies are under way to elucidate whether the formation of elevated levels of etheno adducts occurs in the liver of patients with Wilson’s disease or in other types of virally or chemically induced hepatitis.

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