Lipid Peroxidation-induced Etheno-DNA Adducts in the Liver of Patients with the Genetic Metal Storage Disorders Wilson’s Disease and Primary Hemochromatosis

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Abstract
To assess DNA damage caused by lipid peroxidation due to copper and iron storage disorders in the human liver, the formation of the etheno adducts 1,N\(^2\)-ethenodeoxyadenosine (edA) and 3,N\(^(-)\)-ethenodeoxycytosine (edC) was measured in liver DNA from normal subjects and from patients with Wilson’s disease (WD) and primary hemochromatosis. The mean edA and edC levels per 10\(^7\) parent nucleotides in normal liver were 19.3 ± 4.9 and 27.5 ± 10.0, respectively. The mean edA and edC levels per 10\(^7\) parent nucleotides in WD were 61.03 ± 7.95 and 91.50 ± 36.02, and in primary hemochromatosis, they were 46.62 ± 32.83 and 64.32 ± 11.55, respectively, two to three times higher than those in the normal liver. The etheno adduct levels were highly correlated with the copper content of the liver in the normal and WD samples. This study demonstrates for the first time the formation of promutagenic etheno adducts in humans in association with copper and iron storage-induced lipid peroxidation. Thus, the etheno adducts are implicated as initiating DNA damage in copper/iron-induced carcinogenesis in humans and should also be explored as biomarkers in disease progression and prevention trials.

Introduction
WD\(^3\) is an autosomal recessive disorder of copper transport, resulting in copper accumulation in the liver and brain. The WD gene (ATP7B gene) has been mapped to human chromosome 13q14.3, and its product is characterized as a copper-binding P-type ATPase protein, which acts as an ATP-dependent transporter of copper within hepatocytes (1–3). Copper accumulation in WD patients, if not treated by chelating therapy, leads to liver injury. In LEC rats, an animal model for WD, copper accumulation leads to hepatitis and HCC (4). Similar to LEC rats, HCC has been reported in a WD patient who was poorly compliant to pharmacotherapy (5). PH is a genetic disorder characterized by a progressive accumulation of iron, leading to liver cirrhosis (6). A candidate hemochromatosis gene has been located on chromosome 6, which is related to the MHC class I family (7). PH is 100 times more common than WD in Caucasion (8). A 90–240-fold increase in the relative risk for primary liver cancer has been reported in patients with PH (9–11).

The DNA damage that leads to liver cancer in WD and PH is poorly understood, although evidence exists that the damage is caused by oxidative stress resulting from a high concentration of monovalent copper and bivalent iron ions acting as pro-oxidants (12, 13). The metal-induced oxidative stress can lead to the formation of hydroxyl radicals via the Fenton and Haber-Weiss reactions. A similar process involving transition metal ion-mediated oxygen free radical-dependent DNA damage has been suggested to be responsible for bulky DNA lesions detected in the livers of humans with WD and PH (14). The increased DNA damage may be caused by hydroxyl radicals or via the formation of reactive aldehydes such as trans-4-hydroxy-2-nonal and malondialdehyde as a result of lipid peroxidation (15). Etheno-bridged nucleic acid bases have been shown to be formed as a consequence of lipid peroxidation in vitro, presumably via a reaction between peroxidized trans-4-hydroxy-2-nonal and DNA (16, 17). In LEC rats, age- and copper-dependent increased formation of the etheno adducts edA and edC has been reported (18). To determine whether similar DNA damage could occur in patients with WD and PH, we have measured the levels of edA and edC in the liver DNA of patients with these disorders and compared them with the levels formed in normal human livers. The copper and iron content of the livers was also determined to investigate the relationship between the etheno adduct levels and metal ion concentrations in the liver.

Materials and Methods
Tissue Accrual. WD and PH liver samples were obtained from patients undergoing orthotopic liver transplant at the Queen Elizabeth Hospital (Birmingham, United Kingdom). Control samples were from normal liver donor tissue excess of surgical requirements from the pediatric segmental liver transplant program. Tissue samples were frozen at -85°C until use.

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3 The abbreviations used are: WD, Wilson’s disease; LEC, Long Evans cinnamon; HCC, hepatocellular carcinoma; PH, primary hemochromatosis; edA, 1,N\(^2\)-ethenodeoxyadenosine; edC, 3,N\(^(-)\)-ethenodeoxycytosine; dA, deoxyadenosine; dC, deoxycytidine.
Analysis of Copper and Iron. Copper and iron were determined by atomic absorption spectrometry, after the digestion of tissue with nitric and perchloric acids, using established procedures (19).

Analysis of edA and edC. DNA was prepared from the liver tissue by hydroxylapatite purification with the modification of RNase treatments as described previously (20). A highly sensitive method using immunoaffinity purification with 32P-postlabeling was used to quantitate edA and edC in the liver DNA samples as their 5'-monophosphate (20). In brief, ~25 μg of DNA were hydrolyzed to deoxynucleoside 3'-monophosphates using micrococcal endonuclease and calf spleen phosphodiesterase. edA and edC were determined in enzymatic hydrolysate by high-performance liquid chromatography. edA and edC were enriched on immunoaffinity columns (21) that were prepared from monoclonal antibodies (22). The dried samples were labeled with [γ-32P]ATP and T4 polynucleotide kinase in the presence of 3'-dUMP as an internal standard to nucleoside 5'-monophosphates and resolved on polyethyleneimine cellulose TLC plates using two-directional chromatography (20). The spots corresponding to edA, edC, and 3'-dUMP were quantitated by autoradiography and liquid scintillation counting. The number of adducts/parent nucleotides was obtained from the ratio of the quantity of the etheno adducts measured by TLC:the quantity of the parent nucleotides in the sample obtained from high-performance liquid chromatography analysis (20).

Statistical Analysis. The comparisons of adduct levels and the metal ion concentration between the groups were performed using the Student’s t test. Correlations between the adduct levels and the metal concentration were obtained by simple regression analysis.

Results

Etheno Adducts in Normal, WD, and PH Liver DNA. edA and edC were determined in DNA by a highly sensitive (~4 adducts/10^9 parent nucleotides) and specific immunoaffinity/32P-postlabeling method (20). The reliability of the method has been verified against a RIA method (23), and a duplicate analysis of several DNA samples yielded a coefficient of variation of ~20% (24). The purification of adducts on immunoaffinity columns prepared from monoclonal antibodies that specifically recognize the etheno adducts and the chromatographic properties of the adducts similar to the authentic standards confirm the identity of the adducts as edA and edC. Fig. 1 depicts the autoradiograms of standard etheno adducts and etheno adducts isolated from normal liver DNA and DNA from WD and PH patients.

edA and edC were analyzed in DNA from 10 normal livers, 8 WD livers, and 6 PH livers. The patient details and the etheno adduct levels detected in each sample are given in Table 1. The range and mean ± SD of edA in normal liver DNA were 12.4–28.2 and 19.3 ± 4.9 for edA/10^9 dA and 9.8–38.1 and 27.5 ± 10.0 for edC/10^9 dC. The mean edA and edC levels in WD liver DNA were three times higher than those in the normal livers, and this difference was statistically significant (P < 0.0001). In PH liver, the etheno adduct levels were 2.3 times higher than those in the normal livers (edA, P < 0.02; edC, P < 0.0001; Fig. 2). One of the PH liver samples collected was from a 1-month-old female child who showed the
Table I

Sample details and parameters analyzed in the livers of normal controls and patients with WD and PH

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Age</th>
<th>Sex</th>
<th>Liver status</th>
<th>Cu (ng/mg)</th>
<th>Fe (ng/mg)</th>
<th>edA/10⁹dA</th>
<th>edC/10⁹dC</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>50 y</td>
<td>M</td>
<td>N⁷</td>
<td>6.86</td>
<td>159.96</td>
<td>16.0</td>
<td>38.1</td>
</tr>
<tr>
<td>2</td>
<td>26 y</td>
<td>M</td>
<td>N</td>
<td>9.07</td>
<td>106.53</td>
<td>20.1</td>
<td>41.0</td>
</tr>
<tr>
<td>3</td>
<td>20 y</td>
<td>M</td>
<td>N</td>
<td>5.53</td>
<td>158.55</td>
<td>18.5</td>
<td>9.8</td>
</tr>
<tr>
<td>4</td>
<td>22 y</td>
<td>F</td>
<td>N</td>
<td>5.15</td>
<td>84.47</td>
<td>24.1</td>
<td>18.3</td>
</tr>
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<td>5</td>
<td>35 y</td>
<td>M</td>
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<td>19.28</td>
<td>106.11</td>
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<tr>
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<td>27.5</td>
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<td>7</td>
<td>19 y</td>
<td>F</td>
<td>N</td>
<td>112.52</td>
<td>6.14</td>
<td>28.2</td>
<td>34.5</td>
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<td>8</td>
<td>26 y</td>
<td>F</td>
<td>N</td>
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<td>47.80</td>
<td>22.5</td>
<td>19.5</td>
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<td>9</td>
<td>22 y</td>
<td>F</td>
<td>N</td>
<td>39.68</td>
<td>ND⁸</td>
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<td>28.1</td>
</tr>
<tr>
<td>10</td>
<td>17 y</td>
<td>F</td>
<td>WD</td>
<td>193.86</td>
<td>36.34</td>
<td>49.3</td>
<td>47.0</td>
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<tr>
<td>11</td>
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<td>M</td>
<td>WD</td>
<td>174.84</td>
<td>13.62</td>
<td>54.9</td>
<td>73.3</td>
</tr>
<tr>
<td>12</td>
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<td>M</td>
<td>WD</td>
<td>350.77</td>
<td>ND⁹</td>
<td>55.9</td>
<td>123.6</td>
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<tr>
<td>13</td>
<td>18 y</td>
<td>M</td>
<td>WD</td>
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<td>4.97</td>
<td>66.9</td>
<td>55.9</td>
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<tr>
<td>14</td>
<td>52 y</td>
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<td>ND⁹</td>
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<td>71.8</td>
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<tr>
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<td>M</td>
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<td>74.9</td>
<td>129.0</td>
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<tr>
<td>16</td>
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<td>WD</td>
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<td>7.97</td>
<td>61.6</td>
<td>143.1</td>
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<tr>
<td>17</td>
<td>9 y</td>
<td>F</td>
<td>WD</td>
<td>97.40</td>
<td>ND⁹</td>
<td>60.1</td>
<td>88.4</td>
</tr>
<tr>
<td>Mean</td>
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<td></td>
<td></td>
<td>21.5 ± 33.8</td>
<td>36.34 ± 49.3</td>
<td>49.3 ± 47.0</td>
<td>73.3 ± 73.3</td>
</tr>
<tr>
<td>19*</td>
<td>1 mo</td>
<td>F</td>
<td>PH</td>
<td>18.90</td>
<td>3684.93</td>
<td>2.9</td>
<td>15.8</td>
</tr>
<tr>
<td>20</td>
<td>63 y</td>
<td>M</td>
<td>PH</td>
<td>27.32</td>
<td>7.62</td>
<td>8.4</td>
<td>69.3</td>
</tr>
<tr>
<td>21</td>
<td>31 y</td>
<td>M</td>
<td>PH</td>
<td>23.31</td>
<td>203.00</td>
<td>70.4</td>
<td>69.2</td>
</tr>
<tr>
<td>22</td>
<td>64 y</td>
<td>M</td>
<td>PH</td>
<td>11.37</td>
<td>6.05</td>
<td>13.0</td>
<td>43.9</td>
</tr>
<tr>
<td>23</td>
<td>39 y</td>
<td>M</td>
<td>PH</td>
<td>2.30</td>
<td>124.31</td>
<td>70.7</td>
<td>72.1</td>
</tr>
<tr>
<td>24</td>
<td>45 y</td>
<td>M</td>
<td>PH</td>
<td>10.81</td>
<td>595.80</td>
<td>70.6</td>
<td>67.1</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>15.02 ± 187.36</td>
<td>46.62 ± 64.32</td>
<td>88.4 ± 91.5</td>
<td>73.3 ± 73.3</td>
</tr>
</tbody>
</table>

ᵃ N, normal.
ᵇ ND, not determined.
ᶜ Excluded from the means.

Fig. 2. Mean ± SD of the etheno adduct levels in normal, WD, and PH liver DNA. Statistical significance was determined using Student's t-test: * P < 0.02; ** P < 0.0001 when compared with normal DNA.

Discussion

This is the first report on elevated levels of etheno adducts in the genetic metal storage diseases WD and PH. Because these diseases lead to increased intracellular concentrations of pro-oxidant copper or iron, the enhanced etheno adducts seen may reflect the increased lipid peroxidation-induced DNA damage in the liver. Increased circulating lipid peroxides along with reduced levels of antioxidants (reduced glutathione and...
Etheno Adducts in WD/PH

Copper levels in WD patients have been reported to be increased in both WD and PH (25). Hepatocellular mitochondria have been found to be target organelles for oxidative damage in rats after copper overload (26), in LEC rats (27), in Bedlington terriers with hepatic copper toxicosis (28), and in explanted liver from WD patients (28). It has been suggested that lipid peroxidation is one of the factors responsible for the high incidence of HCC in PH (29).

In the present study, we have shown that the copper content of the liver positively correlates with both edA and edC levels. This confirms a similar finding in LEC rats in which the copper content was shown to correlate with etheno adducts (18). Because LEC rats develop HCC at an older age, and HCC has been reported in a WD patient who was noncompliant to therapy (5), there is a positive, possibly causal association of copper storage and HCC via the formation of etheno adducts. Copper is an essential human micronutrient, playing an important role in the formation of more than 100 metallo-enzymes (30). Copper is also a potent catalyst of the Haber-Weiss reaction in which hydrogen peroxide is converted to the hydroxyl radical, which may react rapidly with the polyunsaturated fatty acids of the cell membrane (31). Besides WD, hepatic accumulation of copper occurs in Indian childhood cirrhosis, idiopathic copper toxicosis, and vineyard sprayers' disease (32, 33), leading to hepatocellular injury, fibrosis, cirrhosis, or fulminant liver disease. Additional studies are required to clarify whether an elevated formation of etheno adducts also occurs in such conditions. Similarly, iron is an essential metal involved in oxygen transport by hemoglobin and in the activity of many enzymes such as catalase and cytochromes (34). Iron may generate reactive oxygen species via Fenton reactions, and several in vitro and in vivo studies have demonstrated that reactive oxygen species can induce lipid peroxidation as well as oxidative DNA damage (29). In our study, the 1-month-old PH patient had the lowest levels of etheno adducts despite having the highest iron content (Table 1), similar to the low levels of etheno adducts detected in the DNA of liver samples obtained from sudden infant death syndrome (20). The low adduct level in the 1-month-old patient indicates that chronic exposure to metal-induced reactive species is required to accumulate DNA adducts.

Etheno adducts can be formed from a broad range of chemical agents (35). However, the most convincing evidence for the involvement of etheno adducts in carcinogenesis is the formation of etheno adducts by vinyl chloride, a potent human and rodent carcinogen, and by urethane, a potent multiorgan rodent carcinogen (23, 24, 35). Vinyl chloride induces angiosarcomas of the liver in humans (36) and in rodents. Besides vinyl chloride exposure, hemochromatosis and the use of copper sulfate for spraying vineyards (one case) are among the etiological factors for developing angiosarcoma of the liver, which is a rare type of cancer in humans (37). Other causative agents are Thorotrast and exposure to inorganic arsenic. The common DNA damage detected after vinyl chloride exposure, in hemochromatosis, and after copper accumulation is the formation of etheno adducts, which suggests that the formation of etheno adducts is a common pathway for the initiation of angiosarcoma of the liver.

Etheno adducts have been shown to be promutagenic lesions in bacterial and mammalian cell systems (35, 38). Their

Fig. 3. Linear regression analysis of copper content versus etheno adducts in normal and WD livers (A) and iron content versus etheno adducts in normal and PH livers (B).
importance in carcinogenesis has been further established from studying mutation patterns in human angiosarcoma as a result of vinyl chloride exposure. GC→AT and CG→AT mutations observed in the Ki-ras gene in this neoplasm corresponded to the expected mutation pattern from edC and N²-3-ethenodeoxyguanosine (35, 39), and the AT→TA transversion observed in the p53 gene in two of five of the same tumors (40) may have originated from edA. In a recent study on the p53 gene mutation pattern in rat liver tumors induced by vinyl chloride, 9 of 13 point mutations involved the A:T bp (41). Specific enzymes that are capable of repairing edA and edC lesions have been characterized in humans (42). Whether the accumulation of these adducts in the liver is due to an inhibition of these repair enzymes by metal accumulation or lipid peroxidation remains to be studied.

In conclusion, using the ultraviolet sensitive 3²P-postlabeling method, we have demonstrated an enhanced formation of lipid peroxidation-induced etheno adducts in WD and PH. The higher liver cancer risk observed in PH and in LEC rats strongly suggests that etheno-DNA adducts are causally associated with metal (iron and copper) storage-induced carcinogenesis. These adducts should therefore be explored as biomarkers in disease progression and in (chemo)prevention trials.

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References
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