Apoptosis and age-dependant induction of nuclear and mitochondrial etheno-DNA adducts in Long-Evans Cinnamon (LEC) rats: enhanced DNA damage by dietary curcumin upon copper accumulation

Jagadeesan Nair1,*, Susanne Strand1,*, Norbert Frank1,*, Jutta Knauft1, Horst Wesch2, Peter R.Galle2 and Helmut Bartsch1

1Division of Toxicology and Cancer Risk Factors, German Cancer Research Center (DKFZ), Heidelberg, Germany; 2First Department of Internal Medicine, Johannes Gutenberg-University Mainz, Germany and 3Division of Oncological Diagnostics and Therapy, German Cancer Research Center (DKFZ), Heidelberg, Germany

*To whom correspondence should be addressed
Email: j.nair@dkfz.de

Long-Evans Cinnamon (LEC) rats, a model for human Wilson’s disease, develop chronic hepatitis and liver tumors owing to accumulation of copper and induced oxidative stress. Lipid peroxidation (LPO)-induced etheno-DNA adducts in nuclear- and mitochondrial-DNA along with apoptosis was measured in LEC rat liver. Levels of etheno-DNA adducts (1, N6-ethenodeoxyadenosine and 3, N4-ethenodeoxyctydine) increased with age reaching a peak at 8 and 12 weeks in nuclear and mitochondrial DNA, respectively. This is the first demonstration that etheno-DNA adducts are also formed in mitochondrial DNA. Apoptosis was assessed by TUNEL+ cells in liver sections. CD95L RNA expression was also measured by in situ hybridization in the same sections. The highest nuclear DNA adduct levels coincided with a reduced apoptotic rate at 8 weeks. Mitochondrial-DNA adducts peaked at 12 weeks that coincided with the highest apoptotic rate, suggesting a link of etheno-DNA adducts in mitochondrial DNA to apoptosis. The DNA damage in liver was further enhanced and sustained by 0.5% curcumin in the diet. Treatment for 2 weeks elevated etheno-DNA adducts 9- to 25-fold in nuclear DNA and 3- to 4-fold in mitochondrial DNA, providing a plausible explanation as to why in our earlier study [Frank et al. (2003) Mutat. Res., 523–524, 127–135], curcumin failed to prevent liver tumors in LEC rats. Our results also confirm the reported in vitro DNA damaging potential of curcumin in the presence of copper ions by reactive oxygen species. LPO-induced adduct formation in nuclear and mitochondrial DNA appear as early lesions in LEC rat liver carcinogenesis and are discussed in relation to apoptotic events in the progression of malignant disease.

Introduction

The Long-Evans Cinnamon (LEC) coated rat is a mutant strain accumulating copper in the liver (1). Because of a deletion in the copper transporting ATPase (Atp7b) gene, which is homologous to the human Wilson’s disease (WD) gene (2), LEC rats spontaneously develop necrotizing hepatitis with jaundice 3–4 months after birth. Around 40% of the animals die of hepatic failure, while surviving animals develop tumors mainly in the liver and kidneys (3).

Curcumin, a chemopreventive phytochemical exhibits antioxidant (4), anti-inflammatory (5) and anticancer properties in a number of chemically induced rodent tumor models (summary in 9). Curcumin induced apoptosis (6) and lowered iron-induced liver toxicity (7,8), as measured by a reduction of lipid peroxidation (LPO) in plasma and liver, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) activities in plasma. In a previous study (9), we had explored the influence of 0.5% curcumin in the diet on tumorigenesis in LEC rats. Unexpectedly, curcumin decreased the median survival time and showed no protective effect against liver and kidney tumors. However, it significantly reduced tumor metastasis compared with untreated animals. Earlier, we had demonstrated that oxidative stress and LPO-induced etheno-DNA adducts [1, N6-ethenodeoxyadenosine (edA) and 3, N4-ethenodeoxycytidine (edC)] were increased in LEC rats in an age- and copper-dependant manner (10). Etheno-DNA adduct levels were also highly correlated with the levels of copper in the liver of human WD patients (11). Oxidative stress induced apoptosis following copper accumulation in the liver has been observed in LEC rats measured by terminal deoxy- nucleotidyl transferase mediated dUTP-biotin nick-end-labeling positive (TUNEL+) cells (12). In an in vitro model of copper-treated hepatoma cells, copper overload resulted in the upregulation of CD95 and the CD95L resulting in hepato- cyte apoptosis. Further analysis of livers of patients with acute WD confirmed the causal relationship of the CD95 system in acute hepatic failure and liver cell damage (13).

These observations prompted us to investigate the role of etheno-DNA adducts as oxidative stress markers in curcumin-fed LEC rats and apoptosis, done in parallel to our earlier chemopreventive studies (9). Etheno-DNA adduct levels were measured in nuclear and mitochondrial DNA; the latter being analyzed in order to explore their role in apoptosis in the liver. In addition, the influence of curcumin on the levels of copper and iron in liver, on liver toxicity as measured by ASAT- and ALAT-activities in serum, on glutathione/glutathione disulfide (GSH/GSSG) status and on apoptotic events in liver was determined. We report here our findings on the changes of these parameters whereby groups of LEC rats with or without curcumin treatment were compared at different ages.

The key observations of our investigation in LEC rats with respect to initiation and temporal progression of liver

Abbreviations:
edA, 1N6-ethenodeoxyadenosine; edC, 3N4-ethenodeoxycytidine; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GSH, glutathione; GSSG, glutathione disulfide; LEC, Long–Evans Cinnamon; LPO, lipid peroxidation; WD, Wilson’s disease.

The first three authors contributed equally to this work.
carcinogenesis are highlighted. The synergistic role of curcumin and copper ions acting as pro-oxidants in this model is discussed.

Materials and methods

Animal treatment

Male LEC rats were purchased from Charles River Germany GmbH (Sulzfeld) imported from Japan. The animals were kept in rooms with controlled temperature (22 ± 1°C), air humidity (55 ± 10%) and 12 h light/darkness cycle. Control animals were fed a standard diet (Altromin, Lage, Germany) and received tap water ad libitum. Curcumin-fed animals received curcumin (95% purity from Schuchard, Hohenbrum, Germany) at 0.5% mixed in the standard diet. The animals were weighed once weekly and were under observation until the end of the experimental time and were killed at the age of 6, 8, 12, 16 and 32 weeks by heart puncture under ether narcosis.

Fifty-two male LEC rats at the age of 4 weeks (40–46 g) were divided into two groups, receiving control diet and curcumin diet, respectively. Four control animals and four experimental animals each were killed at the age of 6, 8 and 16 weeks. For the group of 12 weeks, six animals were used per group and eight animals for 32 weeks. Owing to early death, in the curcumin group for 16 weeks, only one rat survived the 16 weeks. At 32 weeks, eight animals, three survived in the control and five in the curcumin group. The details of effective animals and the treatment length in different groups are given in Table I. Liver and blood samples were collected and subjected to the following investigations.

DNA extraction

Liver tissue was homogenized in 0.25 M sucrose solution using a Potter-Elvehjem homogenizer. Nuclei and mitochondria were separated by gradient centrifugation (14). DNA was extracted from nuclei and mitochondria by a modified hydroxylapatite method (15).

Etheno-DNA adducts

eDA and eDC were analyzed in DNA by immunoaffinity-32-P-postlabeling (15). In brief, DNA (10–25) was hydrolyzed to nucleotide 3-monophosphates using micrococal nuclease and spleen phosphodiesterase. Normal nucleotides were quantitated by high-performance liquid chromatography analysis of normal nucleotides obtained from the DNA digest.

Adduct spots and the internal standard were marked, cut and the absolute eDA: eDC: 25.4 versus 3.3) (Figure 1A).

Results

The liver tissues from the curcumin-treated and untreated LEC rats were immediately snap frozen in liquid nitrogen and stored at –80°C. Cryosections of 4 μm were fixed in 4% paraformaldehyde and stored at –20°C until analysis by TUNEL staining for fragmented DNA. Staining was performed according to the manufacturer’s instructions (Roche, Mannheim, Germany). Counter-staining of nuclei was performed with Hoechst 33342 (Molecular Probes, OR).

Table I. Animal treatment groups and number of effective animals available for the study

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Control</th>
<th>Curcumin treated* (duration of treatment in weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>4/4</td>
<td>4/4 (2)</td>
</tr>
<tr>
<td>8</td>
<td>4/4</td>
<td>4/4 (6)</td>
</tr>
<tr>
<td>12</td>
<td>4/6</td>
<td>4/6 (8)</td>
</tr>
<tr>
<td>16</td>
<td>4/4</td>
<td>1/4 (12)</td>
</tr>
<tr>
<td>32</td>
<td>3/8</td>
<td>5/8 (28)</td>
</tr>
</tbody>
</table>

*0.5% in the diet.

Apoptosis

The liver tissues from the curcumin-treated and untreated LEC rats were immediately snap frozen in liquid nitrogen and stored at –80°C. Cryosections of 4 μm were fixed in 4% paraformaldehyde and stored at –20°C until analysis by TUNEL staining for fragmented DNA. Staining was performed according to the manufacturer’s instructions (Roche, Mannheim, Germany). Counter-staining of nuclei was performed with Hoechst 33342 (Molecular Probes, OR).

CD95L expression

Cryosections (5 μm thick) were prepared from livers of LEC rats and transferred on siliconized glass slides. After fixation with 4% paraformaldehyde, slides were dehydrated in alcohol and air dried. In situ hybridization was performed as described (16) using digoxigenin labeled anti-sense RNA of rat CD95L. Control experiments were performed using a 10-fold excess of unlabeled RNA, resulting in a complete competition of the signal, demonstrating specificity of the in situ hybridization.

Determination of copper and iron

The liver tissues from the curcumin-treated and untreated LEC rats were immediately snap frozen in liquid nitrogen and stored at –80°C. Cryosections of 4 μm were fixed in 4% paraformaldehyde and stored at –20°C until analysis by TUNEL staining for fragmented DNA. Staining was performed according to the manufacturer’s instructions (Roche, Mannheim, Germany). Counter-staining of nuclei was performed with Hoechst 33342 (Molecular Probes, OR).

Determination of ASAT, ALAT

ASAT- and ALAT-activities were determined in plasma using commercial kits (Roche, Mannheim), by photometric determination of NADH (Figure 2A) at 340 nm produced by the transaminase activity.

Determination of glutathione and GSSG

The concentrations of GSH and GSSG in liver homogenates were determined by isocratic HPLC, using N-(1-pyrenyl)maleimide (NPM), which reacts with free sulphydryl groups to form fluorescent derivatives (17). GSSG content was assessed by enzymatic procedure using a l-glutathione reductase/NADPH system (18) to convert GSSG to GSH before NPM derivatization. 2-Vinylpyridine was used to prevent GSH from derivatization before its enzymatic conversion. The protein content was determined according to Lowry (19).

Statistical analysis

The comparison of adduct levels in nuclear and mitochondrial DNA in the same samples were done by the paired t-test. All the other comparisons were done by Mann–Whitney U-test or Rank Sum test.

Results

Etheno-DNA adducts

In the liver, etheno-adduct levels were measured in nuclear and mitochondrial DNA. In untreated 6-week-old rats both etheno-adduct levels (means per 108 parent nucleotides) were higher in mitochondrial DNA compared with nuclear DNA (eDA: 3.2 versus 0.6 and eDC: 25.4 versus 3.3) (Figure 1A).

Both adduct levels in nuclear DNA peaked in 8-week-old rats and in mitochondrial DNA adducts at 12 weeks of age and then decreased at later ages (Figure 1A). When compared with age-matched controls, 2 weeks of curcumin treatment increased eDA levels ~20 times in nuclear DNA and 3 times in mitochondrial DNA (Figure 1B); similarly, eDC levels were increased 10 times and 4 times, respectively. In curcumin-treated rats the adduct levels remained elevated in all age groups except in nuclear DNA of 32-week-old rats where the levels declined (Figure 1B).

Copper and iron content

The content of copper and iron in LEC rat liver and plasma is compared with matched controls, 2 weeks of curcumin treatment increased eDA levels ~20 times in nuclear DNA and 3 times in mitochondrial DNA (Figure 1B); similarly, eDC levels were increased 10 times and 4 times, respectively. In curcumin-treated rats the adduct levels remained elevated in all age groups except in nuclear DNA of 32-week-old rats where the levels declined (Figure 1B).

© 2019 John Wiley & Sons, Inc., Carcinogenesis, 26, 1304–1311
organs investigated; the highest concentration was found in liver, followed by plasma. The amount of iron increased in liver but only marginally in plasma. The major increase of both metals was found in the liver between 8 and 12 weeks: in plasma, the increase of copper was observed from 12 weeks onwards. Treatment of curcumin did not affect the metal concentrations in plasma and liver.

**Apoptosis and CD95L expression**

To assess the temporal and spatial distribution of apoptotic cells with DNA strand breaks, we performed TUNEL staining using cryo sections of liver samples from LEC rats. As shown in Figure 3A, a few apoptotic cells were detected in the livers of 6-week-old LEC rats. To analyze the expression of CD95L mRNA, we carried out in situ hybridization using CD95L antisense oligo-DNA probes. The expression of CD95L mRNA was observed in close proximity to TUNEL+ apoptotic cells at all five time points. This indicates the presence of functional CD95L in the hepatocytes of LEC rats. The number of TUNEL+ cells decreased in 8-week-old rat liver compared with 6-, 12- or 16-week-old rats reaching the highest value at 12 weeks (Figure 4). Enhanced expression of CD95L was spatially and temporally associated with the induction of apoptosis at all time points (representative photomicrographs, Figure 3C–D). The expression of CD95L and the amount of TUNEL+ hepatocytes was slightly higher in the livers of curcumin-fed LEC rats except at 12 weeks of age (Figure 4).

**GSH, GSSG, ASAT and ALAT**

At 12–32 weeks an increase was observed for both GSH and GSSG. The GSH/GSSG ratio was not changed over time. Intake of curcumin had no effect on the GSH and GSSG levels in the liver (Figure 2E and F). In order to monitor inflammatory reactions, ASAT- and ALAT-activities in plasma were measured. Between weeks 8 and 12, the levels of ASAT in plasma of control LEC rats was increased 5-fold and ALAT 24-fold. The feeding of curcumin led to a small reduction in the levels of ASAT and ALAT, which was found to be significant only after 32 weeks (Figure 2G and H).

**Discussion**

**Oxidative stress, LPO-derived DNA adducts and liver injury**

In LEC rat liver a reduced catalase activity, an increase in H₂O₂ concentration, formation of hydroxyl radicals and LPO (measured as TBARS) have been described (20). Our present results on adduct levels in hepatic nuclear DNA reconfirm these and our earlier observation (10). Now we demonstrate for the first time the presence of both etheno-DNA adducts, εdA and εdC in mitochondrial DNA of LEC rats. These DNA adduct levels were highest when copper levels were found to be the highest. The WD-protein, a copper transporting ATPase has been putatively located on mitochondrial membrane and is thought to be involved in mitochondrial copper transport (21). Mitochondria contribute inter alia to programmed cell death.
Increased etheno-DNA adduct levels in mitochondrial DNA may cause mutations, resulting in impaired respiratory-chain function, thus contributing to hepatocellular dysfunction. In LEC rats, copper accumulation occurred predominantly in lysosomes, but also in mitochondria (22). Hepatic mitochondria from WD patients showed increased LPO, and severe mitochondrial dysfunction (21-24) along with increases of mitochondrial copper and of oxidative damage in the liver.

Etheno-DNA adducts probably play a causal role in the initiation and progression of liver carcinogenesis as they produce various types of base-pair substitution mutations in prokaryotic organisms and mammalian cells. edA, edC and N2,3-ethenedeoxyguanosine, which is also formed from LPO products in vivo, can lead to specific transitions and transversions (25-29). These promutagenic properties of etheno-bases strongly implicate them in the initiation of carcinogenesis by vinyl chloride (30), by urethane (ethyl carbamate) (31) and other etheno-adduct forming chemicals. Incorporation of a single edA in either DNA strand transfected in HeLa cells showed a similar miscoding frequency and was more mutagenic than 8-oxo-deoxyguanosine (32). Reduced base excision

Fig. 2. Copper and iron contents of liver (A,B) and plasma (C,D); GSH and GSSG in liver homogenate (E,F); activity of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) in plasma (G,H). The values of copper and iron were determined as mg/ml plasma and converted to µg/g dry weight on the basis of 80 g dry mass per liter of plasma. Black bars represent the values from control animals; light grey bars represent values from curcumin-treated animals.
repair of oxidative DNA damage has been reported in LEC rat liver at the early and chronic hepatitis phase up to 40 weeks of age (33). Such impaired repair may contribute to the persistence of etheno-DNA adducts detected in the early age groups (8- and 12-weeks old) of LEC rats.

Interplay between apoptotic events and DNA adduct formation

Using the TUNEL assay, we observed a trough of the apoptotic DNA fragmentation to coincide with a peak of nuclear etheno-DNA adduct levels, whereas the peak of apoptotic events coincided with the highest mitochondrial DNA adduct levels (Figure 5). This suggests a time window of adduct accumulation in nuclear DNA as the damaged cells may not be removed by apoptosis but are retained as ‘initiated cells’, thus starting the process of carcinogenesis in this model. The subsequent increase of apoptotic rate may incur owing to increased mitochondrial DNA damage paralleled by an increase in compensatory cell division of mutated (initiated) cells. Jia et al. (12) also reported high proliferation rates and relatively low apoptosis in LEC rats occurring in the liver at an early age. Kato et al. (34) found a higher apoptotic level in hepatocytes of 15-week-old LEC rats fed a normal diet in comparison with low apoptotic rate in an iron-deficient group, indicating that accumulated hepatic iron possibly induced apoptosis via radical formation before the onset of the hepatitis.

In our experiments we observed a parallelism in CD95 expression, apoptosis and mitochondrial DNA damage in

Fig. 3. TUNEL staining (A,B) and CD95L expression (C,D) in livers of 6- and 12-week-old control LEC rats and CD95L expression (E,F) in curcumin-treated rats of the same ages. CD95L mRNA (red) in situ hybridization was combined with TUNEL assay (green) for apoptotic cells. Nuclei were stained with Hoechst 33342 (blue) and viewed with a confocal laser scanning microscope.
the LEC rat liver, indicating an interlinked dual pathway of apoptosis induction. The importance of the CD95 system in pathophysiology and homeostasis of the liver is well documented (35,36). In different forms of liver diseases, including viral infections, alcoholic liver damage, hepatocellular carcinoma and WD, we have described that deregulation of CD95/CD95L is causally involved in disease progression (16,35,37). In chronic alcoholism a dual mechanism of hepatic oxidative stress and CD95 ligands upregulation (38). Furthermore, it has been shown in HeLa cells that expression of CD95 is enhanced by dysfunctional mitochondria. Cell lines with mitochondrial DNA carrying a point mutation or a large-scale deletion exhibited cell death when treated with an antibody against CD95, compared with none in wild types, indicating that mitochondrial DNA integrity is essential to avoid CD95-mediated apoptosis (39).

The key observations of our investigation in LEC rats with respect to initiation and temporal progression of liver carcinogenesis are summarized in Figure 6. We demonstrated for the first time elevated etheno-DNA adduct levels in mitochondrial DNA as an early damage in copper-induced hepatocarcinogenesis. The nuclear DNA adducts were highest at the time point of reduced apoptosis suggesting that more damaged hepatocytes escaped apoptosis. Subsequent compensatory cell divisions may fix the damage leading to mutation and genomic instability. At age beyond 12 weeks a decline of apoptotic rate provides another window for accumulating such altered cells before clonal expansion to carcinoma.

Role of hepatic copper, iron and GSH in tissue injury

The content of copper in LEC rat organs found in this experiment was distinctly higher than reported earlier (40); at the age of 8 and 32 weeks, the level in liver was about two times higher. These findings would also be consistent with the observed earlier onset of DNA damage than reported in the literature (41). The iron content was found to be in the same range as described for liver and plasma (34). Whereas in the LEC rat model, the role of copper causing oxidative damage and tumor growth in liver is well supported, the effect of iron is discussed controversially. Sugawara and Sugawara (42) found an earlier onset of hepatitis by an iron-deficient diet, which they explained by an increased hepatic copper deposit when fed an iron-deficient diet. In contrast, Kato et al. (34) proposed that an iron-deficient diet may protect against liver injury. The activities of ASAT and ALAT were in the same order of magnitude as described (43), but again the maximal values were shifted to 12 weeks, indicative of an earlier onset of the liver damage. GSH levels in the liver of our LEC rats did not differ from that in SD rat and LEA rat strains (44–46), whereas the oxidized form, GSSG, was increased to an unusually high level relative to GSH. In common rat strains, the GSSG level is around one-tenth of GSH, but in our rats it was found to be one-third of GSH. This reflects the activity of Cu²⁺, oxidizing GSH to GSSG under simultaneous reduction to Cu⁺. Additionally to this consumption of GSH, Cu⁺ forms a stable complex with the remaining GSH (47,48), which may interact with DNA directly to induce strand breaks (41). Therefore, in the copper overloaded LEC rat the cellular de novo synthesis of GSH may not be sufficient to counteract copper-induced toxicity.

Enhancing effects of dietary curcumin on hepatic DNA adduct levels as clue for the lack of liver injury protection in LEC rats

In our previous study (9), contrary to our expectation curcumin did not protect LEC rats from liver tumorigenesis caused by the inborn gene defect. Neither the body weight nor the death rate by acute liver failure after 12–13 weeks differed in the control and curcumin-fed groups. The median survival time in the long-term experiment was in fact 12% shortened by curcumin in the diet. The main target organs, liver and kidneys, did not show any difference with respect to tumor yield and histological classification. In order to elucidate the mechanisms of curcumin-induced toxicity in LEC rats, which contrasts its well established cancer protecting effects in other rat and mice models (reviewed in 9), further investigations were performed in LEC rats, fed 0.5% curcumin in the diet. In keeping with the lack of tumor protection, curcumin treatment did not lower etheno-adduct formation in LEC rats but increased εdA levels ~20 times in nuclear DNA and three times in mitochondrial DNA; similarly, εdC levels in the two DNAs were increased 10 times and four times, respectively after only 2 weeks of treatment, suggesting an early and strong induction of oxidative stress in curcumin-fed animals. Moreover, adduct levels

![Graph](https://example.com/graph.png)

Fig. 4. Quantitative analysis of TUNEL⁺ cells in the livers of LEC rats after 6, 8, 12, 16 and 32 weeks. Values are expressed as mean ± SD. TUNEL⁺ cells per 200 nuclei were counted in four microscopic fields.

![Graph](https://example.com/graph.png)

Fig. 5. Age-dependant changes in nuclear and mitochondrial etheno-DNA adduct levels in relation to apoptotic events in untreated LEC rat liver.
did not decrease in curcumin-treated animals over time as seen in controls, suggesting the induction of persistent oxidative stress by curcumin, probably causing the shorter survival time of curcumin-treated rats (9). The trend of an overall higher expression of CD95 and a higher rate of apoptosis in the curcumin-fed animals are probably manifestations of a higher level of toxicity induced by curcumin in the LEC rat liver.

**Mechanism of copper-dependent pro-oxidant activity of curcumin**

Research over the last decades has indicated that curcumin acts as a potent antioxidant and anti-inflammatory agent, capable of suppressing tumor initiation, promotion and metastasis in several systems. Pharmacologically, curcumin has been found to be safe and human clinical trials indicated no dose-limiting toxicity when administered at doses up to 10 g/day (reviewed in 49). However, recent studies have presented evidence that curcumin can also induce DNA damage in the presence of Cu(II) and that this reaction is catalyzed by the formation of reactive oxygen species (50, 51). Experiments in vitro showed that (i) curcumin in the presence of Cu(II) causes strand scission in DNA and such degradation is mediated by reactive oxygen species; (ii) Cu(I) appears to be an essential intermediate in the DNA cleavage reaction; and (iii) curcumin is capable of binding to DNA. Probable mechanisms for DNA damage induced by the curcumin/Cu(II) complex is compiled in Figure 7. It appears to involve both the hydroxyl radical as well as singlet oxygen. It was shown that curcumin/Cu(II) generates O$_2^-$ which in turn can give rise to both OH$^*$ and $^1$O$_2$ by a modified Haber-Weiss reaction. O$_2^-$ can give rise to the formation of H$_2$O$_2$ which, through the Fenton reaction, may generate OH$^*$ involving the reduction of Cu(II) to Cu(I) by O$_2^-$. A recent study (52) confirmed that curcumin acts as a pro-oxidant by forming reactive oxygen species through the reduction of Cu(II), causing DNA strand breaks and also the formation of 8-hydroxy-2'-deoxyguanosine in DNA. From previous work it has become clear that several of the

---

**Fig. 6.** Key observations in our study linked to liver pathogenesis and carcinogenesis in LEC rats. Parameters in the shaded boxes were measured in this study. *Age 52 weeks was the time point where moribund animals were killed and liver tumors were detected in our parallel study (9).**
biologically important anti-oxidants are themselves capable of generating reactive oxygen species in the presence of transition metals, such as copper and iron ions (53). These included antioxidants present in extracellular fluids (ascorbate and uric acid) and in dietary plant materials such as flavonoids, other plant phenolics and curcumin (our study). In cells, most transition metal ions are safely bound to storage and transport proteins (ferritin, transferrin, lactoferrin and ceruloplasmin) and are not available to catalyze free radical reactions. Hence in vivo, the antioxidant properties of these compounds predominate over any pro-oxidant effects. However, pro-oxidant effects gain importance under certain disease conditions such as Wilson’s disease, for which the LEC rat is a model. There it was shown that total Cu levels in the liver decreased at the onset of jaundice in LEC rats, while free Cu levels in the liver remained high (54).

Taken together, these reports are consistent with our proposal (Figure 7) that increased levels of redox-active free Cu ions in the livers of LEC rats catalyze Haber–Weiss and Fenton-type reactions, thus producing hydroxyl radicals that attack DNA and initiate LPO. These processes are further enhanced by dietary curcumin generating an excess of etheno-adducts in nuclear and mitochondrial DNA (our study). This may imply that curcumin treatment for preventative measures of patients with cancer-prone liver diseases, such as metal storage diseases and hepatitis C virus infections (55) would be contra-indicative: in the latter, it was shown that accumulation of copper in the liver and hepatic injury, i.e. progression to hepatic fibrosis, was positively correlated (56).

Acknowledgements

We thank S.Fuladlfsch for skilled secretarial help. This work was supported in part by the Deutsche Forschungsgemeinschaft SFB 601 and SFB 553.

Conflict of Interest Statement: None declared.

References


