Relationship between metallothionine and zinc in the protection against DNA damage in zinc-treated Long-Evans Cinnamon rat liver

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The aims of the work presented here were to determine the effect of long term treatment with zinc (Zn) on both total metallothionine (MT) and, in particular, oxidized MT (MTox) concentrations in Long-Evans Cinnamon (LEC) rat liver. We also evaluated semi-quantitatively the cell death index using TUNEL assay as it is a useful method to localize the nuclear fragmentation occurring in oxidative stress conditions. The results demonstrate there were no statistically different MT concentrations between Zn-treated and untreated rats, whereas the Zn treatment was very effective in reducing the percentage of oxidized MT (MTox). MTox is not able to bind metals, so it does not perform its "scavenger" action against copper (Cu) accumulation in LEC rats. The intensity and quantity of fluorescent staining observed in untreated rat sections decreased compared to the treated ones. These findings suggest that in LEC rats one of zinc's roles is to protect from oxidative stress, however, its mode of action remains partially unknown: a hypothesis is competition for Cu binding sites. A new insight is that Zn induced MT can protect efficiently against DNA damage by free radicals.

Key words: metallothionein, copper, zinc, LEC rats

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Long-Evans cinnamon (LEC) rats are an established animal model for Wilson's disease, a hereditary disease caused by a disorder of copper (Cu) metabolism. These animals have a mutation in the gene homologous to the human Wilson's disease and show many features of the disease such as elevated hepatic Cu levels that accumulate mostly in the chemical form of Cu-metallothionein (MT) (Klein et al. 1999). However, when Cu accumulates to levels that exceed the capacity of a cell to synthesize sufficient MT the metal liberated from oxidized-MT (MTox), in which disulfide bonds have been formed, works as a catalyst in the Fenton reaction producing hydroxyl radicals (Suzuki et al. 1999). In this way, MTox is not able to bind any metals, so it does not perform its "scavenger" function. It has also been suggested that free metal ions such as Cu, which is involved in the production of free radicals, plays an important role in the regulation and induction of the apoptotic process. Once the Cu accumulation in the liver exceeds its ability to induce sufficient MT for Cu detoxification, the cupric ions (Cu^{+}) induces hepatic dysfunction, and a large amount of Cu-MT accumulated in the liver is then released into the blood.

Therapy for Wilson's disease (WD) is based on the administration of Cu chelators or competitors such as Zn (Friedman 1993; Sturniolo et al. 1999).

In this paper, we carried out a study aimed at analyzing the effects of 60 days treatment with Zn acetate on total MT and MTox which is an important parameter in evaluating the cytotoxicity of Cu. We also determined the DNA damage using TUNEL assay as a useful method to localize the nuclear fragmentation occurring in oxidative stress conditions in LEC rat liver, an animal model of WD.

Materials and Methods

Animals and Treatment

Twenty six male LEC rats (35 days old, 90g body wt) from Charles River Japan (Tokyo, Japan) were available for this investigation. They were kept on a standard laboratory diet (Morini MIL GLP diets) containing 11.7 mg Cu/Kg and 67.5 mg Zn/Kg and deionized water. They were housed in comfortable cages at 20° C with a 12-h light-dark cycle. Zn acetate supplement was dissolved in 2% glucose solution in distilled water.

We divided the twenty six rats as follows: - thirteen rats received an oral dose of Zn acetate 50 mg/ml by gavage daily for 60 days (treated rats); - thirteen rats received an oral dose of glucose solution 0.02 mg/mL by gavage daily for 60 days (untreated rats).

Rats were sacrificed under diethyl ether and the liver was quickly removed, washed with cold 0.9% NaCl, frozen with liquid nitrogen and stored at -80° C. The tissue was used for TUNEL assay and MT determination.

All procedures on the laboratory rats were carried out according to the regulations of the National Institute's Health Guide for the Care and Use of Laboratory Animals.

Determination of MT content

A part of the hepatic tissue was homogenized in 4 volumes of 20 mM Tris-HCl buffer, pH 8.6, supplemented with 0.006 mM leupeptine, 0.5 mM PMSF (Phenylmethylsulphonylfluoride) as antiproteolitic agents, and 0.01% β-mercaptoethanol as reducing agent. The homogenate was centrifuged at 4°C at 50400 g for 50 min, and the resulting supernatant was used for MT quantification using the silver saturation method (Scheuhammer and Cherian 1991) by atomic absorption spectrophotometry (Perkin-Elmer mod. 4000). The MT amounts in the supernatant were expressed relative to total soluble cell protein assayed by the Lowry method (Lowry et al. 1951).

The reversal of metal ion loss from MT was attempted in accordance with a previous report (Irato et al. 2001), with some modifications. Part of the supernatant was incubated in anaerobic conditions (N_2) for 15 min with 10 mmol/L 2-mercaptoethanol, and then incubated with 6 mmol/L $ZnCl_2$ for 2 h to reconstitute Zn-MT. The MTox content was determined from the difference between the values obtained by the reduction of oxidized MT and those obtained without reduction.

Tunel assay

TdT-mediated fluorescein-dUTP nick-end labeling was used for the detection of DNA strand breaks in LEC rat liver. Frozen hepatic tissue was cut with a Jung CM-1800 cryostat microtome at –20°C; all sections (5 μm) were mounted on slides and fixed in 4% paraformaldehyde for 30 minutes at room temperature and were washed in TPBS supplemented with 10 mM of sodium citrate for 5 minutes at 4°C. The subsequent staining was performed according to manufacturer's instructions (In Situ Cell Death Detection Kit; Roche, Milan, Italy). The specificity of the staining reaction was checked in a negative control experiment, in which TdT was omitted from the procedure. The sections were finally examined under a Leica DMR microscope, and the images were acquired and examined using a charge coupled device camera (Leica DC), model 300F. Images were examined using Casting Imagent for Windows software. The total cells and fluorescent cells were counted for each field examined (at least 10 fields per plate). The percentage of TUNEL-labeled cells was calculated as the number of FITC-stained cells divided by the total number of cells counterstained with Hoechst. The data from at least three independent experiments were collated and standard errors of measurement calculated.

Statistical analysis

All measurements were made in duplicate and the results are reported as means ± SD. Statistical analysis was performed with the Primer statistical program. The data were analyzed with one-way analysis of variance (ANOVA) with a Student-Newman-Keuls follow-up test. In this analysis, treated samples were compared to untreated. The significance level set at $p<0.05$.

Results

Quantification of MT and detection of cell death

There were no statistically different values between the Zn-treated and untreated group in the hepatic MT concentrations (15.2±2.5 vs 13.1± 3.3). In contrast, the percent of MTox in the untreated group was higher than that in the treated one. It was 40.2% whereas the treated group expressed

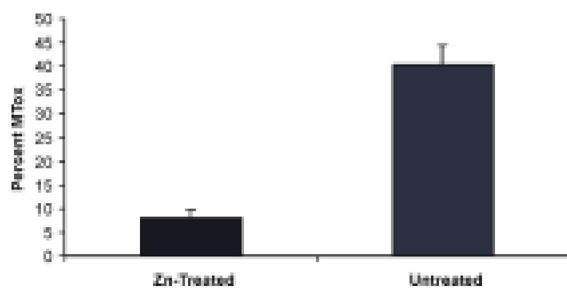

Figure 1. Percentage of oxidized MT (MTox) in liver tissues of Zn-Treated and Untreated Long-Evans Cinnamon (LEC) rats. The results are expressed as mean ± SD (n =13 for the Zn-treated group; n=13 for the Untreated group). The comparison between Zn-Treated and Untreated rats were statistically different (p< 0.05).

8.2% of MTox (Figure 1).

TUNEL staining revealed higher scattering of apoptotic parenchymal cells in liver sections of untreated animals with respect to treated ones. The TUNEL labeling observed in the untreated rats exceeded 70% of cells whereas in the treated rats it was only 4%. The untreated group sections showed a highly fluorescence signal that highlighted the nuclei diffusely and more intensely. In contrast, the intensity and quantity of fluorescence decreased in the treated group (Figures 2A, B). It is also important to point out that in the untreated sections the number of total cells was much lower with respect to the treated ones. Moreover, by a cytological analysis, the untreated sections showed not only morphologic features typical of both apoptotic and necrotic cells such as shrinkage, apoptotic bodies and compactation of chromatin at the nuclear periphery but also the loss of membrane integrity.

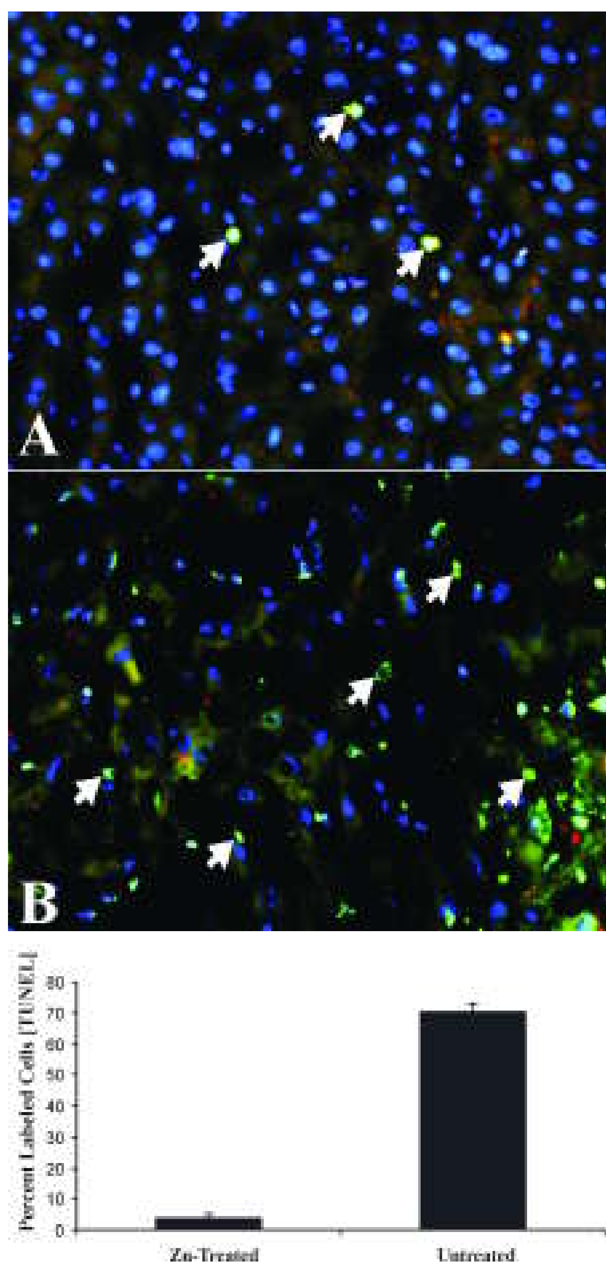

Figure 2. Localization of apoptotic cells (arrows) using TUNEL assay in liver tissues of Zn-Treated (A) and Untreated (B) groups. The figures show hepatic cells stained with fluorescein-dUTP (green) and counterstained with Hoechst 33258 (blue). x40 objective. The results are expressed as mean ± SD (n =13 for the Zn-treated group; n=13 for the Untreated group). The comparison between Zn-Treated and Untreated rats were statistically different (p< 0.05).

Discussion

In this work we studied the relationship between MT and Zn in Zn-treated LEC rat liver in relation to apoptotic processes and MTox expression. The role of Zn in protecting biological structures from excess of heavy metals ions may be due to several factors such as the maintaining of an adequate level of MTs, which are also free radical scavengers (Irato et al. 2001). Moreover, Zn has multiple implications in cellular metabolism, including cell death by apoptosis. The influence of Zn on apoptosis is a well known phenomenon. In both *in vitro* and *in vivo* models, Zn supplementation prevents apoptosis induced by a variety of agents (Thomas and Caffrey 1991; Matsushita et al. 1996). In this context, we investigated the effect of competition between metals in relation to apoptotic processes and MTox expression in LEC rat liver. In our sections, we observed moderately bright fluorescence in treated tissue in compar-

ison with the high intensity and quantity of the green fluorescence of the untreated tissue stained with TUNEL. In fact, in the LEC rat liver, apoptosis is considered to be a major cause of cell death, as substantiated by an increased number of Cuouncilman bodies (Kasai et al., 1990) and by the TUNEL method (Yamate et al., 1999). We also strongly support the hypothesis that, in LEC rats, MT saturated with Cu in hepatocytes may act, not as a protective anti-oxidant, but primarily as a pro-oxidant that promotes the induction of cellular damage leading to apoptosis, as suggested by Deng et al. (1998) in tx (toxic milk) mice, another animal model for the study of WD. In contrast, we believe that the treatment with Zn exerts a protective effect probably attributed to the capacity of this metal to compete for Cu binding sites in proteins and DNA (Bray and Bettger, 1990; Powell, 2000) and to reduce Cu in tissue. In fact the amount of Cu in the Zn-treated rats is 1.6 lower than that untreated one. A new insight is that Zn induced MT can protect efficiently against oxidative stress (Chubatsu and Meneghini 1993). However, if Zn is known to protect from oxidative stress-induced DNA damage (Leccia et al. 1999), its mode of action remains partially unknown. We believe that the protective effect of Zn against oxidative stress is mainly by the induction of MT, rather than a direct action. Our findings also show that both Cu accumulation and Zn supplementation increases tissue MT content, whereas only Zn treatment decreases MTox in LEC rat liver. The elevated amount of MT, induced by Zn and Cu, is probably the expression of a defense mechanism against Cu accumulation while the low levels of MTox can be considered as an index of low oxidative stress resulting in low cell death. All isoforms of zinc-bound MTs (Zn-MTs) are also considered antioxidant agents because the zinc-sulphur cluster is sensitive to changes in the cellular redox state (Mocchegiani et al. 2001). Oxidizing sites induce transfer of Zn from its binding sites in MTs to those of lower affinity in other proteins (Maret et al. 1998). Therefore, the redox properties of Zn-MTs are crucial for their protective role against cytotoxic effect of reactive oxygen species (ROSs) and heavy metal toxicity (Palmiter 1998).

The findings of this work represent an initial approach to the research on the antioxidant properties of Zn in relation to MTox expression and apoptotic processes. Future experiments will be performed using MT null mice cell in order to study the direct action mechanism of Zn. We believe that the study of the relationships between trace elements and their binding proteins will be useful in improving our knowledge on the pathophysiology of WD.

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Cellular localisation of the anti-cancer drug camptothecin in *Camptotheca acuminata* Decne (Nyssaceae)

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In *Camptotheca acuminata*, we studied the cellular sites of accumulation of the alkaloid camptothecin (CPT), in both plants grown in the field and those grown in a greenhouse, subjecting the latter to stress (i.e., draught, nutritional deficit, and pruning). Fresh sections of the leaf, stem, and root were analysed for the presence of CPT by examining the autofluorescence that the CPT molecule emits when exposed to UV radiation. In the plants grown in the field, CPT was observed only rarely. In the greenhouse plants, CPT had accumulated in crystalline form in the vacuole of specialised cells (i.e., segregator idioblasts), which were not morphologically distinguishable from the cells of the surrounding tissues. In the organs examined, the segregator idioblasts were localised in parenchymatic and epidermal tissues. CPT crystals were also detected in the glandular trichomes on both the stem and leaf.

Key words: *Camptotheca acuminata*, camptothecin, indole alkaloids, histochemistry, fluorescence analysis.

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Camptothecin (CPT) is a monoterpene indole alkaloid produced by the Chinese tree *Camptotheca acuminata* Decne (Nyssaceae) and first isolated in 1966 by Wall and coworkers. CPT is known for its remarkable anti-cancer activity, which results from its ability to inhibit the eukaryotic DNA topoisomerase I (Kjeldsen *et al.*, 1992). It also inhibits retroviruses such as the human immunodeficiency virus (HIV) (Priel *et al.*, 1991), apparently as a result of the inhibition of Tat-mediated transcription (Li *et al.*, 1994).

Although much is known about the pharmacological effects of CPT, little is known about its biosynthetic pathway (Silvestrini *et al.*, 2002) or the cellular sites of biosynthesis and accumulation. Recent studies have shown that the biosynthesis and accumulation of alkaloids are tissue-specific and cell-type-specific (De Luca and St-Pierre 2000, Samanani *et al.*, 2002). One of the species that has been most commonly used in the study of alkaloid biosynthesis and accumulation is *Catharanthus roseus*, which produces more than 100 different terpenoid indole alkaloids (St-Pierre *et al.*, 1999), of which the most well known are vinblastine and vincristine. In 1976, Yoder and Mahlberg, using histochemical analysis, observed that the alkaloids in *C. roseus* had accumulated only in "specialized parenchymatic cells" and in laticifers. Immunocytochemical analyses for localising the enzymes involved in alkaloid biosynthesis in *C. roseus* have shown that the site of biosynthesis can differ from the site of accumulation. Moreover, the various stages of alkaloid pathways occur in different tissues. For example, the enzymes involved in the early stage of the vindoline pathway (i.e., tryptophan decarboxylase and strictosidine synthase) are only found in the epidermis of stems, immature leaves, and flowers, and in the apical meristem of the root tips, whereas the enzymes involved in the late stages (i.e., deacetylvindoline-4-hydroxylase and deacetylvindoline-4-O-acetyltransferase) are limited to the laticifer and idioblast cells

of the leaf, stem, and flower bud (De Luca and Laflamme, 2001). At the subcellular level, alkaloids actively accumulate in the vacuole, although their biosynthesis generally occurs in the cytoplasm or in some other cellular compartment (Wink, 1997).

In *C. acuminata*, chemical analyses have demonstrated that CPT accumulates in all organs of the plant. The highest levels of CPT are found in the first young leaves near the shoot apex, and as the leaves mature, the CPT concentration decreases rapidly. In the other vegetative organs, the correlation between the level of CPT and the degree of organ development is not clear (Lopez-Meyer *et al.*, 1994). CPT concentrations have also been observed to vary significantly by specific genotype, season, and plant age (Liu *et al.*, 1998) and depending on whether or not the plant is subjected to environmental stress, specifically, pruning (Liu *et al.*, 1999) and drought (Liu, 2000).

In the present study, we conducted a histological analysis of the vegetative organs of *C. acuminata* and investigated the cellular localisation of CPT in these organs through autofluorescence. The study was conducted both on plants grown in the field and those grown in a greenhouse, subjecting the latter to stress.

Materials and Methods

Plant material

Leaf, stem, and root samples were collected in the Summer of 2003 from three-year-old *C. acuminata* trees, ranging from 1.5 to 2 m in height, which had been grown from seed either in the field or in a greenhouse at the Botanical Garden of "La Sapienza" University (Rome, Italy). The greenhouse plants were grown in 10 L pots with commercial soil and were subjected to the following conditions of stress: beginning in March 2002, they were watered infrequently (i.e., every 15 days) and with scarce amounts of water; the soil was never fertilised; and in March 2003 they underwent drastic pruning. The plants grown in the field were instead subjected to the climatic conditions of the city of Rome, which are illustrated in the Bagnouls-Gaussen's diagram in Figure 1. In the Spring and Summer, they were watered every day.

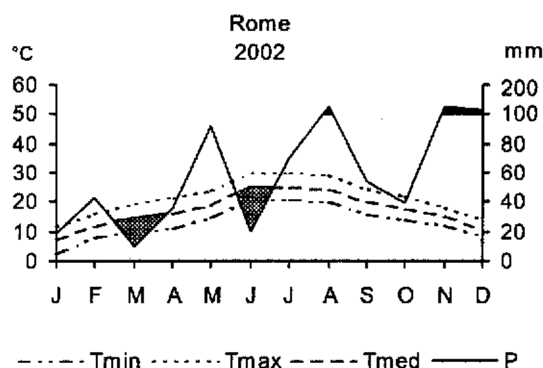

Figure 1. Bagnouls-Gaussen's diagram of the city of Rome: monthly temperature and precipitation in the year 2002.

Chemical analyses

Quantitative chemical analyses were conducted on leaves (i.e., the organ with the greatest CPT accumulation, according to Lopez-Meyer *et al.*, 1994) taken from *C. acuminata* plants. The samples were collected from ten individuals chosen at random (five grown in the field and five in the greenhouse). The samples were extracted following the protocol of Liu *et al.* (1999) and analysed with high-performance liquid chromatography (HPLC). A Waters 2487 Dual λ Absorbance Detector was used, coupled with a 5-μm Symmetry C_{18} 4.6 ×150 column (Waters) operated in the reversed-phase mode using an $MeOH$-H_2O-$MeCN$ mobile phase. The linear gradient elution profile started with $MeOH$-H_2O-$MeCN$ (5:55:40) and ended with $MeOH$-H_2O-$MeCN$ (35:25:40) within 15 min. The flow rate was 1 ml/min, and all chromatograms were plotted at the absorption wavelength 245 nm by a UV detector (Waters). Injection volumes of both standard CPT (Acros Organics) and unknown samples were 50 μL throughout the analysis. CPT was identified by direct comparison of the UV spectrum and retention time of the standard. Standard curves for the quantitative analysis of CPT, run daily, were constructed by plotting the peak area ratios against amount ratios of CPT to the internal standard. The CPT concentrations in the unknown samples were calculated using the curves developed on the standard.

Histologic and histochemical analyses

Leaf, stem, and root samples were embedded in agar (4%) and sectioned (~30 μm thickness) with a vibratome (TPI series 1000). Fresh sections were examined with a Zeiss microscope (Axioscop 2