

Konstantinos N. Tzirogiannis
Georgios I. Panoutsopoulos · Maria D. Demonakou
Rosa I. Hereti · Katerina N. Alexandropoulou
Aristidis C. Basayannis · Michael G. Mykoniatis

Time-course of cadmium-induced acute hepatotoxicity in the rat liver: the role of apoptosis

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Abstract Exposure to toxic metals and pollutants is a major environmental problem. Cadmium is a metal causing acute hepatic injury but the mechanism of this phenomenon is poorly understood. In the present study, we investigated the mechanism and time-course of cadmium-induced liver injury in rats, with emphasis being placed on apoptosis in parenchymal and nonparenchymal liver cells. Cadmium (3.5 mg/kg body weight) was injected intraperitoneally and the rats were killed 0, 9, 12, 16, 24, 48 and 60 h later. The extent of liver injury was evaluated for necrosis, apoptosis, peliosis, mitoses and inflammatory infiltration in hematoxylin–eosin-stained liver sections, and by assaying serum enzyme activities. The number of cells that died via apoptosis was quantified by TUNEL assay. The identification of nonparenchymal liver cells and activated Kupffer cells was performed histochemically. Liver regeneration was evaluated by assaying the activity of liver thymidine kinase and by the rate of ^3H -thymidine incorporation into DNA. Both cadmium-induced necrotic cell death and parenchymal cell apoptosis showed a biphasic elevation at 12 and 48 h and peaked at 48 and 12 h, respectively. Nonparenchymal cell apoptosis peaked at 48 h. Peliosis hepatitis, another characteristic form of liver injury, was first observed at 16 h and, at all time

points, closely correlated with the apoptotic index of nonparenchymal liver cells, where the lesion was also maximal at 48 h. Kupffer cell activation and neutrophil infiltration were minimal for all time points examined. Based on thymidine kinase activity, liver regeneration was found to discern a classic biphasic peak pattern at 12 and 48 h. It was very interesting to observe that cadmium-induced liver injury did not involve inflammation at any time point. Apoptosis seems to be a major mechanism for the removal of damaged cells, and constitutes the major type of cell death in nonparenchymal liver cells. Apoptosis of nonparenchymal cells is the basis of the pathogenesis of peliosis hepatitis. The first peaks of necrosis and parenchymal cell apoptosis seem to evolve as a result of direct cadmium effects whereas the latter ones result from ischemia.

Keywords Apoptosis · Cadmium · Hepatic regeneration · Necrosis · Peliosis · Rat liver · Toxicity

Introduction

The exposure of human populations to a variety of heavy metals has been a public health concern (Goyer 1996). Cadmium is one of the most abundant non-essential elements due to its immense usage in various industrial applications (Page et al. 1986). The health risk to humans from cadmium intoxication and the toxicity from acute and chronic exposure have been well described (Fowler 1991; Goyer 1996).

The toxicity of cadmium, both in experimental animals and in humans, is influenced by a great number of factors such as the route of administration, the dosage, the chemical form of the metal, the duration of the exposure and the age of the experimental animals, etc. (Casalino et al. 1997). Chronic cadmium intoxication results mainly in renal disease (Kotsonis and Klaassen 1977). Acute cadmium exposure in experimental animals primarily results in the accumulation of the metal in the

K. N. Tzirogiannis · G. I. Panoutsopoulos · R. I. Hereti
K. N. Alexandropoulou · A. C. Basayannis
M. G. Mykoniatis (✉)
Department of Experimental Pharmacology,
Medical School, Athens University,
75 Mikras Asias St.,
115 27 Athens, Greece
E-mail: mmykon@cc.uoa.gr
Tel.: +32-10-7462566
Fax: +32-10-7462554

M. D. Demonakou
Histopathology Laboratory,
Sismanogliou G.D. Hospital,
Sismanogliou 1, Marousi,
Attiki 151 27, Greece

liver and in acute hepatotoxicity (Kotsonis and Klaassen 1977; Dudley et al. 1982).

Histopathological characteristics of cadmium toxicity in the liver include focal, zonal and massive necrosis, fatty infiltration and hepatocyte swelling. Fibrosis, cirrhosis and inflammation have also been observed, and liver damage is often accompanied by leukocyte infiltration (Dudley et al. 1982, 1984).

Apoptosis, also known as abortive mitosis, constitutes a mode of active cell death with cellular and morphological features quite distinct from those of lytic necrosis. Morphological features of apoptosis include chromatin margination along the nuclear membrane, nuclear condensation, budding, karyorrhexis, cell shrinkage and cell fragmentation (Searle et al. 1982). The contribution of apoptosis to the genesis of hepatic trauma due to various hepatotoxins has been evident during the last years and it seems to provide a means of eliminating damaged cells without influencing tissue architecture (Ledda-Columbano et al. 1991; Cascales et al. 1994; Leist et al. 1997; Ray and Jena 2000).

Although apoptosis is a well-defined morphological process, the molecular mechanisms implicated in it are still under investigation. Nucleic acids as well as mitochondria, and especially cytochrome oxidase *c*, are often targets of hepatotoxins, and DNA damage and mitochondrial impairment are thought to be the initiating events in inducing apoptosis (Muller and Ohnesorge 1984; Corcoran et al. 1994; Esteve et al. 1999). Additionally, inflammatory mediators, and especially tumor necrosis factor α (TNF α), are thought to play a major role in inducing apoptotic cell death (Leist et al. 1997; Laster et al. 1998).

Acute cadmium intoxication has been reported to induce dose-dependent apoptosis in the mouse liver (Habeebu et al. 1998). Cadmium also induces apoptosis in isolated bovine liver nuclei, and time- and dose-dependent apoptosis in white blood cells (Lohmann and Beyersmann 1994; El-Azzouzi et al. 1994). In relation to the above, it has been suggested that apoptosis is an important but currently understudied process occurring in acute cadmium-induced hepatotoxicity (Habeebu et al. 1998).

In the present study, we investigated the time-course and mechanism of cadmium-induced hepatic injury in male Wistar rats with emphasis being placed on cadmium-induced apoptosis in liver parenchymal and nonparenchymal cells.

Materials and methods

Experimental animal model

Male Wistar rats, up to five months old, weighing 160–180 g, obtained from the Hellenic Pasteur Institute (Athens, Greece), were used in this study. Animals had free access to food and tap water; they were housed in a temperature controlled room, with a 12 h/12 h light/dark cycle and were handled with human care in accordance with the National Institutes of Health guidelines. Animals were

starved for 12 h before any manipulation, and experiments were performed between 0700 and 0900 hours. Cadmium was injected intraperitoneally at the dose of 3.5 mg/kg bodyweight, under light ether anesthesia (diethyl ether per anesthesia, Codex, Carlo Erba, Milan, Italy). Seven groups of animals (12–14 animals in each) were killed under ether anesthesia 0 (control group), 9, 12, 16, 24, 48, and 60 h later. Blood was collected and livers were removed. A standard portion of the liver was excised and placed in 4% neutral buffered formalin. The rest of the liver was flash frozen in liquid nitrogen and stored at -80°C for subsequent biochemical determinations. One hour prior to being killed, animals were injected intraperitoneally with ^3H -thymidine (Amersham Corp. Buckinghamshire, UK) at the dose of 25 $\mu\text{Ci}/100$ g bodyweight. The number of animal deaths varied at different time points and the mean mortality rate was 46%.

Histopathology

Liver tissues were fixed in 4% buffered formalin for 24 h. Sections 5- μm thick were processed routinely, stained with hematoxylin-eosin (HE), and analyzed for necrosis, peliosis, apoptosis, mitoses, and inflammatory infiltration.

Necrosis was analyzed semiquantitatively with a five-point score for severity: 0 no necrosis, 1 necrosis of 1–5% of liver cells, 2 necrosis of 6–25% of liver cells, 3 necrosis of 26–50% of liver cells, 4 necrosis of > 50% of liver cells.

Peliosis was assessed separately by a semiquantitative method and expressed in a four-degree scale (0, 1, 2, 3): 0 none, 1 one zone of hepatic lobule (approximately 33%), 2 two zones (approximately 67%), 3 all the hepatic acinus (approximately 100%).

Apoptosis was demonstrated: (1) in HE-stained sections by total apoptotic index, and (2) in situ using the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling; YLEM, Rome, Italy) by total apoptotic index, apoptotic index for parenchymal and nonparenchymal liver cells. Hepatic cell sub-populations were discriminated by their localization in the acini, and by the immunochemical detection of vimentin. Apoptotic indices were expressed as the number of positive nuclei per 100 cells.

Mitoses were counted in ten randomly selected high-power fields (HPF) and expressed per 100 liver cells.

Inflammatory infiltration was evaluated in ten HPF and expressed as the number of inflammatory cells per 100 liver cells.

Immunocytochemistry was carried out using the APC detection Kit (NeoMarkers, Fremont, CA, USA) for nonparenchymal cells (vimentin 1:50 plus microwave; NeoMarkers) and for Kupffer cells CD68 (1:50; NeoMarkers). Numbers of positive cells were expressed per 100 liver cells.

Biochemical determinations: serum enzyme activity assays

Blood samples were collected from all animals via cardiac puncture. The biochemical evaluation of liver injury was performed by quantifying serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), using a random-access chemistry analyser (RA-1000; Technicon Instruments Corp., Tarrytown, NY, USA).

Liver regeneration

The rate of liver regeneration was evaluated by the mitotic index and by assaying the enzymatic activity of liver thymidine kinase (TK) and the rate of ^3H -thymidine incorporation into hepatic DNA.

Thymidine kinase enzymatic activity

The enzymatic activity of TK was assayed (Kahn et al. 1988) in triplicate aliquots of each sample. The protein content of each

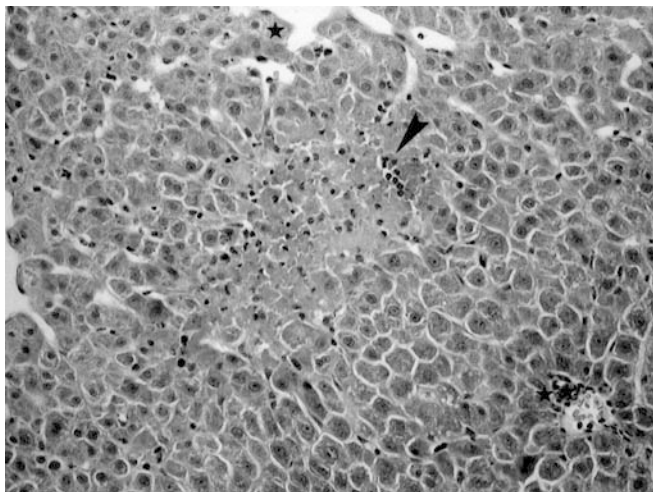


Fig. 1 Zonal necrosis demonstrated (arrowhead) in liver by HE stain, $\times 100$, after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. Zonal necrosis is located in zone 2 of hepatic lobule (asterisk central vein, double asterisk portal tract)

sample was determined (Lowry et al. 1951), and the enzymatic activity expressed as counts per minute, per milligram of protein (cpm/min per mg).

Rate of ^3H -thymidine incorporation into hepatic DNA

The rate of DNA synthesis was evaluated by the uptake of ^3H -thymidine by DNA. Liver DNA was extracted and quantified according to the method of Munro and Fleck (1966) as modified by Kyprianidis et al. (1996). About 250 mg of liver was homogenized in 5 ml cold distilled water, followed by the addition of 2.5 ml cold 0.6 N perchloric acid (HClO_4). The homogenate was left on ice for 10 min and centrifuged at 25,000 g for 20 min at 4°C. Pellets were washed twice with 5 ml 0.2 N HClO_4 and centrifuged at 8,000 g for 20 min at 4°C. The resulting pellets were dissolved in 4 ml 0.3 N KOH, and the preparation was incubated at 37°C for 1 h. After cooling at 4°C, 2.5 ml cold 1.2 N HClO_4 was added, left for 10 min, and the sample centrifuged at 6,500 g for 15 min at 4°C. The supernatant was discarded and the pellets were resuspended in 10 ml 0.3 N KOH and incubated overnight at 45°C until the

precipitate was completely dissolved. An aliquot of each sample was used to determine the amount of DNA by the diphenylamine method (Richards 1974), whereas another aliquot (1 ml) of each sample was added to scintillation vials containing 10 ml scintillation reagent. Samples were counted in a liquid scintillation counter (Wallac LKB 1211, Rackbeta, Sweden). The values were expressed as counts per minute per microgram DNA (cpm/ μg).

Statistical analysis

Data are expressed as means \pm SE. All observations were obtained from at least six animals. The statistical analysis was performed by one-way analysis of variance (ANOVA) and by ANOVA followed by Scheffé's multiple comparison test.

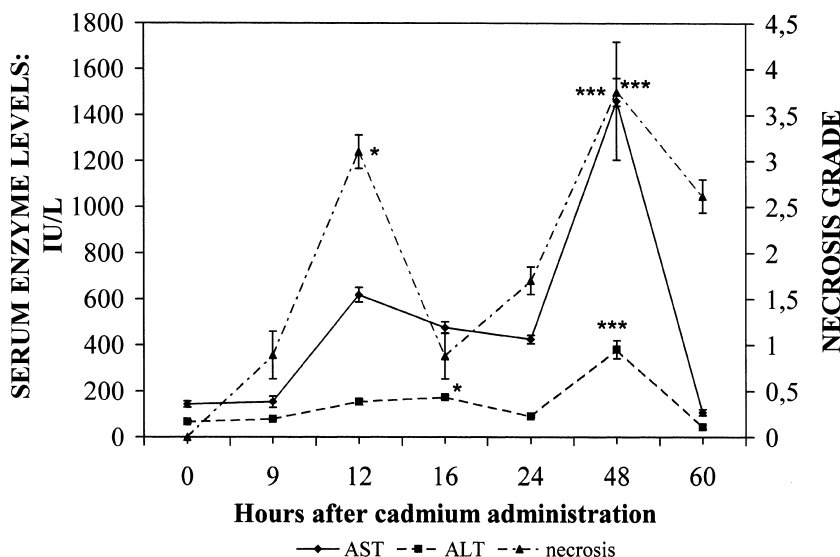
Results

Necrosis was zonal (centrilobular) and mainly located in zone 2 of hepatic acinus (Fig. 1). The kinetics of necrotic cell-death showed a biphasic increase with two peaks at 12 and 48 h with the extent of the lesion being maximal at 48 h (Fig. 2). The serum levels of AST were elevated at 12 h. AST and ALT enzymatic activities peaked at 48 h and were in accordance with the observed peaks of necrosis. AST levels were markedly elevated for all time intervals examined, except those at 9 h, and levels returned to normal at 60 h (Fig. 2).

Apoptosis was present at almost all time points examined and was also mainly observed in zone 2 in the acinus (centrilobular). Apoptosis was observed in necrotic zones, where hepatocytes were mainly affected. Total apoptotic index (HE sections) sharply increased from the value of 0.1 ± 0.03 at 9 h to 1.02 ± 0.11 at 12 h, and peaked to the value of 1.34 ± 0.26 at 48 h, following the same biphasic pattern as necrotic cell death (Fig. 3).

The TUNEL assay (Fig. 4) verified the rapid elevation of the total apoptotic index between 9 h (0.2 ± 0.08) and 12 h (3.22 ± 0.35), and that the index became maximal at 48 h (4.03 ± 0.40) (Fig. 5).

Fig. 2 Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and liver necrosis grade as a function of time after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. Results represent the findings in eight rats killed at 12, 16, 48 and 60 h, and in six rats killed at 0, 9 and 24 h. Values are expressed as means \pm SE. *** $P < 0.001$, * $P < 0.05$, statistically different from control (0 hours)



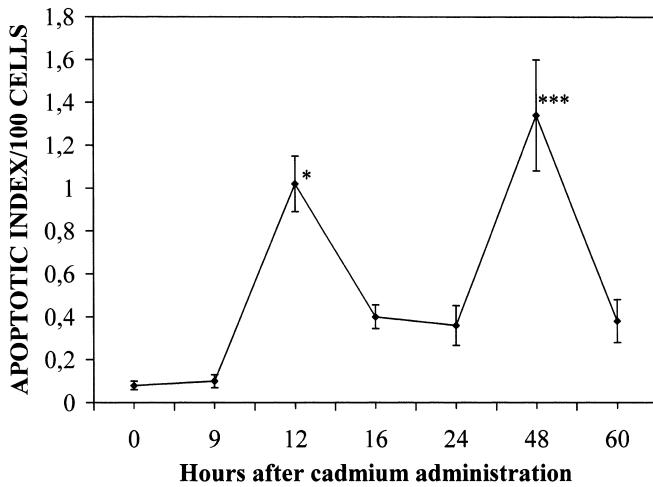


Fig. 3 Total apoptotic index, from HE-stained liver sections, as a function of time after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. Results represent the findings in eight rats killed at 12, 16, 48 and 60 h, and in six rats killed at 0, 9 and 24 h. Values are expressed as means \pm SE. *** $P < 0.001$; * $P < 0.05$, statistically different from control (0 hours)

The apoptotic index was also evaluated for parenchymal and nonparenchymal liver cells. Parenchymal cell apoptosis, as quantified by the TUNEL assay, was maximal at 12 h (2.8 ± 0.34) after cadmium administration (Fig. 5). There was a sharp decrease at 16 h and 24 h, and a second lower peak was observed at 48 h (2.08 ± 0.23) with a subsequent decline at 60 h (0.32 ± 0.075) (Fig. 5).

The kinetics of apoptotic cell death was different for nonparenchymal liver cells (Fig. 6). The apoptotic index was low at 9 h (0.183 ± 0.065), gradually increased from 9 to 48 h and peaked at 48 h (1.95 ± 0.28), while a sharp decrease was observed by 60 h (0.57 ± 0.12) (Fig. 5).

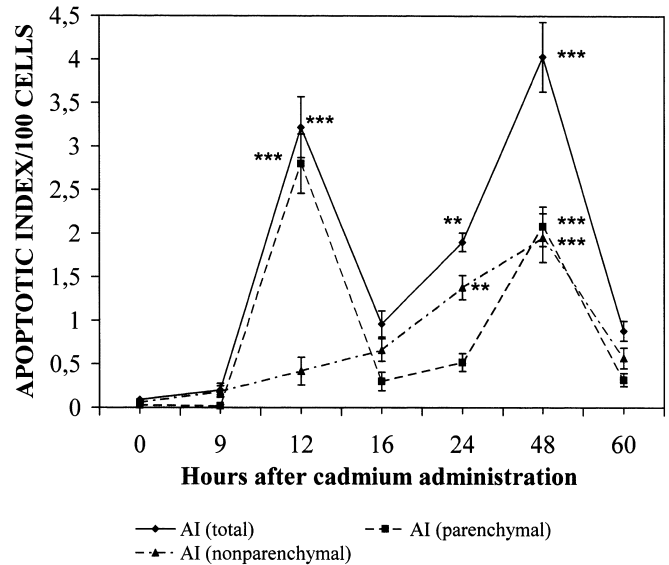


Fig. 5 Time-course of apoptotic index (AI) demonstrated by TUNEL assay, for parenchymal and nonparenchymal liver cells and total apoptotic index after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. Results represent the findings in eight rats killed at 12, 16, 48 and 60 h, and six rats killed at 0, 9 and 24 h. Values are expressed as means \pm SE. *** $P < 0.001$, ** $P < 0.01$, statistically different from control (0 hours)

Peliosis hepatis (Fig. 7) was first observed at 16 h. The extent of peliosis gradually increased from 16 to 48 h, peaked at 48 h, and decreased at 60 h. The time-course of this lesion closely correlated with nonparenchymal cell apoptosis for all time-points examined (Fig. 8). In peliotic cavities, apoptosis was found mainly in nonparenchymal cells but also in hepatocytes. Peliosis was observed throughout the hepatic lobule without showing any of the zonal distribution seen with necrosis and apoptosis.

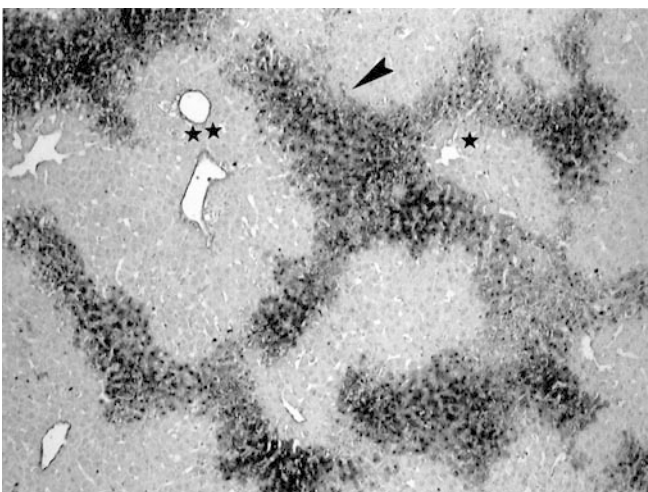


Fig. 4 Apoptosis demonstrated in situ by TUNEL assay, after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. Apoptosis (arrowhead) is located in zone 2 of hepatic lobule (TUNEL, $\times 40$; asterisk central vein, double asterisk portal tract)

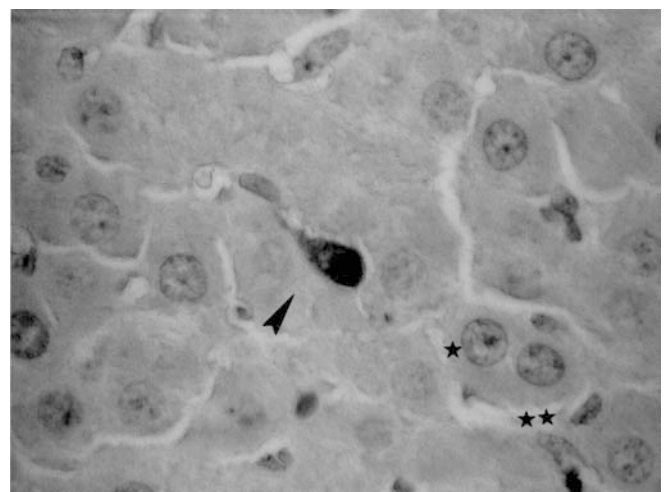


Fig. 6 Apoptosis demonstrated in situ by TUNEL assay ($\times 1000$) in nonparenchymal liver cells (arrowhead), after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. (asterisk hepatocyte, double asterisk hepatic sinusoid)

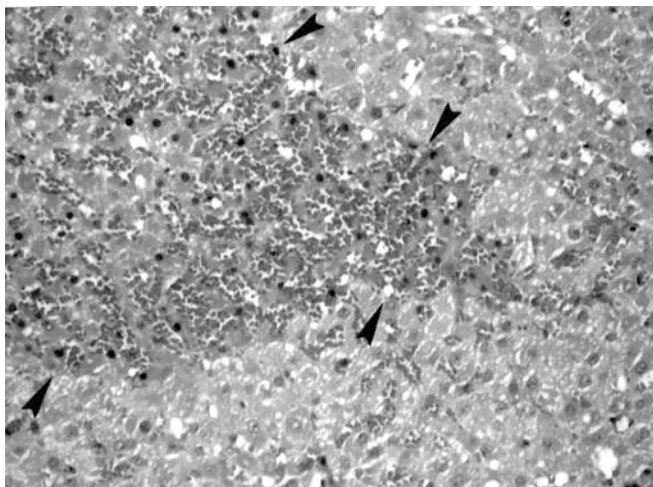


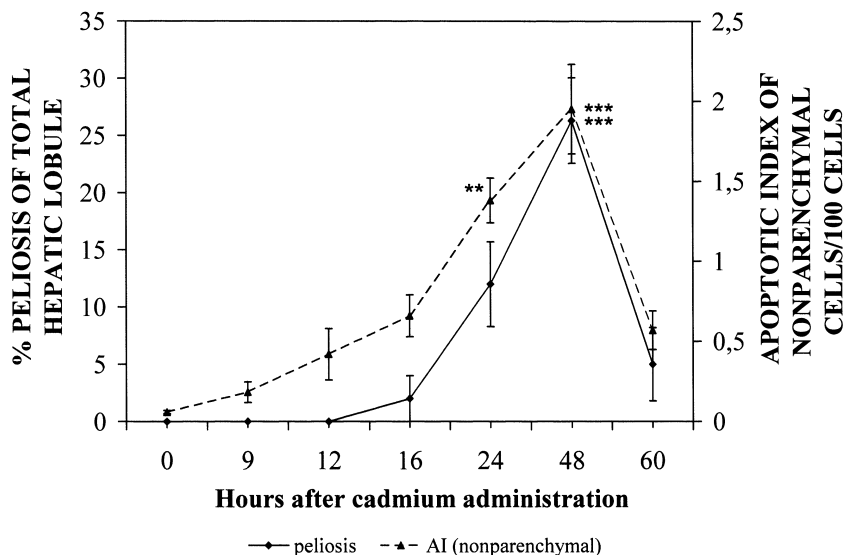
Fig. 7 Peliosis hepatis demonstrated (arrowheads) in HE-stained liver section ($\times 100$) after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight

Kupffer cell activation, as demonstrated by immunohistochemical detection of CD68, was found to be minimal and close to control group values (0.35 ± 0.02) for all time-points examined. Wide variation between the groups of animals was observed at 12, 16 and 48 h. Neutrophil infiltration was also close to control group values (0.06 ± 0.01) for all time-points.

Hepatic regeneration due to cadmium-induced toxic injury, as estimated by the mitotic index in HE sections, was very low during the first 24 h after cadmium intoxication. The mitotic index started increasing at 24 h (0.163 ± 0.068), and this increase was still present at 60 h (0.31 ± 0.1). Mitoses were undetectable in the control group (Fig. 9).

Thymidine kinase activity was sharply elevated between 9 h (47 ± 5) and 12 h, and for all subsequent time-points the activity was markedly elevated compared with control values (35 ± 12). The activity peaked at 12 h (241 ± 17), 48 h, and 60 h (334 ± 14) (Fig. 9).

Fig. 8 Apoptotic index (AI), as demonstrated by TUNEL assay, for nonparenchymal liver cells and the extent of peliosis (percentage of total hepatic lobule) as a function of time after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. Results represent the findings in eight rats killed at 12, 16, 48 and 60 h, and in six rats killed at 0, 9 and 24 h. Values are expressed as means \pm SE. *** $P < 0.001$, ** $P < 0.01$, statistically different from control (0 hours)

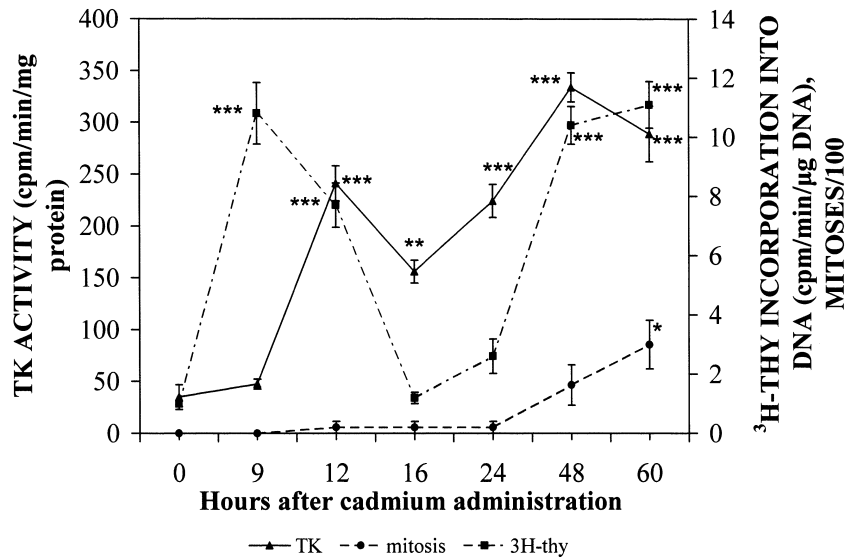


The rate of ^3H -thymidine incorporation into hepatic DNA was increased at 9 h (11 ± 1) and 12 h (8 ± 0.7). A second increase was observed at 48 h (10 ± 0.6) and this increase was still present at 60 h (11 ± 0.8) (Fig. 9). It must be noted that, irrespective of the differences observed, the values were very low compared to the high rates of liver regeneration such as those recorded after partial hepatectomy.

Discussion

Acute hepatotoxicity induced by cadmium has been the subject of numerous reports and today it is considered to be a result of a biphasic process. It has been proposed that acute hepatotoxicity involves two distinct pathways: the first one for the initial injury is caused by the direct toxic effects of the metal and/or ischemia due to endothelial cell injury, and the second process for the latter inflammatory injury is one in which Kupffer cell activation and neutrophil infiltration play a major role through triggering a complicated cascade of inflammatory mediators (Kayama et al. 1995; Sauer et al. 1997; Dong et al. 1998; Rikans and Yamano 2000; Yamano et al. 2000). Endothelial cells are considered the most sensitive cell population to the toxic effects of the metal, and differences in the strain susceptibility have been attributed to this cell population (Liu et al. 1992; Nolan and Shaikh 1986; McKim et al. 1992). The hepatic trauma caused by cadmium has been reported to be age dependent (Yamano et al. 1998); Wistar rats less than 6 months old are resistant to the toxic effects of the metal through reduced activation of Kupffer cells and neutrophil infiltration (Yamano et al. 1998). The role of $\text{TNF}\alpha$, mainly arising as a product of Kupffer cells, in the genesis of acute liver injury induced by cadmium has been recently challenged, though, since $\text{TNF}\alpha$ -null mice do not seem to be protected from the toxic effects of the metal (Harstad and Klaassen 2002).

Fig. 9 Rate of liver regeneration as evaluated by liver thymidine kinase (TK) activity, rate of ^3H -thymidine incorporation (^3H -thy) into hepatic DNA and mitotic index (HE sections) after acute cadmium intoxication in rats given 3.5 mg Cd/kg bodyweight. Ordinate in mitoses $\times 10$ for mitotic index. Results represent the findings in eight rats killed at 12, 16, 48 and 60 h, and in six rats killed at 0, 9 and 24 h. Values are expressed as means \pm SE. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, statistically different from control (0 hours)



In the present study, we investigated the mechanism of cadmium-induced acute liver injury in Wistar rats. Cadmium was injected intraperitoneally at a high dose approaching the LD_{50} (46% mean mortality was observed).

Since acute cadmium-induced hepatic trauma in Wistar rats does not involve inflammation, the lesions observed must be the result of direct metal actions and/or ischemia due to endothelial cell injury.

In relation to the role of apoptosis in the genesis of acute injury induced by cadmium, the results of this study clearly indicate that apoptosis is an essential mechanism for eliminating critically damaged cells in the liver of cadmium-intoxicated rats. Apoptosis was found to be more profound in nonparenchymal cells where it represented the major mode of cell death. This is easily understood given that nonparenchymal liver cells represent less than 15% of the total cell population (a percentage that could be lower in young rats such as those used in this study) (Blouin et al. 1977).

The kinetics of apoptotic cell death was distinct for the two cell populations. Parenchymal cell apoptosis followed a biphasic pattern, with a maximal increase soon after intoxication, and a second peak at the time of maximal necrosis. Nonparenchymal cell apoptosis showed a gradual increase and peaked at the time of maximal necrosis.

Although endothelial cells, which represent almost 50% of nonparenchymal cells (Blouin et al. 1977), have been reported to be the most sensitive cell population to the toxic effects of cadmium and the first cell type to undergo necrosis, this was not verified in the case of apoptosis. Nonparenchymal liver cells seem to be resistant to the apoptotic mode of cell death during the first hours after cadmium intoxication.

The first peak of parenchymal cell apoptosis, as well as the first peak of necrosis, seems to evolve as a result of direct cadmium action on hepatocytes, and this has been confirmed by administration of hepatoprotective

factors, which abrogated the first peak of parenchymal cell apoptosis as well as that of necrosis (unpublished observations). The second peak of parenchymal cell apoptosis cannot be of inflammatory etiology. Nonparenchymal cell apoptosis and subsequent ischemia seem to account for both observed second peaks of parenchymal cell apoptosis and necrosis.

The increase of nonparenchymal cell apoptosis followed the first (and higher) peak of hepatocyte apoptosis. Today it is known that hepatocyte hypoxia through ATP depletion and hydrogen peroxide production induces sinusoidal endothelial cell apoptosis (Motoyama et al. 1998). It is plausible to presume that the initial hepatocyte injury in conjunction with the direct toxic injury of nonparenchymal cells induces apoptotic cell death in nonparenchymal liver cells. This speculation is also in accordance with the fact that mitochondria are considered to be the most sensitive cell organelle to cadmium (Muller and Ohnesorge 1984; Muller and Stacey 1988).

The mechanism by which cadmium induces apoptosis is still unknown. The impairment of respiratory chain proteins in mitochondria and subsequent ATP depletion or chemical alternation of nucleic acids are possible mechanisms (Wyllie et al. 1980; Saplakoglou et al. 1997; Esteve et al. 1999; Lopez-Ortal et al. 1999). Mitochondrial dysfunction as well as direct chemical alternation of nucleic acids are considered to be initiating events of apoptosis (Esteve et al. 1999).

Peliosis hepatis, which refers to the replacement of liver tissue with blood-filled cavities without an endothelial cell lining, was first reported as a feature of cadmium toxicity by Habbebu et al. (1998). This kind of lesion has mainly been reported to be a complication of androgenic/anabolic steroid therapy and, despite being of unknown pathogenesis, it is connected with damage of the sinusoidal endothelium (Taxy 1978; Zafrani et al. 1984).

In the present study peliosis hepatis is connected, for the first time, with nonparenchymal cell apoptosis.

The time-course of peliosis over all time-points examined followed the rate of nonparenchymal cell apoptosis. This finding strongly suggests that the apoptotic cell death of nonparenchymal cells may be the major type of injury implicated in this type of lesion.

Acute cadmium-induced liver injury significantly elevated TK activity at 9 h, and the activity remained at high levels during the whole time period examined. According to our findings, acute cadmium intoxication seems to induce TK activity. This finding is quite unexpected given that acute cadmium intoxication has been reported to suppress liver regeneration after partial hepatectomy through the suppression of TK activity (Margeli et al. 1994). Further investigation is needed in order to elucidate the exact mechanisms of cadmium actions on liver cells and particularly on cell-cycle progression, given that TK represents an induced enzyme at the G1/S transition point in the cell cycle (Gudas et al. 1994).

In conclusion, cadmium-induced acute liver injury in Wistar rats up to 5 months old does not involve inflammation. The liver lesions seem to evolve as a result of direct actions of the metal and ischemia, and two major patterns can be discerned: one biphasic pattern for necrosis and parenchymal apoptosis and another for nonparenchymal cell apoptosis and peliosis. Apoptosis seems to play a major role in eliminating damaged cells, and its participation is profound in nonparenchymal liver cells where it represents the major type of cell death. The pathogenesis of peliosis hepatis is connected with the apoptosis of nonparenchymal liver cells surrounding the sinusoids. Further research is needed in order to fully elucidate the exact actions of the metal on the specific subpopulations of nonparenchymal liver cells.

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