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Rapid Communication

Rapid Induction of Apoptosis in Rat Liver by Cycloheximide

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A single administration of the inhibitor of protein synthesis cycloheximide results in the occurrence of apoptosis in rat liver. The presence of intracellular apoptotic bodies was detected as early as 2 hours after treatment. No evidence of cell necrosis could be observed by histologic and biochemical analysis. Apoptosis was followed by an increased expression of testosterone-repressed prostate message-2 RNA, a gene whose activity has been associated to apoptotic cell death in involuting rat prostate. The finding of* in vivo *induction of apoptosis in nonproliferating cells by an inhibitor of protein synthesis, together with the rapidity and synchrony in the occurrence of cell death make this model potentially useful for the analysis of the kinetics of the apoptotic cycle and in exploring some of the mechanisms of regulation of genes possibly involved in this type of cell death. (Am J Pathol 1992, 140:545–549)

Cell death may occur by at least two distinct modes: necrosis and apoptosis. Although the former type of cell death is considered to be the consequence of cellular acute damage, the latter type, on the contrary, can occur in several circumstances wherein pathologic stimuli could not be demonstrated, i.e., embryogenesis, metamorphosis, suggesting that this type of cell death may have a crucial role in the regulation of cellular turnover in many organs and tissues.[1] Moreover, it is generally believed that apoptosis, unlike necrosis, is an active process requiring protein and RNA synthesis.[2] Genes associated with apoptosis have already been identified in lower organism as well as in mammalians (ced-3 and ced-4, testosterone-repressed prostate message-2, tissue transglutaminase, bcl-2).[3–6]

The importance of synthetic activity is further shown by the finding that the inhibitor of protein synthesis cycloheximide (CHX) can delay or prevent apoptosis.[7–9] CHX does not, however, inhibit apoptosis in all circumstances. In fact, it has no effect on apoptosis induced by cytotoxic T lymphocytes,[10] by mild hyperthermia,[11] or by the fungal metabolite gliotoxin,[12] all conditions in which apoptosis may not be synonymous of programmed or physiologic cell death such as that seen during normal development. Protein synthesis may be essential for initiation of apoptosis by some types of stimuli, but it may not be an absolute requirement for execution of the process when induced by other stimuli (mild injury). On the basis of these observations, we investigated the effect of cycloheximide on rat liver. The present study indicates that a single injection of CHX induces liver apoptosis in the absence of any evidence of cell necrosis. In addition, the expression of the testosterone-repressed prostate message-2 gene (TRPM-2), a gene whose expression increases during apoptosis associated to prostate involution[4] was found to be elevated after CHX treatment.

Materials and Methods

Male Wistar rats (Charles River, Milano, Italy) weighing 200–220 g were maintained on a standard laboratory diet and given water *ad libitum*. CHX dissolved in NaCl 0.9% was injected intravenously or intraperitoneally at a dose of 1.5, 3, and 50 mg/kg body weight. Controls were given an equivalent amount of saline. Rats were killed at various time points after treatment (2, 3, 6, 24 hours).

Histologic Examination

After removal of the liver, small portions were immediately fixed in 4% formalin or Bouin and embedded in paraffin;

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Table 1. *Effect of CHX on the Occurrence of Hepatic Apoptotic Bodies*

Treatment*	Apoptotic bodies/100 nuclei	
	3 hr	6 hr
Controls	0.01 ± 0.01† (4)	0.02 ± 0.01 (4)
CHX 1.5 mg IV	0.65 ± 0.11 (6)	0.35 ± 0.25 (5)
CHX 1.5 mg IP	0.78 ± 0.28 (7)	0.09 ± 0.03 (5)
CHX 3.0 mg IP	0.64 ± 0.09 (4)	0.46 ± 0.29 (4)
CHX 50.0 mg IV	4.40 ± 0.13 (4)	ND

* CHX was given intravenously (IV) or intraperitoneally (IP) at the doses indicated in the table. Rats were sacrificed 3 and 6 hours after treatment. Number of animals is given in parentheses.
† Mean ± SE.

sections 5 μm thick were stained with hematoxylin and eosin.

Electron Microscopy

Immediately after sacrifice, sections from the median lobe of the liver were fixed in Karnowsky solution and postfixed in 1% phosphate-buffered osmium tetroxide (pH 7.4), dehydrated, and embedded in Epon 812. Thin sections (1–2 μm) from 10 to 20 blocks from each sample were stained with toluidine blue and examined by light microscopy; ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Jeol 100S electron microscope. Blocks from livers of untreated animals were processed in the same way.

Serum Glutamate Pyruvate Transaminase Determination

CHX-treated rats were killed at various time intervals (Table 2). The activity of S-GPT was determined according to a standard combination method provided by Boehringer (Mannheim, Germany).

Incidence of Apoptosis

Hematoxylin-eosin stained sections were examined for the scoring of cells undergoing apoptosis detected as membrane-bounded globules (apoptotic bodies, ABs). The number of ABs was randomly counted in 50 to 100 high power microscope fields (×50) with a Laborlux D microscope (Leitz, Germany). Approximately 5000 to 10,000 hepatocytes per rat were counted: four to seven rats per group were used. The number of ABs was expressed as number per 100 nuclei. Only ABs containing nuclear fragments were recorded.

RNA Extraction and Northern (RNA) Blot Analysis

Total mRNA was extracted from frozen tissue by the method of Chirgwin et al.[13] Samples of RNA (20 μg) were electrophoresed on agarose gels, then transferred, and fixed to nylon filters, as described previously.[14] Blots were hybridized to a denatured (^{32}P)labeled probe for beta-actin (Oncor, Inc., Gaithersburg, MD) and TRPM-2,[15] and then exposed to X-ray film for autoradiography. The probe for TRPM-2 was a gift from Martin Tenniswood, University of Ottawa, Canada.

Results

Initial experiments were performed using an intravenous injection of CHX at a dose of 1.5 mg/kg. This dosage

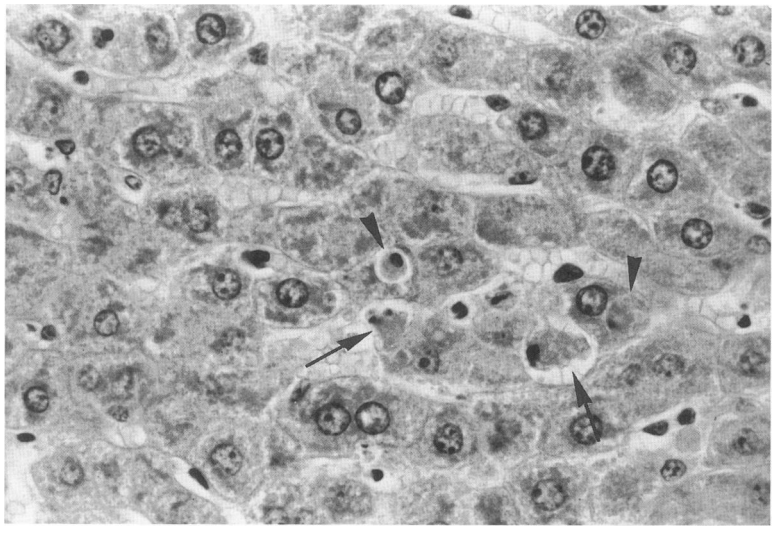

Figure 1. *Rat liver 3 hours after treatment with CHX (1.5 mg/kg). Apoptotic bodies are seen in the intercellular space (arrows) or within the cytoplasm of intact hepatocytes (arrowheads) (H&E ×250).*

Table 2. *Effect of CHX on Levels of Serum Glutamate Pyruvate Transaminase*

Treatment*	SGPT IU/l		
	3 hr	6 hr	24 hr
Controls	17 ± 2† (3)	19 ± 3 (3)	15 ± 1 (3)
CHX 1.5 mg IV	15 ± 2 (7)	14 ± 2 (7)	13 ± 1 (3)
CHX 1.5 mg IP	22 ± 2 (6)	20 ± 3 (6)	16 ± 1 (4)
CHX 3.0 mg IP	27 ± 2 (4)	33 ± 4 (4)	16 ± 3 (4)

* CHX was given intravenously (IV) or intraperitoneally (IP) at the doses indicated in the table. Rats were sacrificed 3, 6, and 24 hours after treatment. Number of animals is given in parentheses.
† Mean ± SE.

although producing an inhibition of protein synthesis ranging from 98% to 90% at 1 and 5 hours after treatment,[16] represents a nonlethal dose. Histologic examination of the liver 3 hours after treatment showed an increased occurrence of membrane-bounded acidophilic globules (apoptotic bodies) scattered throughout the hepatic parenchyma (Table 1). ABs were observed in the extracellular space as well as within the cytoplasm of otherwise intact hepatocytes (Figure 1). Interestingly, while an increased incidence of apoptotic bodies was still present 6 hours after treatment, no evidence of inflammatory response nor of lytic cell necrosis could be detected. Absence of necrosis was also confirmed by the lack of elevation of serum glutamate pyruvate transaminase (S-GPT) a widely used marker of liver necrosis (Table 2). Similar results were found when CHX was given by intraperitoneal route or when a dose of 3 mg/kg of CHX was used (Tables 1, 2).

Our previous studies[17] have shown that after treatment with the hepatotoxicant thioacetamide two types of cell death, namely apoptosis and necrosis are induced in a sequential way, thus posing the question whether apoptosis may simply be the first step of a process ultimately leading to necrosis. To study this question, rats given 1.5 mg/kg of CHX were sacrificed 24 hours after treatment, and the liver was examined. The results obtained indicate that at this time point no evidence of necrosis could be found indicating that the only type of liver cell death induced under our experimental conditions by CHX treatment is apoptosis.

It has been suggested that the capacity of a chemical to induce apoptosis or necrosis may depend on the dosage employed, thus implying that a low dose may have an apoptotic effect with a high dose of the same chemical having a necrogenic capacity. To test this possibility, rats were injected with a dose of 50 mg/kg, and were killed at different time points. The results obtained indicate that this dose of CHX is able to induce apoptosis as early as 2 hours after treatment as shown by the ultrastructural finding of intracellular apoptotic bodies containing nuclear fragments (Figure 2). The incidence of apoptotic bodies induced by this dose of CHX was found to be much higher than that observed with the lower doses (Table 1). Interestingly, when apoptosis was induced by 50 mg of CHX, the vast majority of ABs was found to be localized in the sinusoids (Figure 2). Despite the large extent of cell death occurring under these conditions, no evidence of necrosis could be observed up to 6 hours. No further time points could be examined since this dose of cycloheximide proved to be lethal to the animals after approximately 12 hours from the treatment.

It has been shown that apoptotic cell death occurring in rat prostate after castration and in involuting kidney is associated to an increase in the expression of TRPM-2. It was of interest to us to determine whether increased gene expression could also characterize CHX-induced liver apoptosis. Our results indicate that a) unlike rat kidney or prostate, TRPM-2 is constitutively expressed in

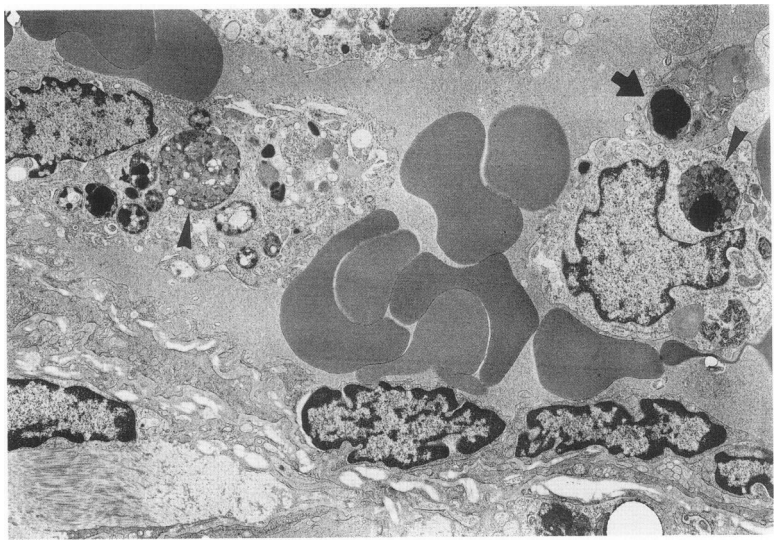

Figure 2. *Rat liver 2 hours after treatment with CHX (50 mg/kg). Apoptotic bodies lie in the sinusoidal lumen (arrows) or within the cytoplasm of sinusoidal cells (arrowhead) (×5800).*

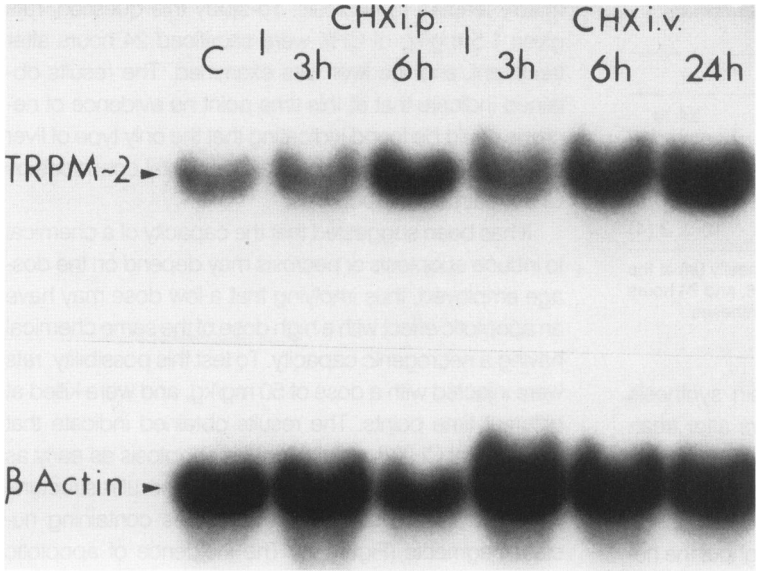

Figure 3. *Expression of TRPM-2 mRNA and beta-actin at various time intervals after IV or IP treatment with CHX.*

control liver, and b) an increased expression of TRPM-2 was observed 6 hours after CHX administration, and persisted up to 24 hours (Figure 3), a time point when apoptosis was not occurring anymore as indicated by histologic examination and S-GPT determination (Table 2).

Discussion

It is evident from the present study that a single administration of the protein synthesis inhibitor CHX alone is able to induce cell death in rat liver cells. The type of cell death observed as early as 2 to 3 hours after CHX occurs in the form of apoptosis as shown by the finding of several morphologic features characteristic of this mode of cell death: shrinkage of the cells, chromatin condensation, membrane blebbing, formation of membrane-bounded globules (apoptotic bodies) and absence of inflammatory response. No evidence of cell necrosis could be observed at any time point.

Apoptosis is believed to be an active process requiring protein as well as RNA synthesis. Delay or inhibition of apoptosis by inhibitors of either RNA or protein synthesis has been reported in several conditions.[7–9] However, other reports have indicated that inhibition of protein synthesis does not prevent apoptosis in all circumstances,[10–12] and furthermore, work by Ijiri et al,[18] and recent studies by Martin et al[19] have shown that inhibitors of RNA as well as protein synthesis per se induce apoptosis *in vivo* or *in vitro* in a range of proliferating cell types including mouse intestinal crypt cells and several human cell lines. Although the latter findings were obtained using experimental models wherein cells were actively proliferating, the present study represents the first *in vivo* evidence of apoptosis induced by inhibitors of protein synthesis in a quiescent organ such as the liver of a young adult rat. Whether cycloheximide-induced apoptosis is a direct consequence of inhibition of protein synthesis or can be due to some other unknown "cytotoxic" effect is not clear. In this respect it is important to note that liver apoptosis can be induced by several other hepatotoxins.[17,20–22]

Finally, our study shows that an increased expression of TRPM-2 (a gene whose activity was found to be associated with apoptosis in various conditions) occurs in CHX-treated rat liver. However, although in prostate and kidney the enhancement in TRPM-2 mRNA preceded the occurrence of apoptosis, the increase in TRPM-2 mRNA seen in rat liver was detected after the onset of apoptotic cell death. In addition, unlike kidney and prostate, rat liver constitutively exhibits high levels of TRPM-2 mRNA. Although the significance of the increase of this gene in relation to apoptosis is unclear, it is possible that CHX-induced apoptosis uses the protein normally present in the liver. The utilization of the protein accompanied by a concomitant inhibition of protein synthesis may trigger a new synthesis of TRPM-2 mRNA to restore the physiologic levels of this protein. Alternatively, the increase in TRPM-2 mRNA may be the consequence of the interference by CHX treatment with the activity of a protein that regulates the expression of TRPM-2 analogously to what suggested for genes such as c-*fos* and c-*myc*.[23,24]

To know more about the normal function of this gene and its possible significance in the apoptotic cell death may be useful in the understanding of the mechanism(s) responsible for this mode of cell death.

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