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BRIEF ARTICLE

Effect of dichloromethylene diphosphonate on liver regeneration following thioacetamide-induced necrosis in rats

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Abstract

AIM: To study the effect of dichloromethylene diphosphonate (DMDP), a selective Kupffer cell toxicant in reference to liver damage and postnecrotic liver regeneration in rats induced by sublethal dose thioacetamide (TA).

METHODS: Rats, intravenously (*iv*) pre-treated with a single dose of DMDP (10 mg/kg), were intraperitoneally (*ip*) injected with TA 6.6 mmol/kg (per 500 mg/kg body weight). Hepatocytes were isolated from rats at 0, 24, 48 and 72 h following TA intoxication and blood and liver samples were obtained. To evaluate the mechanisms involved in the postnecrotic regenerative state, DNA distribution and ploidy time course were assayed in isolated hepatocytes. Circulating cytokine tumor necrosis factor-alpha (TNF-α) was assayed in serum and determined by reverse transcriptase-polymerase chain reaction in liver extract.

RESULTS: The effect of DMDP induced noticeable changes in postnecrotic regeneration, causing an increased percentage of hepatocytes in the cell cycle S phase. The increase at 24 h in S_1 population in rats pretreated with DMDP + TA was significantly ($P < 0.05$) different compared with that of the TA group (18.07% *vs* 8.57%). Hepatocytes increased their proliferation as a result of these changes. Also, TNF-α expression and serum level were increased in rats pre-treated with DMDP. Thus, DMDP pre-treatment reduced TA-induced liver injury and accelerated postnecrotic liver regeneration.

CONCLUSION: These results demonstrate that Kupffer cells are involved in TA-induced liver, as well as in postnecrotic proliferative liver states.

Key words: Dichloromethylene diphosphonate; Kupffer cells; Thioacetamide; Hepatotoxicity; Cell cycle

Core tip: Over the last 20 years, liposomes, useful models for cell membranes, have become a powerful research tool whose study has resulted in many advances in cell physiology. When encapsulated in liposomes, dichloromethylene diphosphonate, a selective Kupffer cell toxicant, completely eliminates large Kupffer cells from the liver, allowing us to elucidate the role of these macrophages in total damage induced by hepatotoxic compounds such as thioacetamide.

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INTRODUCTION

Dichloromethylene diphosphonate (DMDP) is clinically employed for the treatment of osteolytic bone diseases. When encapsulated in liposomes, DMDP is a selective Kupffer cell toxicant that completely eliminates large Kupffer cells from the liver, resulting in their damage and apoptosis[1]. Degree of depletion depends on the injection route and amount of injected DMDP liposomes. In the majority of studies, not only Kupffer cells, but also splenic macrophages have been depleted by a single intravenous (*iv*) injection of DMDP. Kupffer cells, due to the macrophages residing in the liver sinusoids, are the first macrophage population to come into contact with drugs. These cells are anchored to the endothelium in the lumen of the sinusoids[2]. Kupffer cells exhibit intra-acinar heterogeneity because those located in the periportal area are larger and exhibit higher phagocytic activity compared with those localized in the perivenous area[3]. It is well known that the function of these cells (cytokine and protease release, superoxide anion production, *etc*.) plays an important role in the pathogenesis induced by hepatotoxic compounds[4,5]. DMDP is most likely protective because it prevents the release of inflammatory cytokines and toxic oxygen radicals produced by activated Kupffer cells[6,7].

Thioacetamide (TA) is a potent hepatotoxic agent that, when administered at 500 mg/kg body weight doses to rats, gives rise to severe hepatocellular perivenous necrosis[8,9]. The selective destruction of perivenous hepatocytes and the proliferative state of liver cells that immediately follows were employed in the present study as an experimental model by means of which to study the hepatic response against the aggressive attack of a hepatotoxic drug. Thus, this response may be considered from two perspectives: that of hepatocellular necrosis and that of the postnecrotic hepatocellular regeneration linked with restoration of liver function[10,11].

Kupffer cells are also the major source of mitogens such as tumor necrosis factor-alpha (TNF-α) in liver[12,13]. TNF-α is a multifunctional cytokine that in the liver acts as a mediator of the acute phase response and is a cytotoxic agent in many types of hepatic injury. Some authors have suggested that TNF-α may be necessary for hepatocyte proliferation[14]. The observation that TNF-α is required for liver regeneration is surprising because TNF-α is a proinflammatory cytokine and an acute phase response mediator[15]. The proliferative and anti-apoptotic effect of this cytokine appears to take place only under special conditions, such as those existing after partial hepatectomy. Although TNF-α appears to be beneficial and required for liver regeneration after partial hepatectomy, the need for this factor has not been as clearly established after liver injury, a more common regenerative stimulus. In fact, a number of studies have suggested that TNF increases liver injury after toxic damage[16,17]. Moreover, Fujita *et al*[18] demonstrated that the absence of TNF-α does not impair liver regeneration.

The purpose of the present study was to elucidate the role of Kupffer cells in regeneration after liver injury, specifically blocking Kupffer cell function by DMDP. The proliferative postnecrotic response was assayed by evaluating ploidy and DNA distribution in the cell cycle phases in isolated hepatocytes by flow cytometry.

MATERIALS AND METHODS

Reagents

DMDP (dichloromethylene diphosphonate) was provided by Roche Diagnostics (Mannheim, Germany), phosphatidylcholine by Lipoid EPC, LIPOID (Ludwigshafen, Germany) and monoclonal ED-1 (MCA1018G) and monoclonal ED-2 antibodies were provided by Serotec, Hilversum (The Netherlands). Enzymes were obtained from Boehringer Mannheim (Germany). Substrates and coenzymes were from Sigma Chemical Co. (St. Louis, MO, United States). Standard analytical grade laboratory reagents were obtained from Merck (Darmstadt, Germany).

Liposome-encapsulated DMDP

Liposomal clodronate was prepared as previously described[19]. Briefly, 86 mg phosphatidylcholine and 8 mg cholesterol were dissolved in chloroform in a round-bottom flask. The thin film that formed on the interior of the flask after high vacuum rotary evaporation was dispersed by gentle rotation under low vacuum conditions for 10 min in 10 mL phosphate buffered saline (PBS) (control liposomes) or in 10 mL of a 0.6 mol/L DMDP (2.5 g DMDP in 10 mL distilled water and clodronate-containing liposomes). After swelling, sonication and washing in PBS, the liposomes were resuspended in 4 mL PBS. The resulting liposomal formulation contained clodronate at a concentration of 0.7 mol/L.

Animal treatment and sample processing

Two month old male Wistar rats (weighing 200-220 g) were obtained from the Bioterio, Instituto de Ciencias de la Salud, Universidad Autónoma del Estado de Hidalgo (UAEH), Mexico, and acclimated to our animal room for 2 wk, during which time the rats were supplied with food (Purina de México, S.A.) and water ad libitum, exposed to a 12 h light-dark cycle, and administered intraperitoneally (*ip*) with a single necrogenic dose of thioacetamide (TA) 6.6 mmol (500 mg/kg body weight) (TA) freshly dissolved in 0.9% NaCl. The TA dose was chosen as the highest dose with survival of > 90%[20,21]. Experiments were performed on two different groups.

Rats were treated with a single dose of TA and rats pre-treated with DMDP 24 h prior to TA (DMDP + TA). DMDP encapsulated in liposomes was injected into tail vein (10 mg/kg). Untreated animals received 0.5 mL of 0.9% NaCl. Rats were cervically dislocated and blood and liver samples were obtained and processed as previously described[21]. Blood was collected from hearts and maintained at 4 ℃ for 24 h, centrifuged at 3000 *g* for 15 min, and serum was obtained as the supernatant. Hepatocytes were isolated from rats by the classic perfusion method[22] at 0, 24, 48 and 72 h following TA (24 h). The viability of isolated hepatocytes (> 90%) was assessed by trypan blue exclusion as previously described[10].

Each experiment was performed in duplicate on four different animals and following the International Criteria of Experimental Animals outlined in Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 85-23, 1985, and all procedures involving experimental animals were conducted according to our Federal Regulations for Animal Experimentation and Care (Ministry of Agriculture; SAGAR, Mexico) and The Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology in 1989.

Determination of parameters of injury and TNF-α in serum

Enzymatic determinations were carried out in serum under optimal conditions of pH, temperature, substrate and co-factor concentrations. Aspartate aminotransferase (AST) and isocitrate dehydrogenase (ICDH) were determined in serum as a biochemical indicator of hepatocellular necrosis according to the manufacturer's protocol. AST (EC 2.6.2.1) activity was assayed following the method of Rej and Horder[25]. ICDH (E.C 1.1.1.39) was determined as described previously[26]. Concentrations of immunoreactive TNF-α was determined by the enzyme-linked immunosorbent assay (ELISA) system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. In brief, the extracted plasma was reacted with the assay reagents in the TNF-α kit and analyzed spectrophotometrically at 450 nm absorbance. TNF-α levels were calculated from kit standards and expressed as pg/mL of plasma.

RNA extraction and reverse transcriptase-polymerase chain reaction analysis of TNF-α

Total RNA was isolated from rat liver following the guanidinium thiocyanate/phenol reagent method[27]. For reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA (1 μg) was subjected to random primer first-strand complementary DNA (cDNA) synthesis in 40 μL reactions composed of 50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L DTT, 1 mmol/L dNTP (each), 50 ng of random hexamer and 0.5 IU/μL Mo-Mu-LV reverse transcriptase (Super-Script Pre-Amplification System; Gibco-BRL, Life Technologies). The reactions were incubated for 60 min at 42 ℃ and terminated at 65 ℃ for 15 min. First-strand cDNA were subsequently amplified by PCR; β-actin cDNA was utilized as an internal control. Sequences of the primers were as follows: TNF-α sense: 5'-TGG CCC AGA CCC TCA CAC TC-3'; TN-α antisense: 5'-CTC CTG GTA TGA AAT GGC AAA TC-3'; β-actin sense: 5'-TAC AAC CTC CTT GCA GCT CC-3'; and β-actin antisense: 5'-GGA TCT TCA TGA GGT AGT CAG TC-3'. The PCR reaction mixture contained PCR buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl], 1.5 mmol/L $MgCl_2$, 100 mmol/L dNTP (each), 0.4 mmol/L primers and 0.0025 U/μL of Taq polymerase in a final volume of 50 μL. Number of PCR cycles was adjusted to avoid saturation of the amplification system [at 94 ℃ for 1 min, 59 ℃ for 1 min and 72ºC for 1 min (35 cycles) for TNF-α, and at 94 ℃ for 30 s, 58 ℃ for 45 s and 72 ℃ for 30 s (24 cycles) for β-actin], with a final elongation at 72 ℃ for 10 min. Amplification products were visualized on 1.8% agarose gels containing ethidium bromide (1 μg/mL), TNF-α product, 281 bp, and β-actin product, 630 bp. A 100 bp DNA ladder was used as a marker. The products were quantified by laser densitometry.

Flow cytometry analysis of DNA content

DNA content was obtained from 10^6 isolated viable hepatocytes stained with propidium iodide following the multistep procedure of Vindeløv *et al*[28]. The fluorescence emitted from the DNA-propidium iodide complex was assayed in a FACScan flow cytometer (Becton-Dickinson) in the FL2-A channel. A double discriminator module was employed to distinguish between signals deriving from a single nucleus and nuclear aggregation products. Data analysis was carried out by evaluation of single inputs (10^4 nuclei/assay) and was expressed as the percentage of DNA distribution in cell cycle phases G_0/G_1 (2N), S_1, G_2 + M (4N), S_2, $(G_2 + M)_2$ (8N) and hypodiploid peak (< 2N).

Statistical analysis

The results were calculated as the mean ± SD of four experimental observations in duplicate (four animals). Differences between groups were analyzed by analysis of variance (ANOVA) following Snedecor F ($\alpha = 0.05$). The Student's *t* test (statistical significance $P < 0.05$) was performed for statistical evaluation as follows: (1) all values against their control; and (2) differences between two groups: DMDP + TA *vs* TA.

RESULTS

Effect of DMDP on parameters of liver necrosis

Liver damage induced by xenobiotics is characterized by the release in serum of hepatic enzymes due to the necrosis of hepatocytes. AST is randomly distributed in the hepatic acinus and is the enzyme activity utilized as the marker of necrosis. The increase in AST and ICDH in serum reached the maximum at 24 h (Figure 1). The extent of TA-induced necrosis was detected by a peak of 30 and 15 times baseline values for AST and ICDH activity, respectively. When rats were pre-treated with DMDP, the

Table 1 Quantitative analysis of the DNA ploidy in hepatocytes of adult rats following different treatments

Group	Hypodiploid (< 2N)	Diploid (2N)	S1 Phase (2N → 4N)	Tetraploid (4N)	S2 Phase (4N → 8N)	Octoploid (8N)
Control	0.98	12.3	2	75.31	2.48	3.97
Control DMDP	0.74	18.86	0.98	71.09	5.6	2.67
TA 24 h	1.61	41.74[a]	8.57[a]	39.0[a]	7.82[a]	0.9
TA-DMDP 24 h	2.01	25.45[a,c]	18.07[a,c]	49.20[a,c]	3.92	0.76
TA 48 h	1.59	52.87[a]	11.95[a]	22.86[a]	10.18[a]	0.2
TA-DMDP 48 h	2.35	42.77[a]	14.92[a]	28.0[a]	10.25[a]	1.7
TA 72 h	3.78[a]	47.61[a]	7.21[a]	35.6[a]	4.29[a]	1.12
TA-DMDP 72 h	2.65[a,c]	45.99[a]	1.44[c]	41.71[a]	5.98	1.83

The values are expressed as the percentage of DNA in the following: hypodiploid population (< 2N); diploid population, 2N (cells in G0-G1); tetraploid population, 4N (cells in G2 + M); octaploid population, 8N, and cells synthesizing DNA, S1 phase (from G1-G2, 2N→4N) and S2 phase (from 4N→8N). Data are expressed as the means of four experimental observations (four rats) ± SD, [a]$P < 0.05$ *vs* the untreated control group; [c]$P < 0.05$ *vs* changes due to dichloromethylene diphosphonate (DMDP). TA: Thioacetamide.

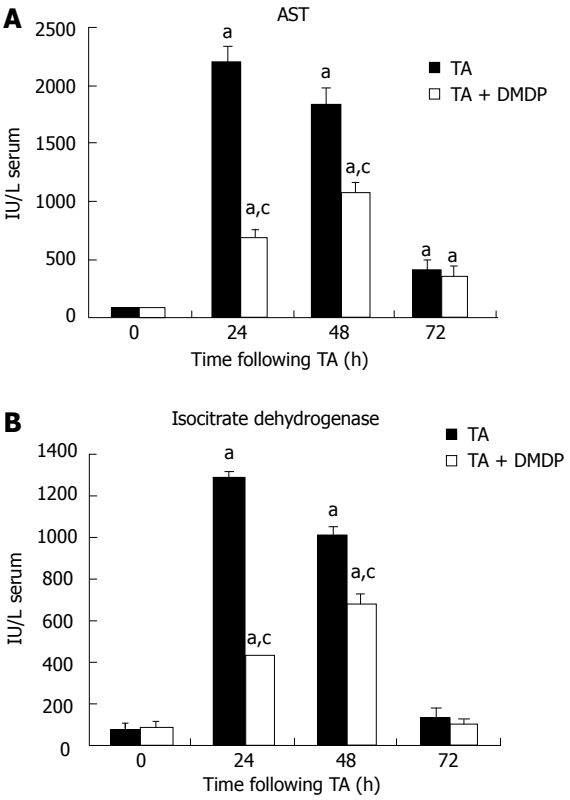

Figure 1 Enzymatic activity after dichloromethylene diphosphonate pre-treatment in rats intoxicated with one sublethal dose of thioacetamide. A: Effect of dichloromethylene diphosphonate (DMDP) pre-treatment on aspartate aminotransferase (AST) activity in the serum of rats intoxicated with one sublethal dose of thioacetamide (TA); B: Illustrates the effect of DMDP pre-treatment on isocitrate dehydrogenase activity in the serum of rats intoxicated with one sublethal dose of TA. Samples were obtained at 0, 12, 24, 48 and 72 h following TA 6.6 mmol (per 500 mg/kg body weight). The results, expressed as nmol per min per mL of serum, are the mean ± SD of four determinations in duplicate from four rats. [a]$P < 0.05$ *vs* the respective control, [c]$P < 0.05$ *vs* differences due to DMDP.

24 h peaks were reduced to 30% and 40%, respectively. However, at 48 h of intoxication, the DMDP-associated difference was 58% for AST activity, which indicates that DMDP delays TA-induced liver injury because maximal necrosis appeared at 48 h of intoxication. No effects were detected on serum activities when empty liposomes were administered (data not shown).

Effect of DMDP pre-treatment on the time course of genomic DNA ploidy and distribution in hepatocytes isolated from TA-treated rats

Table 1 shows the percentages of cell cycle populations related with ploidy and DNA content, as associated with histograms determined on the basis of fluorescence emission at 623 nm by the DNA propidium iodide complex. Following TA, liver cells exhibit marked variations in the pattern of DNA distribution, which can be summarized as a sharp decrease at 48 h in tetraploid population parallel to an increase in diploid population, followed by restoration to nearly normal values at 72 h. It can also be observed how the S_1 population is increased from 24 h, reaching maximal increase at 48 h. When rats were pre-treated with DMDP, variations in the pattern of DNA distribution is very similar to that observed in the TA group. However, we are able to detect an important difference: the highest increase in S_1 population is reached at 24 h (18.07% *vs* 8.57%) instead of at 48 h; thus, the proliferative state in hepatocytes is reached 24 h prior to that obtained in single dose TA-treated rats. No changes were detected in DNA ploidy when empty liposomes were administered (data not shown).

Effect of DMDP pre-treatment on serum TNF-α levels and expression in liver of rats following liver intoxication with TA

TNF-α is a multifunctional cytokine that, in the liver, acts as a mediator of the acute phase response and is a cytotoxic agent in many types of hepatic injury. TNF-α determination was performed in serum and liver. In TA-intoxicated rat serum, the level of this cytokine increased at 24 h of intoxication and when DMDP was pre-administered; this increase was significant (Figure 2).

Figure 2B and C depict the levels of TNF-α messenger RNA (mRNA) assayed by RT-PCR. As observed in

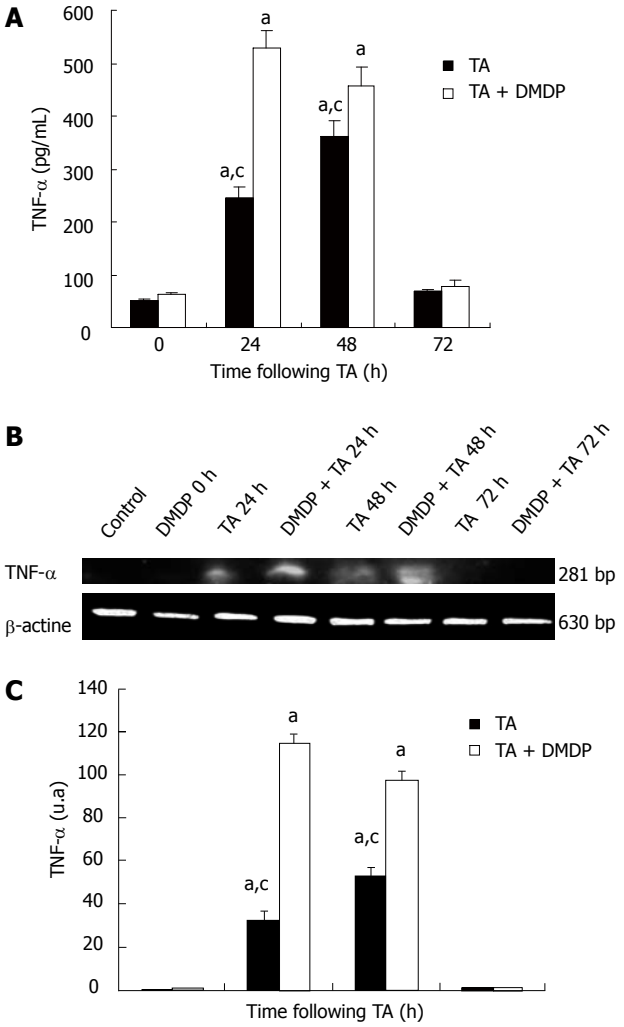

Figure 2 Effects of dichloromethylene diphosphonate in protein levels, gene expression and messenger levels tumor necrosis factor-alpha. A: Effects of dichloromethylene diphosphonate (DMDP) pre-treatment on serum tumor necrosis factor-alpha (TNF-α) levels determined by enzyme-linked immunosorbent assay tests on serum samples. Columns and vertical bars represent mean ± SD evaluated in four determinations from four rats. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* the DMDP-treated group; B: Effects of DMDP in gene expression profile of TNF-α assayed by reverse transcriptase-polymerase chain reaction analysis; C: Illustrates the effect of DMDP pre-treatment on the levels of TNF-α messenger RNA (mRNA) in liver homogenates of rats intoxicated with a sublethal dose of thioacetamide (TA). Samples were obtained at 0, 24, 48 and 72 h. The results, expressed in arbitrary units, are the mean ± SD of four determinations from four rats. $^{a}P < 0.05$ *vs* the respective control, $^{c}P < 0.05$ *vs* differences due to DMDP.

serum levels of TNF-α , mRNA follow the same pattern, which corroborates with the results obtained by ELISA.

DISCUSSION

Macrophages such as Kupffer cells in liver are multifunctional cells. They are involved in host defense mechanisms and possess a regulatory role in many biomedical processes. Their selective depletion[6], employing liposome-encapsulated drugs, forms a widely accepted approach to studying their functional aspects *in vivo*. There is evidence that liposome-mediated DMDP delivery actually depleted Kupffer cell in rat liver. We and others[7,29] found this "suicide" approach highly effective in depletion of Kupffer cell in liver tissue.

On the other hand, TA-induced liver injury is a well-established area of considerable pharmacological interest because reactive oxygen species (ROS) and free radicals, generated in microsomal drug oxidation, participate in the mechanisms of cell death[20,21,30]. Xenobiotics may act directly on hepatocytes, causing toxicity by interacting with target molecules, and may also act indirectly by means of activating phagocytic cells. The active phagocytes participate in the pathogenesis of tissue injury by releasing, among others, inflammatory cytokines that upregulate the expression of adhesion molecules. Tissue damage initiates an inflammatory response characterized by an accumulation of neutrophils at the site of injury[31].

Kupffer cells and infiltrating neutrophils contribute to liver injury in different experimental models of hepatotoxicity[32-34]. In our experiments, DMDP significantly attenuates TA-induced liver damage. Blockade of Kupffer cell function by DMDP appears to result in a disruption of a part of the sequence of events leading to hepatotoxicity.

In addition, the role of DMDP in TNF-α expression by Kupffer cells has been widely debated. Depletion of Kupffer cells, the major source of TNF-α production in liver, should give rise to a decrease in serum and in the mRNA TNF-α level in liver, a fact that has been described and corroborated by several authors[5,35,36]. However, other authors have reported opposite data[37,38] after partial hepatectomy in rats pre-treated with gadolinium, another inhibitor of Kupffer cells. Additionally, depletion of Kupffer cells with DMDP appears to increase hepatocyte proliferation and liver regeneration following partial hepatectomy; however, the responsible mechanism remains unknown.

On the other hand, it has already been reported that DMDP protects the liver from a number of toxicants that require biotransformation to elicit toxicity[39,40]. Badger *et al*[41] demonstrated in hepatocytes isolated from DMDP pre-treated rats that CYP-450 activity was reduced and the susceptibility of hepatocytes was altered. It has also been shown that hepatic injury induced by ischemia/reperfusion is modulated by the Kupffer cells[42].

In previous reports, we described that when TA was administered to rats, necrosis developed and peaked at 24 h of intoxication and that a synchronous proliferative response was immediately initiated, reaching a peak of DNA synthesis at 48 h. Postnecrotic proliferative response after experimental liver cell death constitutes an interesting area in which to study the factors involved in the regulation of hepatocyte proliferation.

Regarding postnecrotic regeneration, the peak of DNA synthesis was similar in both groups, although it is noteworthy that initial DNA synthesis levels were significantly higher due to the effect of DMDP, indicating that in our experiments, this compound also exerts mitogenic action, which can lead to liver hyperplasia.

After depleting Kupffer cells with DMDP, we explain that elevation of serum TNF-α levels and enhanced mRNA levels in the liver by hepatic cells other than Kupffer cells may contribute to cytokine synthesis or that TA-inducible cells residing in the liver contribute to cytokine levels in plasma. Endothelial cells may be a hepatic source of cytokines because this cell type readily responds to TA stimulation[35,43] and may not be affected directly by clodronate.

Following TA, liver cells exhibit marked variations in the DNA distribution pattern, which can be summarized as a sharp decrease at 48 h in tetraploid population parallel to an increase in diploid population, followed by restoration to nearly normal values at 72 h. It can also be observed how the S_1 population increases from 24 h, reaching the maximum at 48 h. When rats were pretreated with DMDP, variations in the pattern of DNA distribution are very similar to those observed in the TA group. However, we can detect an important difference: the highest increase in S_1 population is reached at 24 h (17.17% *vs* 10.01%) instead of at 48 h; thus, the proliferative state in hepatocytes is reached 24 h prior to that obtained in rats treated with the single dose of TA.

Our results clearly indicate that administration of DMDP + TA in rats results in stimulated tissue repair. From these results, we are able to speculate that Kupffer cells may play a crucial role in inducing DNA synthesis by secreting the priming factors (TNF-α) in the early phase of oval cell-mediated liver regeneration[44].

We conclude that DMDP pre-treatment significantly attenuates TA-induced hepatotoxicity. These results demonstrate that Kupffer cells are involved in TA-induced liver, as well as in postnecrotic proliferative liver states.

Modulation of Kupffer cell function by DMDP may serve as a potential target for therapeutics and could be useful for preventing drug-induced liver damage.

COMMENTS

Background

Thioacetamide (TA)-induced liver injury is a well-established area of considerable pharmacological interest because reactive oxygen species (ROS) and free radicals generated in microsomal drug oxidation participate in the mechanisms of cell death. In the present study, TA-induced hepatotoxicity was used to investigate the effect of a single dose of dichloromethylene diphosphonate (DMDP) (clinically employed for the treatment of osteolytic bone diseases); but in the present study, when encapsulated in liposomes, DMDP is a selective Kupffer cell toxicant.

Research frontiers

The aim of this study was to elucidate the role of Kupffer cells in regeneration after liver injury, specifically blocking Kupffer cell function by DMDP. The effect was assayed on an experimental model of liver injury induced by a single sublethal dose of TA.

Innovations and breakthroughs

Macrophages such as Kupffer cells in the liver are multifunctional cells. They are involved in host defense mechanisms and possess a regulatory role in many biomedical processes. Their selective depletion, utilizing liposome-encapsulated drugs, forms a widely accepted approach of studying their functional aspects *in vivo*.

Applications

As it is generally accepted that Kupffer cell function is involved in the severity of drug-induced liver damage and that DMDP induces a selective blockade of Kupffer cell function when administered intravenously, the purpose of the present study was to elucidate the role of Kupffer cells in regeneration after liver injury, opening a window to novel therapeutic strategies.

Terminology

Liposomes can be used for intracellular drug delivery into macrophages. In the present study, the authors utilized a liposome-mediated macrophage "suicide" technique based on intraphagocytic accumulation of the liposomes delivered.

Peer review

This is a nice experimental study showing that Kupffer cells play an important role in experimental hepatotoxicity by TA and in the regenerating process. The paper is well written.

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