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Short Communication

Evaluation of oxidative stress during apoptosis and necrosis caused by D-galactosamine in rat liver

Fang Sun[a], Eri Hamagawa[a], Chihiro Tsutsui[a], Naomi Sakaguchi[a], Yuri Kakuta[a], Sadako Tokumaru[b], Shosuke Kojo[a,*]

[a]*Department of Food Science and Nutrition, Nara Women's University, Nara 630-8506, Japan*
[b]*Department of Life and Health Sciences, Joetsu University of Education, Joetsu, Niigata 943-8512, Japan*

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Abstract

Eighteen and twenty-four hours after intraperitoneal administration of D-galactosamine (1 g/kg body weight) to rats, the activity of caspase-3-like protease in the liver increased significantly compared with that in the control group given saline. Histological examinations including the *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method found apoptotic hepatocytes 18 hr after the administration of D-galactosamine. Caspase-3 activity was barely detectable in the plasma of control rats, but increased significantly 24 hr after drug administration along with a dramatic increase in glutamate-oxaloacetate transaminase (GOT). These results indicated that D-galactosamine causes apoptosis in the liver by activating caspase-3, which is released to the plasma by secondary necrosis. The concentration of lipid hydroperoxides in the liver increased significantly 24 hr after D-galactosamine administration. In contrast, the concentration of vitamin C in the liver decreased significantly 18 and 24 hr after D-galactosamine administration. These results suggest that D-galactosamine induces severe oxidative stress in the liver, leading to extensive necrosis.

Keywords: Apoptosis; Caspase-3; Galactosamine; Necrosis; Vitamin C; Vitamin E

1. Introduction

A high dose of D-galactosamine causes necrosis of the liver by UTP depletion and inhibition of protein synthesis [1], although D-galactosamine is often used in combination with lipopolysaccharide or tumor necrosis factor (TNF) [2–5]. In the necrotic process induced by D-galactosamine, it is suggested that reactions involving free radicals play a role [6,7] based on an increase in TBARS, which had been the most commonly used indicator of lipid peroxidation [8]. However, the level of TBARS was found to have limited usefulness, even for the peroxidation of a simple oil [9]. Oxidative mediators are postulated to be another index of oxidative stress [10–16]. Lipid hydroperoxides are probable candidates, because they are formed by reactions in the membrane involving free radicals, have a sufficient lifetime to migrate in the cell, and modify both protein and DNA.

We developed a specific and sensitive method of determining the total level of lipid hydroperoxides, involving the chemical conversion of lipid hydroperoxides into aromatic phosphine oxide followed by the measurement of oxide by HPLC [10]. The efficiency of lipid hydroperoxides as an index of oxidative stress has been confirmed by their increase in typical pathologic conditions such as vitamin C deficiency [11], vitamin E deficiency [12], iron overload [13], diabetes [14], and thioacetamide [15] and carbon tetrachloride [16] intoxication. In this paper, we applied this method to evaluate oxidative stress in the liver of D-galactosamine-treated rats.

To prevent oxidative damage in the cell, a variety of antioxidants scavenge free radicals. The primary defense against oxidative stress in the tissue rests with antioxidants, including vitamins C and E, and GSH. Therefore, these antioxidants are expected to be consumed by enhanced

* Corresponding author. Tel.: +81-742-20-3459; fax: +81-742-20-3459.
E-mail address: kojo@cc.nara-wu.ac.jp (S. Kojo).

Abbreviations: Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide); AMC, aminomethylcoumarin; GOT, glutamate-oxaloacetate transaminase; TBARS, thiobarbituric acid-reactive substances; TUNEL, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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radical reactions. We determined the concentrations of vitamins C and E in the plasma and liver of rats as another type of index of oxidative stress induced by D-galactosamine.

Although D-galactosamine has long been known as a typical hepatotoxin causing necrosis [1], it has been reported to induce apoptosis in the livers of rats [17] and mice [18], based on histochemical observations and DNA laddering. To shed more light on the toxic mechanism of the chemical, we investigated the possible involvement of caspase-3 [19] in chemically induced apoptosis. In this paper, we report that caspase-3 is indeed activated in the liver by D-galactosamine and released in the plasma as in the case of thioacetamide [15,20] and carbon tetrachloride [21].

2. Materials and methods

2.1. Animals

Guidelines from the Prime Minister's Office of Japan (No. 6 of 27 March 1980) for the care and use of laboratory animals were followed. Male rats (SLC: Wistar strain) were obtained from the Japan SLC Co. The animals were housed in a room at 24 ± 2°, with a 12 hr/12 hr light–dark cycle. Animals were fed commercial laboratory chow (MF, Oriental Yeast Co.) and water *ad lib.* Six-week-old rats were administered D-galactosamine (1 g/kg body weight) intraperitoneally. The control rats received saline.

2.2. Analytical methods

Rats were anesthetized with diethyl ether and killed by collecting the blood from the inferior vena cava, using a syringe containing sodium heparin as an anticoagulant. After perfusion of ice-cooled saline through the portal vein, the organs were removed. The excised tissue was homogenized in 5 vol. of phosphate-buffered saline (10 mM, pH 7.4) under ice cooling. All determinations were made in duplicate experiments with 6–7 animals in each group. Vitamin C was measured according to a specific and sensitive method [11,22] involving chemical derivatization and HPLC. The concentration of α-tocopherol was determined by HPLC [23]. The conditions of HPLC and fluorescence detection (Shimadzu RF-535) have been reported previously [9]. Blood was centrifuged at 13,000 *g* for 5 min at 4° to separate the plasma. The activity of plasma GOT (EC 2.6.1.1) was determined using a diagnostic kit (GOT-UV Test Wako, Wako Pure Chemicals Co.) and is expressed as Karmen units.

Caspase-3 activity was measured as described previously [15,20]. In brief, liver homogenate was centrifuged at 15,000 *g* for 5 min at 4°, and the supernatant, diluted 3- to 4-fold with PBS, was assayed for caspase-3 activity. Plasma diluted 5-fold with PBS was used as an enzyme source. An enzyme solution (1.0 mL) was mixed with 790 μL of a mixture containing 100 mM Tris–HCl, 2 mM EDTA, and 20 mM EGTA. To the resulting mixture, 200 μL of dithiothreitol (final concentration 1 mM) solution and 10 μL of Ac-DEVD-MCA (final concentration 50 μM) solution were added. The reaction was performed at 37°, pH 7.5. After 0, 10, and 20 min, aliquots (190 μL) were taken from the reaction mixture, and 10 μL of $HClO_4$ was added to terminate the reaction. After centrifugation at 15,000 *g* for 5 min at 4°, the fluorescence of the supernatant containing released AMC was determined using a fluorescence spectrophotometer (Shimadzu, RF-1500) with excitation at 380 nm and emission at 460 nm. All assays were essentially linear during this time interval. A standard curve was prepared using solutions of AMC at various concentrations in the assay solution containing 5% $HClO_4$. The activity of the enzyme was expressed as picomoles of AMC liberated per milligram of protein per minute.

Protein concentrations were determined according to the method of Lowry *et al.* [24], using bovine serum albumin as the standard.

Data expressed as means ± SEM were analyzed using StatView software (Abacus Concepts). Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test (PLSD) generated by this program.

2.3. Histological analysis

Eighteen hours after the administration of D-galactosamine or saline (control), the liver was fixed in 4% formalin and embedded in paraffin, and then 5-μm serial sections were stained with hematoxylin-eosin. Apoptotic hepatocytes were detected by the TUNEL method according to Sgonc *et al.* [25]. For the TUNEL method, a kit using fluorescein-dUTP produced by the Roche Diagnostics Co. (Catalogue No. 1684 817) was used, and all treatments were done according to the procedures described by the provider.

3. Results and discussion

3.1. Liver necrosis and apoptosis caused by D-galactosamine

A necrogenic dose [7,18] of D-galactosamine (1 g/kg) was administered intraperitoneally to the rats. After 12 hr, plasma GOT activity did not differ significantly from that of the control group, which was administered saline; however, after 18 hr, it was significantly higher than that of the control group (Table 1). After 24 hr, this activity had increased dramatically compared with that of the control group. These results show that the necrotic process was initiated at around 18 hr and proceeded thereafter.

Table 1
Activities of caspase-3-like protease and GOT 12, 18, and 24 hr after D-galactosamine administration in rats

Treatment	Caspase-3-like protease (pmol/mg protein/min)		Plasma GOT (Karmen units)
	Liver	Plasma	
D-Galactosamine			
After 12 hr	18.1 ± 3.3	0.17 ± 0.07	246 ± 132
After 18 hr	49.3 ± 5.3*	3.79 ± 0.95	1445 ± 191**
After 24 hr	59.8 ± 12.9*	16.7 ± 9.1**	2454 ± 721*
Control	18.3 ± 2.3	0.06 ± 0.02	83.7 ± 3.6

D-Galactosamine (1 g/kg) was administered intraperitoneally to rats. After 12, 18, and 24 hr, activities of caspase-3-like protease in the liver and plasma and of plasma GOT were determined as described in the text. Control rats received saline, and the enzyme activity was determined after 24 hr. Values are means ± SEM for 6–7 rats.

* Indicates a significant differences from the corresponding control group [ANOVA Fisher's protected least significant difference test (PLSD)]: $P < 0.01$.

** Indicates a significant differences from the corresponding control group [ANOVA Fisher's protected least significant difference test (PLSD)]: $P < 0.05$.

Muntane *et al*. [17] and Stachlewitz *et al*. [26] reported that apoptosis was observed histochemically in the rat liver after D-galactosamine administration. To elucidate the mechanism of apoptosis, the activity of caspase-3-like protease, a cysteine protease specifically involved in apoptosis [19], was examined. Caspase-3-like protease activity in the liver, determined using a specific peptide substrate (Ac-DEVD-MCA), increased significantly compared with that in the control group 18 and 24 hr after D-galactosamine administration (Table 1). The maximal activity (59.8 pmol/mg protein/min) of the enzyme was considerably higher than that caused by carbon tetrachloride (24.2 pmol/mg protein/min) [21] or thioacetamide (31.2 pmol/mg protein/min) [20]. These results suggest that D-galactosamine causes a higher apoptosis/necrosis ratio in the liver than either carbon tetrachloride or thioacetamide based on plasma GOT values (an indicator of necrosis), since the activity of caspase-3 is assumed to reflect the extent of apoptosis. The high incidence of apoptosis in D-galactosamine intoxication may be partly explained on the grounds that the toxicity of D-galactosamine is mediated through serum TNF-α [17,27], which causes apoptosis in liver cells by activating caspases [28,29].

Since Ac-DEVD-MCA is also a substrate of caspase-7 [30], the DEVDase activity arises from caspases, which are proteases specifically activated in apoptosis. Therefore, the activity of caspase-3-like protease is a good indicator of apoptosis in animal tissues.

In plasma, caspase-3 activity was barely detectable in the control rats but it increased significantly 24 hr after drug administration, along with a dramatic increase in GOT (Table 1). It is surprising that caspase-3, a protease, showed activity in plasma, which has a strong anti-protease activity. These results indicate that the activity of caspase-3 in the liver and plasma is a reliable biochemical indicator of apoptosis under pathological conditions. Increases in caspase-3 activity in the liver and plasma were reported previously, when typical necrogenic toxins such as thioacetamide [15,20] or carbon tetrachloride [21] were administered to rats, or when liver regeneration after two-thirds partial hepatectomy was inhibited by an α-blocker [31].

These results indicate that D-galactosamine causes apoptosis in the liver and that it involves the activation of caspase-3. Additionally, they demonstrate that apoptosis and necrosis proceed simultaneously in the liver, as caspase-3 is released by secondary necrosis, occurring around 24 hr, as indicated by the high plasma caspase-3 and GOT activities. Itokazu *et al*. [27] reported that liver caspase-3 activity did not increase after D-galactosamine was administered to mice at 3.0 g/kg, an extremely high dose. The discrepancy between their study and ours may be explained by not only the difference in animal species and the method of analyzing the enzyme activity but also by a difference in the dose. It is conceivable that liver necrosis proceeded quickly at 3 g/kg; therefore, liver caspase-3 was released rapidly from the liver into the plasma.

Jaeschke *et al*. [29] reported that endotoxin, a hepatotoxin leading to necrosis, causes a 17-fold activation of caspase-3-like protease in the mouse liver 7 hr after administration. Activation caused by endotoxin was much higher than that observed in the present study, although the animal species was different. The extent of apoptosis caused by D-galactosamine seemed to be much less than that caused by endotoxin. Therefore, it is suggested that D-galactosamine mainly causes necrosis and partial apoptosis leading to secondary necrosis, rather than apoptosis and necrosis in a sequential fashion.

Since measurable caspase-3 activity was detected in the control rat liver, it may be argued that the increase in plasma caspase-3 activity is not caused by apoptosis but is the result of leakage of basal levels of caspase-3 from damaged hepatocytes into the plasma in the same way that GOT is increased. To evaluate the relative contribution of apoptosis to plasma caspase-3 activity, we calculated the activity ratio of plasma caspase-3 and GOT, based on the data in Table 1. If plasma caspase-3 activity originates solely from necrotic cells and not from apoptotic cells, this ratio is assumed to be constant. The ratios $\times 10^4$ were calculated to be 7.2 for the control rats and 6.9, 26.2, and 68.1, respectively, after 12, 18, and 24 hr for the D-galactosamine-treated rats. As the ratios for D-galactosamine-intoxicated rats after 18 and 24 hr were considerably higher than that of the control group, it is concluded that plasma caspase-3 activity is also derived from apoptotic cells in rats given D-galactosamine. Similar results were reported previously in the intoxication of rats by carbon tetrachloride [21] and thioacetamide [15,20].

Histological examinations with hematoxylin-eosin staining (Fig. 1A and B) revealed apoptotic cells in the liver 18 hr after the injection of D-galactosamine. The

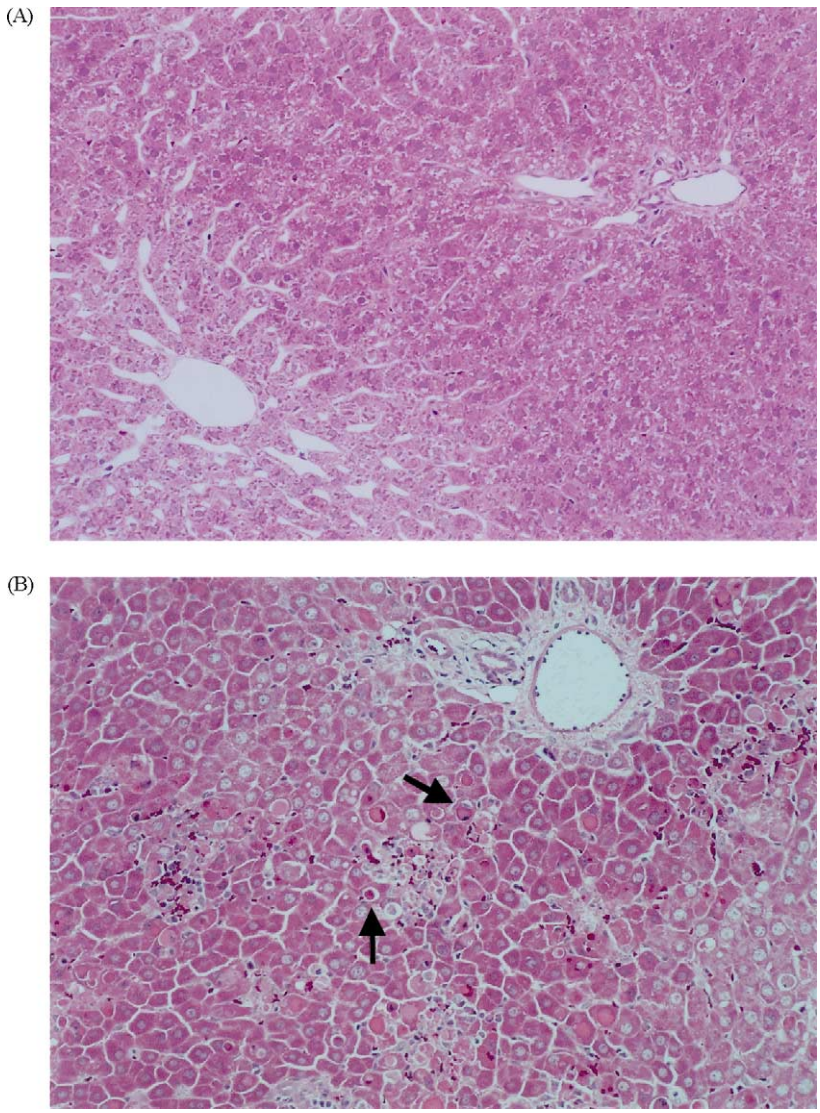

Fig. 1. Histological changes (hematoxylin-eosin staining) in the rat liver 18 hr after saline (control) or D-galactosamine injections. (A) Control. (B) D-Galactosamine-treated. Among degenerated hepatocytes, typical apoptotic cells (shown by arrows) were observed. (Magnification of panels A and B: 150×).

TUNEL method clearly demonstrated many positive cells (Fig. 2A and B), thus confirming the occurrence of apoptosis in the liver 18 hr after the administration of D-galactosamine.

3.2. Changes in the levels of lipid hydroperoxides and vitamins C and E in rats treated with D-galactosamine

Twelve hours after the administration of D-galactosamine, the liver concentration of lipid hydroperoxides, mediators of free radical reactions [11–16], in the treated group did not differ from that in the control group (Table 2). Eighteen hours after the injection of D-galactosamine, when apoptosis and necrosis of the liver cells started based on the increase in liver caspase-3 and plasma GOT levels (Table 1), the concentration of lipid hydroperoxides in the treated liver still did not differ from that in the control group (Table 2). After 24 hr, when extensive necrosis had developed, the liver concentration of lipid hydroperoxides was increased to 1.5-fold of that of the control rats (Table 2). These observations suggest that free radical reactions, i.e. lipid peroxidation, are caused by D-galactosamine, and that necrosis is free radical-mediated. An increase in TBARS [6,7], aldehydic products of lipid peroxidation, and the decrease in glutathione [6,7] induced by D-galactosamine administration also support the involvement of lipid peroxidation.

Twelve hours after galactosamine administration, the hepatic vitamin C level in the treated group did not differ from that in the control group; however, it decreased dramatically to 60% of the level in the control group after 18 hr (Table 2). After 24 hr, the vitamin C level in the liver remained at 58% of the level in the control group (Table 2). Although vitamin C is not an essential vitamin for Wistar rats, excessive liver damage and oxidative stress caused by D-galactosamine may deplete vitamin C. Recently, we reported that the concentration of vitamin C is decreased in the tissues of Wistar rats with streptozotocin-induced

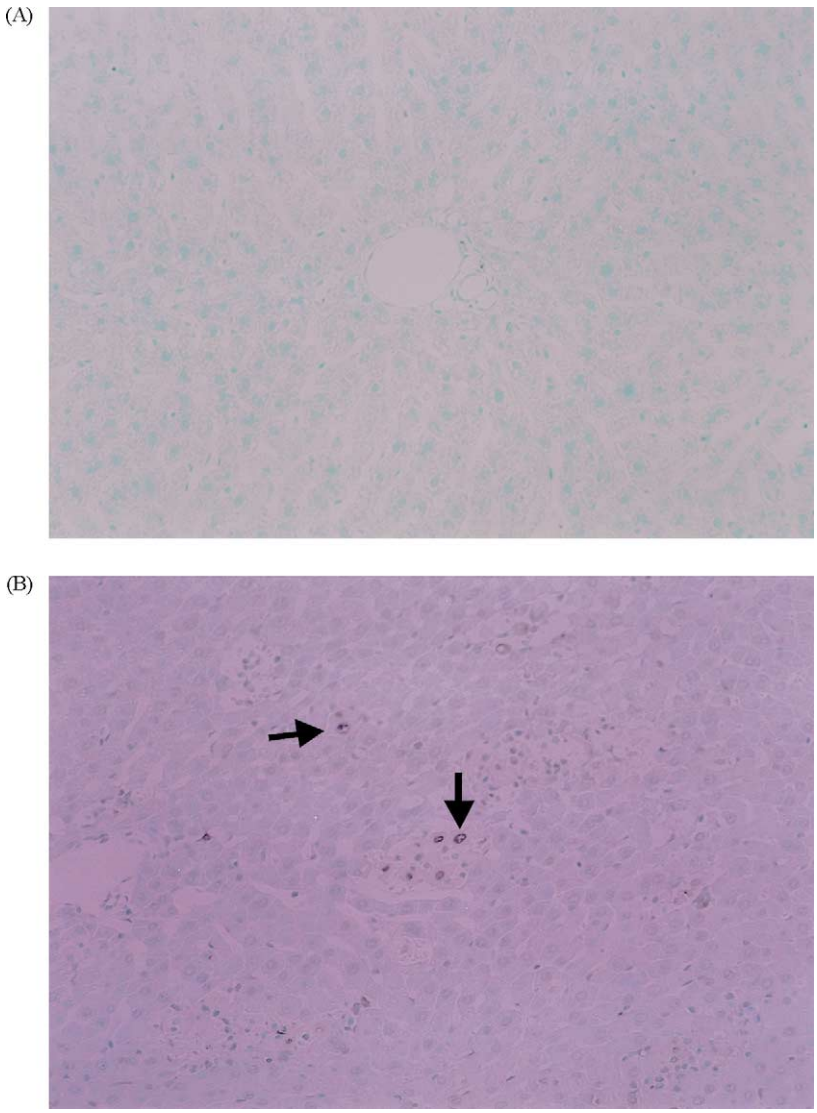

Fig. 2. Histological changes (the TUNEL method) in the rat liver 18 hr after saline (control) or D-galactosamine injections. (A) Control. (B) D-Galactosamine-treated. Many apoptotic cells were observed, typical examples of which are indicated by arrows. (Magnification of panels A and B: 150×).

diabetes [14], and also after the administration of thioacetamide [8] or carbon tetrachloride [12], while the liver concentration of vitamin E is not always decreased under these conditions [8,12,14]. These results indicate that the tissue concentration of vitamin C is a good indicator of oxidative stress even in Wistar rats. The plasma concentration of vitamin C was not changed by D-galactosamine administration (Table 2).

Table 2
Liver lipid hydroperoxides and concentrations of vitamin C and vitamin E in the liver and plasma in rats 12, 18, and 24 hr after D-galactosamine treatment

	Liver lipid hydroperoxides (pmol/mg protein)	Vitamin C (nmol/g tissue)		Vitamin E (nmol/g tissue)	
		Plasma	Liver	Plasma	Liver
D-Galactosamine					
After 12 hr	216.2 ± 23.7	50.6 ± 3.0	1514 ± 191	10.0 ± 1.6	27.1 ± 1.2
After 18 hr	247.6 ± 15.1	42.1 ± 1.8	807 ± 50*	6.2 ± 0.6*	29.8 ± 2.4**
After 24 hr	322.6 ± 25.0*	54.8 ± 6.1	784 ± 41*	8.5 ± 1.4	28.3 ± 2.4
Control	212.7 ± 13.0	44.9 ± 3.7	1344 ± 40	11.9 ± 1.1	23.6 ± 1.5

D-Galactosamine (1 g/kg) was administered intraperitoneally to rats. After 12, 18, and 24 hr, concentrations of lipid hydroperxides in the liver, and of vitamin C and vitamin E in the plasma and liver were determine as described in the text. Twenty-four hours after the administration of saline, determinations were also made for control rats. Values are means ± SEM of 6–7 rats.

* Indicates a significant difference from the control group [ANOVA Fisher's protected least significant difference test (PLSD)]: $P < 0.01$.

** Indicates a significant difference from the control group [ANOVA Fisher's protected least significant difference test (PLSD)]: $P < 0.05$.

The hepatic α-tocopherol level increased 18 hr after D-galactosamine administration (Table 2). Since vitamin C is decreased markedly in the liver by D-galactosamine, oxidative stress should be increased; however, liver vitamin E increased as has been reported previously [32]. The elevation of the liver α-tocopherol level may be explained partly on the grounds that vitamin E is mobilized from plasma to alleviate the oxidative stress as evidenced by the significant decrease in the plasma α-tocopherol level 18 hr after D-galactosamine administration (Table 2). Twenty-four hours after D-galactosamine injection, oxidative stress increased further, based on the increased level of lipid hydroperoxides and the decrease in vitamin C (Table 2). However, the hepatic concentration of α-tocopherol did not change (Table 2). These results also support the view that the tissue concentration of vitamin C is a better indicator of oxidative stress than that of vitamin E.

3.3. Oxidative stress, apoptosis, and necrosis

Eighteen hours after the administration of D-galactosamine, caspase-3 was activated and plasma GOT was elevated. Simultaneously, vitamin C was decreased in the liver. The involvement of reactive oxygen species in apoptosis has been suggested [33]. Recently, we reported [33,34] that hydroxyl radicals are involved in the activation of caspase-3 during apoptosis of HL-60 cells induced by anticancer drugs, based on a systematic evaluation of different kinds of antioxidants. We reported further [33] that oxidative stress during the apoptotic process was not as extensive and did not markedly decrease the cellular concentration of vitamin E. This result is consistent with the report describing that low concentrations of hydrogen peroxide induce apoptosis, while necrosis occurs when millimolar amounts of the oxidant are present [35].

Therefore, it is suggested that the decrease in vitamin C in the liver 18 hr after D-galactosamine administration is related to necrosis, judging from the large increase in GOT at this time. Twenty-four hours after the injection of D-galactosamine, plasma GOT was increased further and lipid hydroperoxides in the liver increased significantly. This is consistent with the case of thioacetamide intoxication [15], which typically increases lipid hydroperoxides and decreases vitamin C in the liver. Whether oxidative stress is a cause or a result of necrosis is an important issue. In a previous paper [21], we reported that necrosis caused by carbon tetrachloride intoxication does not involve extensive lipid peroxidation. This result indicates that necrosis is not a cause of extensive oxidative stress. Rather, our present observations indicate that D-galactosamine-induced necrosis is the result of extensive free radical-generating reactions or oxidative stress, as is the case following thioacetamide treatment [15].

It is well accepted that, like actinomycin D, D-galactosamine causes the loss of uridine nucleotides, leading to inhibition of RNA synthesis [1]. Recently, we reported [33,34] that actinomycin D causes apoptosis in HL-60 cells involving the generation of hydrogen peroxide and activation of caspase-3. It is possible that the inhibition of synthesis of housekeeping enzymes results in enhanced oxidative stress. The relationship between the inhibition of enzyme synthesis and oxidative stress is an important problem to be solved in the future.

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