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**SHORT COMMUNICATION**

# Change in Caspase-3-Like Protease in the Liver and Plasma during Rat Liver Regeneration Following Partial Hepatectomy

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**ABSTRACT.** Recent studies have shown that many factors orchestrate liver regeneration after a two-thirds partial hepatectomy (PH). However, the termination mechanism in liver regeneration has not been thoroughly studied. In this paper, we report that the activity of liver caspase-3-like protease, which is specifically activated in apoptosis, increases 18, 36, and 48 hr after PH during maximal hepatocyte proliferative activity. This is the first study that shows the activation of an apoptosis-executing enzyme during physiological liver regeneration. These results suggest that apoptosis is induced in each surge of DNA synthesis as the termination mechanism. When phenoxybenzamine, an α-blocker that has been reported to inhibit DNA synthesis during liver regeneration, was injected 8 hr after PH, the caspase-3-like activity in the liver peaked at 15 hr after PH and the enzyme activity also increased in plasma at 18 and 24 hr after PH in sharp contrast to the case of normal regeneration. These results indicate that extensive apoptosis is caused by phenoxybenzamine and that the secondary necrosis of apoptotic cells results in the increase of caspase-3-like protease activity in the plasma. BIOCHEM PHARMACOL **60**;12:1883–1886, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** apoptosis; caspase-3; liver regeneration; partial hepatectomy; $\alpha_1$-receptor; TGF-β

Liver regeneration after PH† in a rat is remarkably rapid, attaining its original size in 7–10 days [1], and DNA synthesis is almost complete in 72 hr [2]. The events involved in the regenerative response are precise, carefully orchestrated, and highly regulated [3]. Growth-promoting factors, such as HGF, EGF, TGF-α, TNF-α, interleukin-6, norepinephrine, and insulin, have been shown to regulate liver regeneration [2, 4]. Paradoxically, the increased expression of TGF-β, a growth-inhibitory factor, occurs during maximal hepatocyte proliferative activity [5]. It has been suggested that TGF-β plays a critical role in terminating the regenerative response to PH once recovery of the liver mass has been accomplished [6]. It is well documented that TGF-β1 causes apoptosis in hepatocytes [7]. Although Fan *et al*. [8] suggest that apoptosis is involved in the remodeling of the regenerating liver based on the change in transcripts of apoptosis-associated genes, no biochemical evidence showing apoptosis during physiological liver regeneration has been obtained. Helvering *et al*. [9] reported that PH significantly decreases the number of hepatocytes undergoing apoptosis based on histochemical study. It is very important to evaluate the role of apoptosis during liver regeneration in relation to the termination mechanism.

To evaluate the extent of apoptosis during liver regeneration, a convenient method is necessary. We recently reported [10, 11] that hepatic caspase-3-like protease activity, which is specific to the apoptotic process [12], is a reliable indicator of apoptosis caused by thioacetamide. In this study, we determined the change in the enzyme activity and demonstrated that caspase-3-like protease is activated significantly when a large body of hepatic cells is in the S phase. Furthermore, we demonstrated that apoptosis and necrosis are enhanced significantly when DNA synthesis is inhibited by the administration of phenoxybenzamine, an α-blocker reported to strongly inhibit DNA synthesis during liver regeneration [13, 14].

## MATERIALS AND METHODS

### *Animals and Treatments*

Guidelines from the Prime Minister's Office of Japan (No. 6; 27 March 1980) for the care and use of laboratory animals were followed. Eight-week-old 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*.

PH was performed under diethyl ether anesthesia by the

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† *Abbreviations*: PH, two-thirds partial hepatectomy; HGF, hepatocyte growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; and GOT, glutamate-oxaloacetate transaminase.

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procedure of Higgins and Anderson [1]. Phenoxybenzamine in 50% propylene glycol (10 mg/kg body weight) was injected i.p. at 8 hr after PH, as described [13].

### *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, at various times after PH. After perfusion of ice-cooled saline through the portal vein, organs were removed. The excised tissue was homogenized in 5 vol. of ice-cold PBS (10 mM, pH 7.4). All determinations were made in duplicate experiments with 3–5 animals.

Blood was centrifuged at 10,000 *g* for 5 min at 4° to separate the plasma. The activity of plasma GOT (EC 2.6.1.1) was determined using diagnostic kits (GOT-UV Test, Wako Pure Chemicals, Co.) and expressed as Karmen units. Caspase-3-like protease activity was determined using a specific peptide substrate, acetyl-DEVD-α-(4-methyl-coumaryl-7-amide) and expressed as picomoles per milligram protein per minute as described previously [10, 11]. Protein concentrations were determined according to the methods of Lowry *et al*. [15] using bovine serum albumin as the standard.

Data, expressed as means ± SD, were analyzed by ANOVA, using StatView software (Abacus Concepts). Differences between the group means were considered significant at $P < 0.05$, using the Fischer procedure generated by this program.

## RESULTS AND DISCUSSION

### *Change in the Activity Of Caspase-3-Like Protease in the Liver and Plasma Following PH*

Recently, two different modes of cell death, apoptosis and necrosis, have received much attention, and biochemical mechanisms leading to apoptosis have been studied extensively using a cultured cell system. In contrast, only limited studies are available on the occurrence of apoptosis in animal tissues [16]. The termination mechanism for liver regeneration has not been elucidated well, and apoptosis caused by TGF-β1 has been suggested as one possibility [8]. However, definite experimental evidence has not been obtained. Therefore, a convenient biochemical method to detect apoptosis in animal tissues is necessary to evaluate the role of apoptosis during liver regeneration. We recently reported [10, 11] that the hepatic activity of caspase-3-like protease, which is finally activated among caspases in apoptosis [17], was a reliable biochemical indicator of apoptosis caused by thioacetamide. In this study, we followed the change in the activity of caspase-3 during liver regeneration.

At 2, 4, 6, 12, and 15 hr after PH, the activity of hepatic caspase-3-like protease did not change (Fig. 1). After 18 hr, caspase-3-like protease activity (19.5 ± 2.1 pmol/mg protein/min) was significantly higher than that of the regenerating liver at 2, 4, 6, 12, and 15 hr after PH and was also higher than that of sham-operated rats 18 hr after the operation (15.2 ± 2.1 pmol/mg protein/min). The activity of caspase-3-like protease in the liver decreased at 24 hr (Fig. 1) to a level that was not significantly different from that of sham-operated rats (12.5 ± 1.2 pmol/mg protein/min) 24 hr after the operation. At 36 and 48 hr after PH, the activity of caspase-3-like protease showed a significantly higher peak value than at 24 hr after PH (Fig. 1). These results demonstrate that the activation of caspase-3-like protease takes place significantly at 18, 36, and 48 hr after PH, when a large body of hepatic cells is in the S phase [4]. This study is the first that shows the activation of the apoptosis-executing enzyme during physiological liver regeneration. The present results suggest that apoptosis is induced in each surge of DNA synthesis as the termination mechanism possibly to prevent excessive response of the liver, although it has been assumed that apoptosis plays a critical role when recovery of the liver mass is fully accomplished [6]. It should be noted that the fluctuation pattern of caspase-3-like activity in the liver after PH resembles the patterns of mRNA of TGF-β1 [5, 18] and bax [19], which promote apoptosis [20, 21]. Furthermore, it has also been reported [19] that the major 3.0 kb transcript of *bcl-2*, an apoptosis inhibitory gene, is decreased dramatically by 18 hr after PH. It is generally assumed that apoptotic cells in animal tissues are phagocytosed rapidly by neighboring cells to prevent inflammation. It is conceivable that the clearing mechanism hampered the histologi-

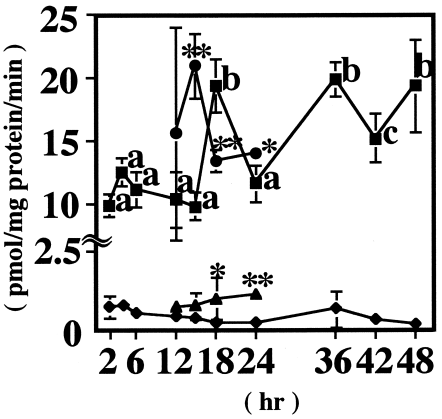


**FIG. 1. Change in the activity of caspase-3-like protease in plasma and liver. The control group of rats was subjected to PH. The experimental group of rats was injected with phenoxybenzamine at 8 hr after PH. At indicated times after PH, caspase-3-like protease activity was assayed in the liver and plasma as described in the text. Values are means ± SD of 3–5 rats. Where there is no bar shown, the SD was smaller than the symbol. Different letters by the bars indicate significant differences among values of the control group at various time points after PH, determined by the Fischer procedure ($P < 0.05$). Asterisks indicate significant differences between the control and the experimental groups compared at the same time after PH by the Fischer procedure (*$P < 0.05$, **$P < 0.01$). For the control group: liver (■) and plasma (♦); for the experimental (phenoxybenzamine-treated) group: liver (●) and plasma (▲).**

cal detection of apoptotic cells during the early phase of liver regeneration as reported [9].

The activity of caspase-3-like protease in the plasma of partially hepatectomized rats was negligibly low (Fig. 1) and similar to that in plasma of sham-operated rats 18 and 24 hr after the operation (0.15 ± 0.041 and 0.13 ± 0.13 pmol/mg protein/min, respectively). These results suggest that the rate of apoptosis during physiological regeneration is regulated in the region where apoptotic cells are eliminated effectively by neighboring cells to prevent secondary necrosis that leads to an elevation of caspase-3 activity in plasma [10, 11].

### *Change in the Activity of Caspase-3-Like Protease in the Liver and Plasma of Rats Administered Phenoxybenzamine 8 hr after PH*

It is well established that catecholamines are important physiological regulators of liver regeneration after PH [2, 4, 13, 14]. We reported [13, 14] that administration of phenoxybenzamine, an α-blocker, to rats 8 hr after PH strongly inhibits DNA synthesis in the liver by preventing the induction of thymidylate synthase and thymidine kinase, which are rate-determining enzymes of DNA synthesis [22]. As a result, the DNA content and the liver weight 24 hr after PH are completely suppressed to a level almost identical to the levels just after PH [13]. We observed that β-blocker had no effect [13]. Calcium ion involvement in the regulation of liver regeneration was indicated by a study [23] that demonstrated that hypocalcemia caused by a thyroparathyroidectomy strongly inhibits DNA synthesis during liver regeneration and also by an experiment [24] showing that calcium channel blockers prevent liver regeneration effectively. Since calcium ion is a second messenger of $\alpha_1$-receptors [25], we suggested that $\alpha_1$-receptors among catecholamine receptors of the liver were involved in the regulation of liver regeneration [23, 24]. However, the role of $\alpha_1$-receptors in liver regeneration has not been clarified.

When phenoxybenzamine, which is known to inhibit DNA synthesis during liver regeneration [13, 14], was injected into rats at 8 hr after PH, the hepatic activity of caspase-3-like protease peaked at 15 hr (21.0 ± 2.5 pmol/mg protein/min) after PH. In addition, the enzyme activity was also significantly higher at 24 hr after PH than that of rats subjected to only PH (Fig. 1).

The caspase-3 activity *in the plasma* (where strong antiprotease activity exists) of phenoxybenzamine-treated rats was significantly higher at 18 and 24 hr (0.93 ± 0.67 and 1.08 ± 0.08 pmol/mg protein/min, respectively) after PH than in rats not administered the drug (Fig. 1). Caspase-3-like activity in plasma of normal and partially hepatectomized rats was always negligible (Fig. 1). It has been reported [13, 14] that caspase-3-like protease activity increases in the plasma of rats that were administered thioacetamide and that the enzyme activity was released from the liver into the plasma by secondary necrosis of apoptotic liver cells. The present findings support the idea that the administration of phenoxybenzamine causes extensive apoptosis involving the activation of caspase-3 approximately 15 hr after PH and that apoptosis caused by the blocker results in secondary necrosis to augment plasma caspase-3 and GOT (*vide post*) activities approximately 18–24 hr after PH. These results suggest that catecholamines play an important role in controlling the extent of apoptosis during the rapidly proliferating phase, when an apoptogenic factor such as TNF-β1 is increased simultaneously [5, 18]. This idea may also be supported by a study [26] which reported that norepinephrine decreases the hepatocyte sensitivity to TGF-β1 between 12 and 18 hr after PH. We could not detect a typical DNA ladder at 12, 15, 18, and 24 hr after PH or in the liver of phenoxybenzamine-treated rats at 15, 18, and 24 hr after PH (data not shown). It has been reported [27] that apoptosis induced by TGF-β1 takes place within 5 hr without significant DNA fragmentation. This might be a characteristic of apoptosis caused by TGF-β1.

### *Plasma GOT values*

Plasma GOT values after 24 hr were 123 ± 37, 520 ± 354, and 1930 ± 950 Karmen units for the sham-operated, partially hepatectomized, and phenoxybenzamine-treated partially hepatectomized rats, respectively. The plasma GOT value in the α-blocker-treated rats was significantly higher than that of the other groups, demonstrating that more extensive necrosis took place by treatment with the drug.

In conclusion, the present study suggests that apoptosis is induced in each surge of DNA synthesis as the termination mechanism during liver regeneration after PH. When phenoxybenzamine was injected 8 hr after PH, the caspase-3-like activity in the liver peaked at 15 hr after PH and the enzyme activity also increased in plasma at 18 and 24 hr after PH, in sharp contrast to the case of normal regeneration. These results indicate that extensive apoptosis is caused by phenoxybenzamine and that the secondary necrosis of apoptotic cells results in the increase of caspase-3-like protease activity in the plasma.

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