Expression of γ -Glutamylcysteine Synthetase mRNA in Regenerating Rat Liver

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Changes in the level of GSH and γ -Glutamylcysteine Synthetase (γ -GCS) activity with time were determined during regeneration of rat liver after partial hepatectomy. The GSH level and γ -GCS activity increased in regenerating rat liver, reaching a maximum (2.1- and 1.5-fold that in sham-operated controls) on day 2, respectively. Furthermore, γ -GCS mRNA expression increased within 24 h and reached maximum on day 2. These results suggest that the increased GSH level in regenerating rat liver after partial hepatectomy is associated with the transcriptional activation of γ -GCS gene and the sequence elevation in γ -GCS activity.

Key words glutathione; γ -glutamylcysteine synthetase; regenerating rat liver

Glutathione (GSH) is a tripeptide, L- γ -glutamyl-cysteinyl-glycine, which is a critical factor for homeostasis of the cellular redox state and protects cells from oxidative injury such as that caused by ionizing radiation, heavy metals, and alkylating chemotherapeutic agents.^{1,2)} GSH is synthesized in most mammalian cells by the action of two ATP-requiring GSH-synthesizing enzymes, γ -glutamylcysteine synthetase (γ -GCS) and glutathione synthetase. γ -GCS catalyzes the rate-limiting step of GSH synthesis and undergoes feedback inhibition by GSH.³⁾

In previous paper, we reported that the level of GSH increased during the regeneration process of rat liver, due to an increase in cysteine content and activation of γ -GCS.⁴⁾ It has been reported the induction of de novo GSH synthesis by oxidative stress is associated with the transcriptional activation of the γ -GCS gene and the sequence elevation in γ -GCS activity.^{5–7)} To investigate the physiological role of the elevation of GSH level in regenerating rat liver in more detail, we decided to examine if γ -GCS mRNA is expressed in regenerating rat liver. We found that the activation of γ -GCS activity in regenerating rat liver is associated with the transcriptional activation of γ -GCS gene.

MATERIALS AND METHODS

Animals and Treatments Male Wistar rats (180–200 g) were obtained from Shizuoka Laboratory Animal. They were given tap water and food (MF, Oriental Yeast) *ad libitum* and were housed at 25–27 °C on a 12 h light/dark cycle. Partial hepatectomy (corresponding to 65–70% resection of the liver) was performed under ether anesthesia according to the procedure of Higgins and Anderson.⁸⁾ Control animals were subjected to a sham operation. Immediately after operation and again 20 h later, penicillin in saline (20000 units/kg i.p.) was administered to the hepatectomized and sham-operated animals. The rats were killed under ether anesthesia at indicated times after operation, the livers were resected and then stored at –80 °C until use.

Measurement of GSH Content GSH content in rat liver was measured according to the modified method of Griffith.⁹⁾

Assay of γ -GCS Activity The liver (1 g) was homogenized in 5 volumes 150 mM KCl, 1 mM MgCl₂ containing 5 mM 2-mercaptoethanol using a Potter-Elvehjem homogenizer

with a Teflon pestle. After centrifugation at 15000 *g* for 20 min at 4 °C, the supernatant was used as the enzyme solution. The enzyme activity was measured as described in the literature.¹⁰⁾

Northern Hybridization A cDNA probe for the catalytic subunit of γ -GCS was kindly donated by Dr. D. Eaton (University of Washington, U.S.A.).¹¹⁾ Methods for isolation of total RNA and Northern blot analysis were essentially based on those of Sambrook *et al.*¹²⁾ Briefly, total RNA (10 μ g/lane as determined by absorbance at 260 nm) isolated from rat liver was subjected to electrophoresis in 1.0% agarose containing formaldehyde, subsequently transferred to a nylon membrane filter, and hybridized at 42 °C with random-primed ³²P-labeled probe for γ -GCS. After hybridization, the filter was washed in 2 $\times$ SSPE (0.75 M NaCl, 43 mM sodium phosphate, 6 mM EDTA) containing 0.1% SDS at room temperature for 30 min and sequentially in 0.2 $\times$ SSPE containing 0.1% SDS at 50 °C for 1 h. The filter was autoradiographed and analyzed using a Fujix Bio-Analyzer BAS-2000 (Fuji Photo Film, Japan).

Protein Determination Protein concentrations were determined by the method of Lowry *et al.* using bovine serum albumin as a standard.¹³⁾

RESULTS AND DISCUSSION

Liver regeneration has been used as a model for regulated proliferation for many years. In rats, following surgical removal of 70% of the liver, the remaining hepatocytes behave as a synchronized cell population, and are activated to undergo mitosis. While the factors signaling both the initiation and termination of proliferation have not been conclusively established, a number of growth factors, including hepatocyte growth factor, insulin, epidermal growth factor, glucagon, and vasopressin, appear to be necessary for this process. In addition, a number of intracellular signaling events have been shown to occur following partial hepatectomy. It has been reported that there is an increase in the concentration of free amino-nitrogen in regenerating liver,¹⁴⁾ and this rise may be due to a change in the rate of protein and urea synthesis.

We have also shown that the GSH level increased in regenerating rat liver, due to the activation of γ -GCS.⁴⁾ Since GSH synthesis is thought to be an important factor in cellular de-

Fig. 1. Glutathione Content (A) and γ -GCS Activity (B) in Rat Liver after Partial Hepatectomy or Sham Operation

GSH content (A) and γ -GCS activity (B) in rat liver were assayed by the methods described in materials and methods. Values are means $\pm$ S.E. ($n=3-5$).

●, partial hepatectomy; ○, sham operation.

fense against some forms of oxidative stress, it is important to prevent any activation of γ -GCS in the regenerating rat liver after partial hepatectomy.

In this experiment, we investigated if the activation of γ -GCS in regenerating liver was associated with transcriptional activation of the γ -GCS gene. Figures 1(A) and (B) show the GSH content and γ -GCS activity in rat liver after partial hepatectomy or sham operation, respectively. After partial hepatectomy, the GSH content in the liver increased within 24 h and reached a maximum (2.1-fold that in sham-operated controls) on day 2 (Fig. 1(A)). The activity of γ -GCS, which is the rate-limiting enzyme for GSH synthesis, also increased in regenerating liver, with a maximal (1.5-fold that in sham-operated controls) occurring on day 2 (Fig. 1(B)).

Fig. 2. Northern Blot of γ -GCS mRNA in Rat Liver after Partial Hepatectomy or Sham Operation

After partial hepatectomy, total RNA was extracted at various periods of time and fractionated by 1% agarose gel electrophoresis. Northern blot hybridization (A) was performed using the 32 P-labeled γ -GCS cDNA.

The relative level of γ -GCS mRNA was determined using a scanning densitometer and normalized to the level of the control (B).

●, partial hepatectomy; ○, sham operation.

Nextly, in order to examine if the activation of γ -GCS in regenerating liver was due to expression of γ -GCS mRNA, γ -GCS gene expression was measured by Northern analysis probed with 32 P-labeled γ -GCS cDNA (Fig. 2). The γ -GCS probe hybridized with a single band around 3.7 kilobases in total RNA isolated from rat liver, which is consistent with the published size of rat γ -GCS mRNA. As shown in Fig. 2, γ -GCS mRNA expression increased within 24 h and reached a maximum (3.7-fold that in sham-operated controls) on day 2, and then returned to the control level on day 4 after partial hepatectomy. These results suggested that the increase in GSH in regenerating rat liver after partial hepatectomy was associated with the transcriptional activation of the γ -GCS gene and the sequence elevation in γ -GCS activity.

Agents known to cause oxidative stress have been shown to increase γ -GCS activity in a variety of cells and organs. In all cases, the increase in γ -GCS activity is associated with an increase in the transcription of γ -GCS. Wood *et al.* demonstrated that both renal GSH and γ -GCS mRNA were markedly elevated following 3 weeks of administration of methylmercury hydroxide to male F344 rats.⁵ They sug-

gested that the increase in GSH biosynthesis was an adaptive response to mercury-induced oxidative stress in the kidney. In 1991, Utley and Mehendale also suggested that the change in GSH content occurs as a protection mechanism in response to chemical-induced oxidative stress.⁶⁾ Recent efforts have focussed on elucidating the molecular mechanisms of the oxidative stress-induced increase in γ -GCS expression. The 5'-flanking region of the gene that encodes human γ -GCS has been cloned and sequenced.^{15,16)} The region from -817 to +45 on the nucleotide sequence of human γ -GCS contains AP-1 (activator protein-1) or AP-1-like responsive elements that have been found to be critical in mediating the effects of hydrogen peroxide and tumor necrosis factor on γ -GCS promoter activity.¹⁷⁻¹⁹⁾ A recent study by Sekhar *et al.* further confirmed the importance of AP-1 in mediating the effect of oxidative stress on γ -GCS transcription.²⁰⁾ In addition, Mulcahy *et al.* described a critical distal antioxidant response element located between -3802 and -2752, which mediates constitutive and β -naphthoflavone-inducible expression in Hep G2 cells.¹⁵⁾ Oxidative stress also induces NF- κ B (nuclear factor κ B) activity, and Urata *et al.* showed that blocking activation of NF- κ B by antisense strategies prevented the cytokine-induced increase in γ -GCS transcription in mouse endothelial cells.²¹⁾

From these pieces of evidence, it seems likely that the increase in GSH content and activation of GSH synthesis in regenerating rat liver after partial hepatectomy are due to some form of oxidative stress which is caused by partial hepatectomy.

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