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Elevation of Glutathione Level in Rat Hepatocytes by Hepatocyte Growth Factor *via* Induction of γ-Glutamylcysteine Synthetase[1]

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Hepatocyte growth factor (HGF) was found to cause a dose- and time-dependent increase in intracellular glutathione (GSH) level (2.0-fold in 24 h with 5 ng/ml) in rat hepatocytes. The activity of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme of GSH biosynthesis, was also increased by HGF (1.7-fold in 24 h with 5 ng/ml). Northern blot analysis revealed the induction of γ-GCS mRNA. These increases by HGF were synergically stimulated by the addition of insulin. In contrast, epidermal growth factor had no effect on GSH level and γ-GCS activity in hepatocytes. These results suggested that the induction of GSH synthesis by HGF is associated with the transcriptional activation of the γ-GCS gene and the subsequent elevation of γ-GCS activity.

Key words: acetaminophen, γ-glutamylcysteine synthetase, glutathione, hepatocyte, hepatocyte growth factor.

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is present in most mammalian cells and plays an important role in the cellular defense system against oxidative stress (*1*). GSH is synthesized intracellularly by the consecutive reactions of two ATP-dependent enzymes, γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (*2*). The first reaction, which is catalyzed by γ-GCS, is the rate-limiting step and undergoes feedback inhibition by GSH (*3*). Increased GSH synthesis has been suggested to be an adaptive response to oxidative stress. Since γ-GCS is the rate-limiting step in GSH synthesis, its induction by sublethal oxidative stress could play a key role in maintaining or elevating cellular GSH content. In addition to oxidative stress, several other conditions, such as drug resistance, hormones, plating density, and treatment with certain antioxidants, are also known to influence the γ-GCS activity and the steady-state γ-GCS mRNA level.

In a previous paper, we reported that the level of GSH increased during the regeneration of rat liver, due to the increase of cysteine content and the activation of γ-GCS activity (*4*). This increase of GSH level in regenerating rat liver has also been reported by other workers (*5, 6*).

Liver regenerates in response to partial hepatectomy or liver injury, and the regeneration is thought to be triggered by humoral hepatotropic factors. Hepatocyte growth factor (HGF) is considered to be a major humoral hepatotropic factor that induces mitosis of hepatocytes during liver regeneration (*7, 8*). In experimental animals, the mRNA level of HGF has been shown to increase in partially hepatectomized rats (*9-11*) and hepatotoxin-treated rats (*12*), and the level of endogenous HGF in the plasma of rats increased after partial hepatectomy and liver injury (*10-13*). In addition, the level of HGF was found to increase significantly in acute hepatitis and other liver diseases which are known to require liver regeneration for recovery. These observations suggest that HGF plays an important role in liver regeneration.

Against this background, we examined the effects of HGF on the GSH level and γ-GCS activity in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Animals—Male Wistar rats (180-200 g) were obtained from Shizuoka Laboratory Animal. They were provided tap water and food (MF, Oriental Yeast) *ad libitum* and housed at 25-27°C on a 12 h light/dark cycle.

Chemicals—Recombinant human HGF was purchased from Funakoshi. Insulin was from Sigma. [α-^{32}P]dCTP was from Du Pont-New England Nuclear. Cell culture reagents were from Dainippon Pharmaceutical. Other compounds were from Wako Pure Chem.

Preparation and Primary Culture of Isolated Hepatocytes—Hepatocytes, having more than 85% initial viability as measured by trypan blue exclusion, were isolated from rats by *in situ* perfusion technique with collagenase (*14*). The isolated hepatocytes were suspended in William's medium E supplemented with 5% fetal bovine serum, 1 μg/ml insulin, 1 μM dexamethasone, and 100 μg/ml kanamycine, inoculated at a cell density of 4×10^4 cells/0.2 ml/cm^2 onto 16- or 35-mm diameter Corning plastic dishes, which had been coated with rat tail collagen, and cultured in a humidified atmosphere of 5% CO_2:95% air at 37°C. After

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Abbreviations: GSH, glutathione; γ-GCS, γ-glutamylcysteine synthetase; HGF, hepatocyte growth factor; EGF, epidermal growth factor; APAP, acetaminophen; CCl_4, carbon tetrachloride; PBS, phosphate buffered saline.

the attachment period of 2 h, the medium was replaced by a serum-free medium containing 100 μg/ml kanamycine, and culture was continued for a further 21 h. HGF (0, 2.5, or 5 ng/ml), EGF (5 ng/ml), and/or insulin (1 μg/ml) were added to the medium, and culture was continued for various periods of time.

Determination of Levels of GSH in Hepatocytes—For the determination of levels of intracellular GSH, cells cultured in 16-mm dishes were washed three times with ice-cold phosphate buffered saline (PBS), then 250 μl of 2% perchloric acid was added to the cell pellet, which was kept at 4°C for 30 min. Two hundred microliters of 2% perchloric acid solution was transferred to an Eppendorf tube, neutralized with 40 μl of 4 M K_2HPO_4, then centrifuged at 5,000 × *g* for 5 min. The supernatant was used for determination of GSH. GSH was measured by a modification of the method of Griffith (*15*).

Assay of γ-GCS Activity in Hepatocytes—Hepatocytes cultured in 35-mm dishes were washed three times with ice-cold PBS, then scraped off the dishes with a silicon policeman. The hepatocytes were homogenized in 1.2 ml of 150 mM KCl, 1 mM $MgCl_2$ containing 5 mM 2-mercaptoethanol using a Potter-Elvehjem homogenizer with a Teflon pestle. After centrifugation at 15,000 × *g* for 20 min at 4°C, the supernatant was used as enzyme solution. The enzyme activity was measured as described (*16*).

Northern Hybridization—A cDNA probe for the catalytic subunit of γ-GCS was kindly donated by Dr. D. Eaton (University of Washington, USA) (*17*). Methods for isolation of total RNA and Northern blot analysis were essentially based on those of Sambrook *et al.* (*18*). Briefly, total cellular RNA (10 μg/lane as determined by absorbance at 260 nm) isolated from rat hepatocytes was subjected to electrophoresis in 1.0% agarose containing formaldehyde, then transferred to nylon membrane filter and hybridized at 42°C with random-primed ^{32}P-labeled probe for γ-GCS. After hybridization, the filter was washed in 2×SSPE (0.75 M NaCl, 43 mM sodium phosphate, 6 mM EDTA) containing 0.1% SDS at room temperature for 30 min, then in 0.2×SSPE containing 0.1% SDS at 50°C for 1 h. The filter was autoradiographed and analyzed using a Fujix Bio-Analyzer GAS-2000 (Fuji Photo Film, Tokyo).

Hepatotoxicity Induced by Acetaminophen (APAP)—Hepatotoxicity was induced by addition of 1 mM APAP into culture medium. APAP was dissolved directly into the medium. The degree of hepatotoxicity was judged by measurement of aspartate aminotransferase (AST) leakage into the medium. AST activity was assayed using a commercially available kit (Transaminase CII-test Wako). HGF (5 ng/ml) was added into the medium 1 h prior to exposure to 1 mM APAP. After incubation for 24 h, the levels of intracellular GSH were determined.

Protein Determination—Protein concentration was determined by the method of Lowry *et al.* using bovine serum albumin as a standard (*19*).

RESULTS

Effect of HGF on GSH Levels—Rat hepatocytes were treated with HGF in fresh culture medium for 6, 12, 24, and 30 h, and the cellular GSH content was determined (Fig. 1). HGF (2.5 or 5 ng/ml) caused a dose- and time-dependent increase in intracellular GSH content. Intracellular GSH increased to 2.0 times the control level after 24 h.

Effect of HGF on γ-GCS Activity—Cell extracts (15,000 × *g* supernatant) were used to test the effect of HGF on the γ-GCS activity. Conditions for HGF treatment were the same as above. Treatment of cells with HGF caused dose- and time-dependent increases of γ-GCS activities in comparison with controls (Fig. 2). The maximum increase of γ-GCS activity was about 1.7 times (33.7±2.12 mU/mg protein) higher than the control level (19.4±2.73 mU/mg protein) 24 h after the addition of HGF (5 ng/ml).

Effects of Insulin and EGF on GSH Level and γ-GCS Activity in Response to HGF—It has been reported that the growth-promoting activity of HGF on hepatocytes was stimulated additively or synergistically by EGF or insulin, respectively (*20*). As shown in Fig. 3A, the effects of insulin and EGF on HGF-caused GSH elevation in hepatocytes were examined. Insulin alone caused a small increase (35%

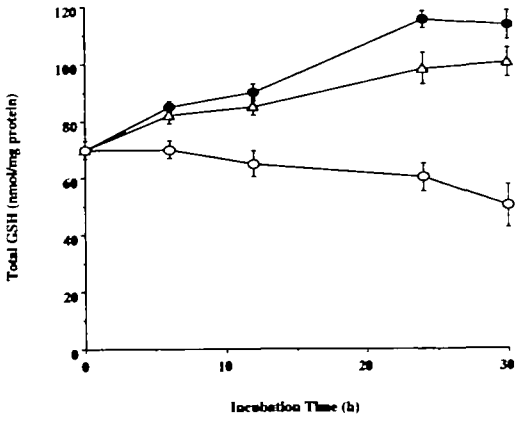

Fig. 1. **Effect of HGF on GSH levels in rat hepatocytes.** Cells were treated with 0 (○), 2.5 (△), or 5 (●) ng/ml HGF in fresh culture medium for 0, 6, 12, 24, and 30 h. Cells were then washed with ice-cold PBS and lysed with 2% perchloric acid for measurement of GSH. Values are means±SD of six experiments.

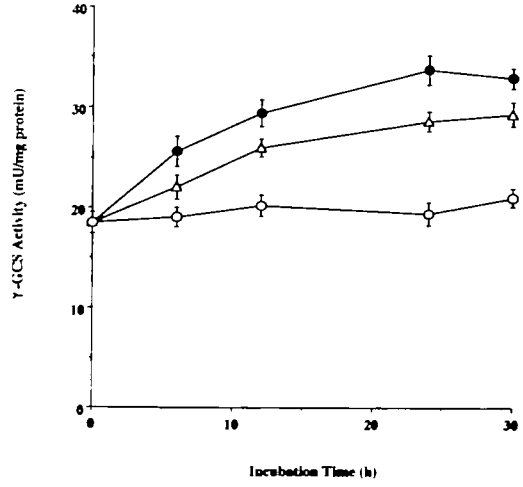

Fig. 2. **Effect of HGF on γ-GCS activity.** Cells were incubated with 0 (○), 2.5 (△), or 5 (●) ng/ml HGF in fresh culture medium at 37°C for the indicated times. Cells were homogenized, then used as enzyme solution for measurement of γ-GCS. Values are means±SD of six experiments.

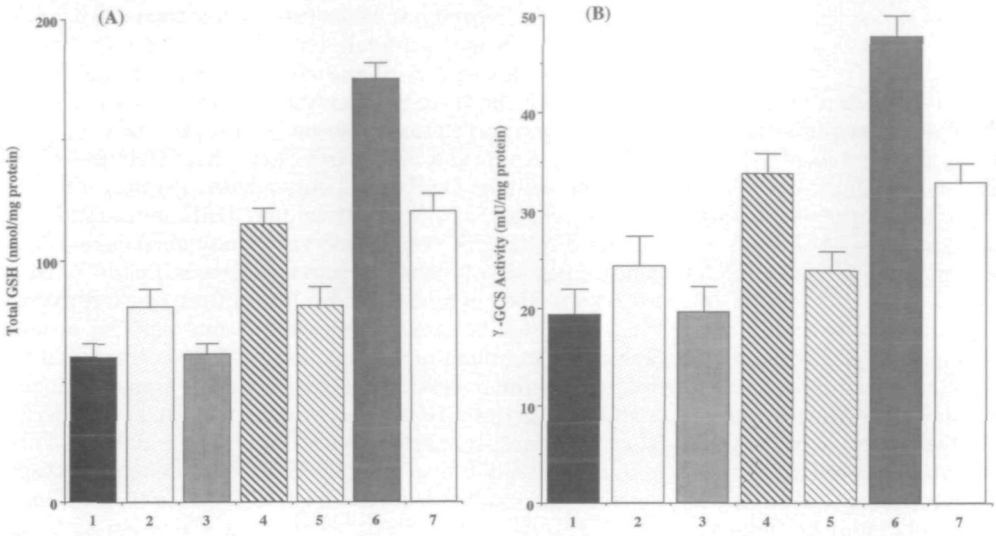

Fig. 3. **Effects of insulin and EGF on GSH level and γ-GCS activity in response to HGF.** Cells were incubated at 37°C for 24 h with insulin, EGF and/or HGF, then the GSH level (A) and the γ-GCS activity (B) were measured. Lane 1, control; lane 2, insulin (1 μg/ml); lane 3, EGF (5 ng/ml); lane 4, HGF (5 ng/ml); lane 5, insulin and EGF; lane 6, insulin and HGF; lane 7, HGF and EGF. Values are means±SD of six experiments.

TABLE I. **Effects of HGF and EGF on APAP-induced hepatotoxicity and GSH contents in hepatocytes.** Hepatocytes were incubated in the culture medium containing HGF (2.5 or 5 ng/ml) or EGF (5 ng/ml) 1 h prior to addition of 1 mM APAP. After incubation for 24 h, GSH level in hepatocytes and AST leakage into the medium were measured as described in "MATERIALS AND METHODS." Values are means± SD of six experiments.

	AST		GSH	
	U/ml	Inhibition (%)	nmol/mg protein	% of control
Control	150± 5.71		60.0±5.02	100
APAP	1087±34.7		17.8±2.05	29.7
APAP+HGF (2.5 ng/ml)	385±10.3	75.7	42.5±4.15	70.8
+HGF (5 ng/ml)	161± 7.98	98.9	54.8±3.97	91.3
+EGF (5 ng/ml)	1056±47.6	3.31	18.3±3.64	30.5

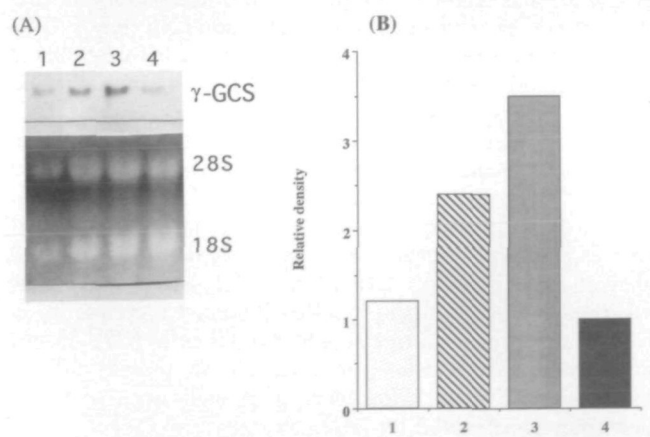

Fig. 4. **Northern blots of γ-GCS mRNA in rat hepatocytes.** After incubation of hepatocytes with or without insulin and HGF for 24 h, total cellular RNA was extracted and fractionated by 1% agarose gel electrophoresis. Northern blot hybridization (A) was performed using the ^{32}P-labeled γ-GCS cDNA. Lane 1, insulin (1 μg/ml); lane 2, HGF (5 ng/ml); lane 3, insulin and HGF; lane 4, control. The relative level of γ-GCS mRNA was determined using a scanning densitometer and normalized to the level at control (B). Each lane in (B) corresponds to that in (A).

over control) of the GSH level in hepatocytes. In the presence of HGF, however, the addition of insulin in the culture medium caused the synergic elevation of the GSH level (175 nmol/mg protein) in hepatocytes in comparison with controls (60.0 nmol/mg protein). Insulin also increased the HGF-caused elevation of γ-GCS activity in hepatocytes by 1.4 times (Fig. 3B). EGF, in contrast, had no effect on the GSH level or γ-GCS activity in hepatocytes, even in the presence of HGF (Fig. 3).

Altered γ-GCS mRNA Expression by HGF—γ-GCS gene expression was measured by Northern analysis probed with ^{32}P-labeled γ-GCS cDNA (Fig. 4). γ-GCS probe hybridized with a single band around 3.7 kilobases in total RNA isolated from rat hepatocytes, which is consistent with the published rat γ-GCS mRNA size. The addition of HGF (5 ng/ml) to hepatocytes caused an increase in γ-GCS mRNA expression in comparison with control (Fig. 4, lanes 2 and 4). Expression of γ-GCS mRNA caused by HGF was stimulated by insulin (Fig. 4, lanes 2 and 3).

Protection against APAP-Induced Hepatotoxicity and GSH Depletion in Hepatocytes by HGF—The protective effect of HGF against APAP-induced hepatotoxicity was examined. As shown Table I, AST activity in the culture medium increased markedly (about 7.2-fold over the control) following APAP treatment of hepatocytes. The pretreatment of HGF dose-dependently prevented the elevation of AST activity in the medium. However, EGF had no protective effect against APAP-induced hepatotoxicity. A similar effect was observed on GSH depletion in APAP-treated hepatocytes. The GSH level in hepatocytes decreased to 29.7% by APAP treatment; and HGF pretreatment significantly prevented the GSH depletion produced by APAP.

DISCUSSION

GSH synthesis is thought to be an important factor in cellular defense against radiation and drug resistance. Godwin *et al.* reported the elevation of the levels of γ-GCS in drug-resistant malignant cells in accordance with the levels of GSH and indicated the significance of GSH synthesis for cells to acquire drug resistance (*21*). Woods *et al.* demonstrated that both renal GSH and γ-GCS mRNA content were markedly elevated following 3 weeks of administration of methylmercury hydroxide to male F344 rats (*22*). They suggested that the increase in GSH biosynthesis was an adaptive response to mercury-induced oxidative stress in the kidney. Utley and Mehendale suggested that deviation of GSH content occurs as a protection mechanism in response to chemically induced oxidative stress (*23*). Furthermore it has been reported the induction of *de novo* GSH synthesis by naphtoquinone-induced oxidative stress is associated with the transcriptional activation of the γ-GCS gene and the subsequent elevation of γ-GCS activity (*24*).

The present study demonstrated that treatment with HGF elevated the GSH content in rat hepatocytes (Fig. 1). The increase in GSH in response to HGF was associated with increased activity of γ-GCS (Fig. 2), the rate-limiting enzyme of GSH biosynthesis. It has been reported that γ-GCS normally functions at substantially less than its maximal rate because of feedback inhibition by GSH (*3*). The decrease of cellular GSH content can release feedback inhibition of γ-GCS activity; however, the continuous rise of GSH during the first 24 h of exposure to HGF and sustained GSH content for 24 h above the control content suggested that release of feedback inhibition by GSH depletion did not play a major role in enhanced synthesis of GSH (Fig. 1).

In the elevation of GSH level by HGF we examined the effects of insulin and EGF on the GSH level and γ-GCS activity (Fig. 3). Lu *et al.* reported that insulin, which was commonly used as a growth factor in culturing hepatocytes, increased GSH level of cultured hepatocytes (*25*). Gohda *et al.* reported EGF and insulin stimulated the growth-promoting activity of HGF on hepatocytes additively and synergistically, respectively (*20*). As shown in Fig. 3A, insulin synergistically stimulated the elevation of GSH level in hepatocytes induced by HGF, but EGF had no effect on the GSH level or γ-GCS activity in hepatocytes.

It has been reported that the increase in GSH precedes the increase in DNA synthesis during liver regeneration (*26*). The significance of this increase in hepatic GSH is open to speculation. Previous studies involving lymphocytes and fibroblasts showed that an increased GSH level was associated with an early proliferation response and was essential for the cell to enter the S phase (*27, 28*). The requirement for increased GSH prior to DNA synthesis may be related to the fact that proliferating cells require increased amounts of pentoses and thiols. DNA synthesis depends absolutely on the formation of pentoses and on their conversion into deoxyribose by ribonucleotide reductase (*29*). The activity of this rate-limiting enzyme in DNA synthesis requires reduced glutaredoxin or thioredoxin, which are maintained by GSH with concomitant oxidation to GSSG *via* glutathione reductase or oxidation of NADPH *via* thioredoxin reductase, respectively. Alternatively, an increase in the cellular GSH content may change the thiol-redox status of the cell, which activates genes essential for the G_1 to S transition. However, other studies did not find a correlation between GSH levels and cell cycle (*30, 31*). Kang and Enger reported that EGF failed to increase cellular GSH level but induced a mitogenic response, and the elevation of cellular GSH level failed to induce a mitogenic response in rat kidney fibroblasts (*32*). The exposure of cells to tumor necrosis factor (TNF) increases DNA synthesis and cell proliferation, indicating that this cytokine acts as a mitogenic stimulus. TNF is also known as an inflammatory cytokine. TNF was reported to increase cellular GSH level, mediated by transcriptional regulation of the γ-GCS gene, which attenuated the generation of hydrogen peroxide and lipid peroxidation. This elevation of GSH level induced by TNF is due to protection against the oxidative stress elicited by TNF (*33*). From these results, it was assumed that the elevation of GSH level induced by HGF in hepatocytes is associated with protective mechanism against oxidative stress rather than a proliferation response.

Agents known to cause oxidative stress have been shown in a variety of cells and organs to increase γ-GCS activity. In all cases, the increase in γ-GCS activity was associated with an increase in the transcription of γ-GCS. Recent efforts have focussed on elucidating the molecular mechanisms of 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 (*34, 35*). The region from −817 to +45 on the nucleotide sequence of the human γ-GCS contains AP-1 or AP-1-like responsive elements that have been found to be critical in mediating the effects of hydrogen peroxide and TNF on γ-GCS promoter activity (*33, 36, 37*). A recent study by Sekhar *et al.* confirmed the importance of AP-1 activity in mediating the effect of oxidative stress on γ-GCS transcription (*38*). On the other hand, 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 (*34*). Oxidative stress also induces NF-κB activity, and Urata *et al.* showed that blocking activation of NF-κB by antisense strategies prevented the cytokine-induced increase in γ-GCS transcription in mouse endothelial cells (*39*). In our experiment, HGF caused an increase in γ-GCS mRNA expression (Fig. 4). However, it is still unclear which promoter is activated by HGF.

APAP caused liver injury by GSH depletion, which successively leads to oxidative stress (*40, 41*). Accordingly, the hepatotoxicity of APAP is prevented by compounds such as *N*-acetylcysteine or 2-oxothiazolidine-4-carboxylate, which act to retain the GSH level in the liver and promote the redox cycle of GSH (*42, 43*). As shown in Table I, the pretreatment of HGF inhibited the hepatotoxicity and the GSH depletion in hepatocytes caused by APAP. It was also reported that the administration of HGF prevented carbon tetrachloride (CCl_4)-induced liver injury in rats (*44*). From this evidence, it was suggested that the protective effect of HGF on APAP or CCl_4-induced hepatotoxicity was due, at least in part, to the maintenance of hepatic GSH level. Thus the results of these experiments indicate a new role of HGF in liver protection and suggest

important clinical implications for developing new therapeutic modalities in the treatment of liver failure.

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