Knock Down of γ-Glutamylcysteine Synthetase in Rat Causes Acetaminophen-induced Hepatotoxicity*

Sho Akiyama, Hikro Hosomi, Keiichi Minami, Koichi Tsuneyama, Miki Katoh, Miki Nakajima, and Tsuyoshi Yokoi

From the 4Division of Pharmaceutical Sciences, Graduate School of Medical Science, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan and 5Department of Diagnostic Pathology, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama, Sugitani 930-0194, Toyama, Japan

Drug-induced hepatotoxicity is mainly caused by hepatic glutathione (GSH) depletion. In general, the activity of rodent glutathione S-transferase is 10 to 20 times higher than that of humans, which could make the prediction of drug-induced hepatotoxicity in human more difficult. γ-Glutamylcysteine synthetase (γ-GCS) mainly regulates de novo synthesis of GSH in mammalian cells and plays a central role in the antioxidant capacity of cells. In this study, we constructed a GSH depletion experimental rat model for the prediction of human hepatotoxicity. An adenovirus vector with short hairpin RNA against rat γ-GCS heavy chain subunit (GCSh) (AdGCSh-shRNA) was constructed and used to knock down the GCSh. In in vitro study in H4IIE cells, a rat hepatoma cell line, GCSh mRNA and protein were significantly decreased by 80% and GSH was significantly decreased by 50% 3 days after AdGCSh-shRNA infection. Using this GSH knockdown rat model, acetaminophen-induced hepatotoxicity was shown to be significantly potentiated compared with normal rats. This is the first report of a GSH knockdown rat model, which could be useful for highly sensitive tests of acute and subacute toxicity for drug candidates in preclinical drug development.

Glutathione (5-L-glutamyl-L-cysteinylglycine, GSH) is one of the most abundant tripeptides, consisting of glycine, glutamic acid, and cysteine. It serves an important function in protecting tissues against the degenerating effects of oxidative damage by scavenging free radicals from endogenous or exogenous compounds (1, 2). GSH is synthesized from its precursor amino acids in two steps of enzymatic reactions. γ-Glutamylcysteine synthetase (γ-GCS) (3) catalyzes the formation of γ-glutamylcysteine from glutamic acid and cysteine. GSH synthetase couples glycine to γ-glutamylcysteine to form GSH. γ-GCS is a rate-limiting step in GSH biosynthesis, and GSH is a feedback inhibitor of γ-GCS activity. γ-GCS is a heterodimeric enzyme composed of a catalytic subunit (heavy chain, 73 kDa) (4) and a modulatory subunit (light chain, 27.7 kDa) (5). Studies performed by purified γ-GCS suggested that the active site exists in the catalytic subunit, whereas the modulatory subunit increases the affinity of the catalytic subunit for glutamic acid and decreases the sensitivity to feedback inhibition by GSH (4).

In mice, embryos homozygous for the γ-GCS heavy chain (GCSh) mutation fail to gastrulate and die (6). In contrast, homozygous knock-out mice with targeted disruption of the γ-GCS light chain are viable and fertile although the GSH level is decreased by 87% in the liver, and thus this model could be used as a GSH depletion mouse model in vivo (7).

Rat is the most frequently used experimental animal for pharmacological and toxicological studies in the drug development process because of their body weight and ease of sampling blood or urine. A standard technique of gene knock out in rat has not been established yet. Recently, a nuclear transfer method has been established and this method may be able to produce conditional knock out and gene replacement in the future (8), but this method is very difficult and not available for general use. Recently, recombinant adeno-virus methods are being developed and used for the purpose of clinical therapy or gene delivery in vivo (9–11). Furthermore, a small interfering RNA strategy, which has been proven to be more specific and efficient than the full-length antisense cDNA strategy, has been established (12). In addition, an adeno-virus-mediated short hairpin RNA (shRNA) knockdown approach could reduce the target gene specifically in the liver in mice, resulting in the expected phenotype (13). However, to our knowledge, there is no report that adeno-virus-mediated shRNA knock down was successfully applied in rats in vivo. In the present study, we constructed a recombinant adeno-virus (AdGCSh-shRNA) that could knock down rat GCSh mRNA efficiently in vitro and in vivo. We established the GSH-depleted rats and this rat model, when treated with acetaminophen (APAP), which is known to be biotransformed to quinoneimine (14), or some other radical species (15), demonstrated hepatotoxicity with high sensitivity compared with normal rats.

The activity of rodent glutathione S-transferase (GST) is about 10 to 20 times higher than that in human (16). Therefore,
active metabolites produced in vivo in rat would be immediately detoxified by GSH conjugation, which would make the prediction of drug-induced hepatotoxicity in human more difficult. From this perspective, the AdGCSh-shRNA-mediated GSH depletion rat model could be useful for predicting the hepatotoxicity caused by unknown active metabolites of drug candidates produced by Phase I enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—APAP and GSH were obtained from Wako Pure Chemical Industries (Osaka, Japan). β-NADPH and glutathione reductase were from Oriental Yeast (Tokyo, Japan). ISOGEN was from Nippon Gene (Tokyo, Japan). ReverTra Ace (Moloney Murine Leukemia Virus Reverse Transcriptase RNaseH Minus) was from Toyobo (Tokyo, Japan). The Adenovirus Expression Vector kit (Dual Version), random hexamer and SYBR Premix Ex Taq were from Takara (Osaka, Japan). The QuickTiter Adenovirus Titer Immunoassay kit was from Cell Biolabs (Tokyo, Japan). Lipofectamine 2000 and minimum essential α medium were from Invitrogen. The GeneSilencer shRNA Vector kit was from Gene Therapy Systems (San Diego, CA). Dulbecco’s modified Eagle’s medium and Ham’s F12 medium were from Nissui Pharmaceutical (Tokyo, Japan). All primers and oligonucleotides for shRNA were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Standard metabolites of APAP, such as APAP-glucuronide, APAP-sulfate, APAP-mercapturate, APAP-cysteine, and APAP-GSH, were kindly provided by McNeil Consumer Products (Washington, PA). Other chemicals were of analytical grade or the highest commercially available.

**Animals**—Male Fisher 344 rats (7 weeks old, 130–150 g) were obtained from SLC Japan (Hamamatsu, Japan). Animals were housed in a controlled environment (temperature 25 ± 1 °C, humidity 50 ± 10%, and 12-h light/12-h dark cycle) in the institutional animal facility with access to food and water ad libitum. Animals were acclimatized for a week before use for the experiments. Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, as approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan.

**Design of Short Hairpin RNA**—Rat GCSH (Gene Bank™ accession code J05181 Gene bank) knock down was achieved by RNA interference using an adenovirus vector-based shRNA approach. The sequences of shRNA-targeted GCSH cDNA were designed by B-Bridge (Mountain View, CA). The sequences of GCSH-shRNA are: top strand, 5′-gatecGTGTGAAATGTTCCAGAGTTAagaacctgtAACTCTGTGACATTCCACAActttgaggac-3′, and bottom strand, 5′-ggccgcttccaaaaaGTGTGAATGTCCAGAGTTAagctgtAACTCTGTGACATTCCACAG-3′. As a negative control, the oligonucleotide sequences of the shRNA target for luciferase from a GeneSilencer shRNA Vector kit were used.

**Recombinant Adenovirus**—To generate the recombinant adenovirus vector expressing GCSH-shRNA, pGSU6-GFP plasmids were recombined into the pAXcwt vector using the cosmid-terminalsequence method according to the manufacturer’s instruction. In brief, double strand oligo DNA for shRNA of GCSH and luciferase were inserted into the BamHI and NotI sites of the pGSU6-GFP vector. This product was digested by HincII and inserted into the Swal site of the pAXcwt vector. This pAXcwt vector and the parental adenovirus DNA terminal protein complex were co-transfected into 293 cells by Lipofectamine 2000. The recombinant adenovirus was isolated and propagated into the 293 cells. An adenovirus containing shRNA of GCSH (AdGCSh-shRNA) and one containing shRNA of luciferase (AdLuc-shRNA) were constructed. The titer was determined by a QuickTiter Adenovirus Titer Immunoassay kit. The titers of AdGCSh- or AdLuc-shRNA were 4.95 × 10⁶ pfu/ml and 2.98 × 10⁵ pfu/ml, respectively. The viral stock solution was concentrated with the Amicon Ultra-15 filtration system (Millipore, Billerica, MA) for the in vivo study.

**Cell Culture**—The 293 cell line and rat hepatoma cell lines BRL3A and H4IIE were obtained from American Type Culture Collection (Manassas, VA). The human hepatoma cell line HLE was obtained from the Japanese Collection of Research Biosources (Tokyo, Japan). The mouse hepatoma cell line Hepa–6 was kindly provided by Dr. S. Kaneko (Kanazawa University, Japan). The 293 cell line was maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (BioWhitaker, Walkersville, MD), 3% glutamine, 16% sodium bicarbonate, and 0.1 mm nonessential amino acids (Invitrogen) in a 5% CO₂ atmosphere at 37 °C. BRL3A cells were maintained in Ham’s F12, HLE and Hepa–6 cells were maintained in Dulbecco’s modified Eagle’s medium, and H4IIE cells were maintained in α-minimal essential medium. All cell lines were infected by the adenovirus in medium containing 5% fetal bovine serum.

**Real-time Reverse Transcription PCR Analysis**—RNA from the hepatoma cells or from liver specimens was isolated using ISOGEN. Rat GCSH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time reverse transcription PCR. Primer sequences used in this study were as follows: rat GCSH, 5′-ATG CAGTATTCTGAACACTAC-3′ and 5′-ACAATCTAGTATCCCTAC-3′; mouse GCSH, 5′-TCTAACAGAACATCCGGCA-3′ and 5′-GTCAGGGTCGGTCACTGTTA-3′; human GCSH, 5′-ATTAGAAGAAATTACGGCCCT-3′ and 5′-GTAGCAACTGTCAATAAAGG3′; mouse GAPDH, 5′-GTTCACCAAGGGCTGTTTCTC-3′ and 5′-GGTTTTCCCGTGTGAC-3′; mouse GAPDH, 5′-TCACCGGCGTTCATTTTGGGA-3′ and 5′-CTACCCCAATTGATTGGTAGT-3′; human GAPDH, 5′-CCAGGGCTGTTTCTACTC-3′ and 5′-GCTCCCCCTTACAAAG-3′. For the reverse transcription process, total RNA (2 µg) and 150 ng of random hexamer were mixed and incubated at 70 °C for 10 min. RNA solution was added to a reaction mixture containing 100 units of ReverTra Ace, reaction buffer, and 0.5 mm dNTPs in a final volume of 40 µl. The reaction mixture was incubated at 30 °C for 10 min, 42 °C for 1 h, and heated at 98 °C for 10 min to inactivate the enzyme. The real-time PCR was performed using the Smart Cycler (Cepheid, Sunnyvale, CA). PCR mixture contained 1 µl of template cDNA, SYBR Premix Ex Taq solution, and 10 pmol of sense and antisense primers. The PCR condition for GAPDH and GCSH were as follows. After an initial denaturation at 95 °C for 30 s, the amplification was performed by denaturation at 94 °C for 4 s, annealing and extension at 64 °C for
Knockdown Effects of γ-Glutamylcysteine Synthetase in Rat

20 s for 45 cycles. Amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I (Molecular Probes, Eugene, OR) that binds to double strand DNA amplified by PCR.

Western Blot Analysis—The H4IIE cell lysates, 1.5 µg, were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membrane (Immobilon-P; Millipore). The specific proteins were detected by rabbit anti-human GCSh polyclonal antibody, cross-reacting to rat GCSh (sc-22755; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200. The protein bands were developed by biotinylated second antibody-peroxidase reaction. The quantitative analysis of protein expression was performed using ImageQuant TL Image Analysis software (Amersham Biosciences).

GSH Level—Cell lysates were mixed in 5% (w/v) metaphosphoric acid and incubated on ice for 10 min. After the addition of 0.125 M sodium phosphate buffer containing 6.3 mM EDTA, pH 7.5, the cell lysates were centrifuged at 13,000 × g at 4 °C for 5 min. Livers (100 mg) were homogenized with ice-cold 5% sulfosalicylic acid and centrifuged at 8,000 × g at 4 °C for 10 min. The GSH concentration in the supernatant was measured as described previously (17).

Adenovirus Infection and APAP Administration in Rats—Fourteen days after one intravenous injection of AdGCSh-shRNA or AdLuc-shRNA at 2 × 10¹¹ pfu/ml/body, the rats were orally administered APAP suspended in 0.5% carboxymethylcellulose (0, 300, 1000 mg/kg body weight). Blood samples were collected at 0, 30, 60, 120, and 180 min after the APAP treatment. Twenty-four hours after the administration of APAP, serum samples were collected for assessment of transaminase levels and for APAP metabolite analysis. The liver was fixed in buffered neutral 10% formalin. The fixed samples were embedded in paraffin and sectioned at a thickness of 2 µm and stained with hematoxylin-eosin for microscopic examination. Rat liver cytosol and microsomes were prepared as described previously (18). In all experiments, the rats were not treated by fasting prior to the APAP treatment or sacrifice.

GST Activity and Cytochrome P450 (CYP) Content—The cytosolic GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate according to the method of Habig et al. (19). The microsomal cytochrome P450 content was determined by the method of Omura and Sato (20).

Determination of Plasma Concentrations of APAP and Its Metabolites—The plasma concentrations of APAP and its metabolites were measured using a high performance liquid chromatography method described previously (21). In brief, plasma was mixed with an aliquot of acetoniitrile containing theophylline as an internal standard. After extraction and centrifugation, the resulting supernatant was evaporated under nitrogen. The residue was diluted with distilled water as necessary before being injected into high performance liquid chromatography. APAP and its metabolites, APAP-GSH, APAP-cysteine, APAP-mercapturate, APAP-glucuronide, and APAP-sulfate, were separated in a Mightysil RP-18 column (4.6 × 150 mm; 5 µm; Kanto Chemical, Tokyo, Japan). APAP and the metabolites, eluted with 1.8% aqueous acetic acid-methanol-H₂O (66:9:100) at a flow rate of 1.0 ml/min, were monitored at 248 nm.

Statistical Analysis—Statistical analyses were performed with the GraphPad Instat version 2.0 computer program (GraphPad Software, San Diego, CA) by Student t-test, Dunnett’s post hoc test, or Bonferroni test.

RESULTS

Changes of GCSH mRNA Expression and GSH Level in Various Hepatoma Cell Lines—To investigate the knockdown effect on the cells, various hepatoma cells were infected with AdGCSh-shRNA or AdLuc-shRNA (negative control adenovirus) at a multiplicity of infection (m.o.i.) of 20 for 3 days. Real-time reverse transcription PCR analysis and GSH assay were performed to examine the GCSH mRNA expression and GSH suppression (Fig. 1). The expression level of GCSH mRNA was significantly reduced to 20–30% in BRL3A, H4IIE, and Hepa1–6 cells. In contrast, AdGCSh-shRNA was less potent, reducing GCSH mRNA to 45% in HLE cells. The GSH level was suppressed only in H4IIE cells by 50%, despite the efficient GCSH mRNA knock down in BRL3A and Hepa1–6 cells. Based on these results, H4IIE cells were used in the next experiments.

Time-dependent Knockdown Effect of AdGCSh-shRNA in H4IIE Cells—To investigate the most efficient condition for infection, H4IIE cells were infected with AdGCSh-shRNA at m.o.i. 10 or 20 for 1, 2, 3, and 5 days. GCSH mRNA was reduced after 24 h of infection, and an 80% decrease of GCSH mRNA was achieved after 2 days of infection (Fig. 2A). The decrease of GCSH mRNA was accompanied by a decrease in GCSH protein (Fig. 2B). The GSH level was significantly reduced by 50% after 3 days of AdGCSh-shRNA m.o.i. 20 infection (Fig. 2C). There was no difference between 3 and 5 days of infection. These results suggested that a m.o.i. of 20 and 3 days of infection could be an appropriate condition for cytotoxicity experiments.

Effect of APAP Treatment in H4IIE Cells Infected with AdGCSh-shRNA—To investigate the effect of GSH depression on the cytotoxicity of APAP, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed. H4IIE cells were infected with AdGCSh-shRNA at m.o.i. 20 or with AdLuc-shRNA in the same conditions as a negative control. After 3 days of infection, H4IIE cells were exposed to various concentrations of APAP of 0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 µm. After 24 h of APAP treatment, AdGCSh-shRNA-infected cells showed no toxic effects compared with control (data not shown).

Infection of AdGCSh-shRNA to Rat—To further investigate in vivo in rat, a single tail vein injection to Fisher 344 rats was made to deliver AdGCSh-shRNA. The effects of GCSH knock down were examined 14 days after infection. Control animals were treated with PBS or AdLuc-shRNA. The hepatic GCSH mRNA expression was remarkably decreased dose dependently (Fig. 3A). At the dose of 2 × 10¹¹ pfu/ml/body, GCSH mRNA was significantly decreased by 90%. Consistent with the decrease of GCSH mRNA, the hepatic total GSH level was also reduced to 20% at doses above 2 × 10¹¹ pfu/ml/body (Fig. 3B). The hepatic GSH level in AdLuc-shRNA-infected rat was slightly increased compared with the PBS-treated rats (Fig. 3B).

To examine the hepatotoxic effect of the adenovirus infection, serum AST and ALT were measured 14 days after infection. As shown in Fig. 3C, AST was significantly elevated in the
4.0 and $8.0 \times 10^{11}$ pfu/ml/body infection by 1.6- and 4.1-fold, respectively, compared with the control. ALT was significantly elevated in only the $8.0 \times 10^{11}$ pfu/ml/body infection by 2.2-fold compared with the control. The dose of $2.0 \times 10^{11}$ pfu/ml/body did not affect the AST and ALT, and thus this condition was adopted in the next experiments. The cytochrome P450 content and GST activity slightly increased in AdGCSh-shRNA-treated rats (data not shown).

APAP-induced Hepatotoxicity in AdGCSh-shRNA-infected Rat—To determine whether APAP-induced hepatotoxicity was potentiated by the suppression of hepatic GSH, rats were tail vein-injected once with $2.0 \times 10^{11}$ pfu/ml/body AdGCSh-shRNA or AdLuc-shRNA. After 14 days, APAP was orally administered without previous fasting. The serum AST and ALT levels are shown in Figs. 4, A and B. Twenty-four hours after APAP administration, 300 mg/kg treatment did not result in hepatotoxicity. In contrast, the AdGCSh-shRNA-infected rats treated with 1000 mg/kg APAP demonstrated a significant increase of AST (2159 ± 1156 units/liter) and ALT (924 ± 667 units/liter) compared with AdLuc-shRNA infected rats. Without fasting treatment, the AdLuc-shRNA and normal rats administered 1000 mg/kg APAP did not show hepatotoxicity. The results of the histological examination in 1000 mg/kg APAP-administered rats are shown in Fig. 4C. Remarkable hepatic necrosis, especially around the central vein, was observed in AdGCSh-shRNA-treated rats given 1000 mg/kg APAP, consistent with the elevation of AST and ALT. There was no histological change in the other groups.

Metabolism of APAP in Rats Infected with Adenovirus—Changes in the plasma concentration of APAP and its metabolites are shown in Fig. 5. For APAP, APAP-glucuronide, and APAP-sulfate, the maximum plasma concentration was observed 30 min or 1 h after APAP administration. The concentration of APAP-glucuronide was significantly elevated in rats infected with AdGCSh-shRNA compared with AdLuc-shRNA-infected rats (Fig. 5B). On the other hand, APAP-sulfate, a major detoxification product in rats generated directly from APAP, was decreased (Fig. 5C). For APAP-GSH, APAP-cysteine, and APAP-mercapturate, the maximum plasma concentration was observed 1 h after APAP administration in rats infected with AdGCSh-
As for the rats infected with AdLuc-shRNA, the concentration of APAP-GSH was gradually decreased, whereas the concentrations of APAP-cysteine and APAP-mercapturate were slightly increased.

Continuation of the Depletion of GSH Level—To examine the continuation of the hepatic GSH depletion, rats were tail vein-injected once with 2 $\times$ 10$^{11}$ pfu/ml/body AdGCSh-shRNA. After 2, 3, 4, and 5 weeks, the hepatic GSH level was measured (Fig. 6). Hepatic GSH was significantly decreased by 80% at 2 to 3 weeks after infection. The hepatic GSH was reduced by 66 and 45% at 4 and 5 weeks after infection, respectively. In addition, at 7 and 10 days after infection of AdGCSh-shRNA, the hepatic GSH was decreased by 20 and 50%, respectively (data not shown). The effects of the circadian rhythm were also examined 2 weeks after infection with AdGCSh-shRNA (data not shown). The hepatic GSH level was lower than those from PBS-treated rats at all the time points examined, and no effect of the circadian rhythm on the GSH level was observed in rats infected with AdGCSh-shRNA.

**DISCUSSION**

In this study, a recombinant adenovirus vector expressing an shRNA-directed rat GCSh was generated (AdGCSh-shRNA). The GSH level was efficiently decreased by 50% only in H4IIE cells infected with AdGCSh-shRNA (Fig. 1). The target sequence of the rat GCSh is the same as mouse, but it differs from that of human. This would probably affect the mRNA knockdown efficiency. The lack of a decrease of GSH in BRL3A cells may be due to differences in the expression levels of coxsackie and adenovirus receptor (22).

In the cytotoxicity study, APAP treatment did not show a toxic effect in AdGCSh-shRNA-infected cells compared with AdLuc-shRNA-infected cells. APAP is mainly metabolized by UDP-glucuronosyltransferases and sulfotransferases, partly by CYP enzymes (23, 24). APAP toxicity is highly dependent upon bioactivation by CYP enzymes to the reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI), and a depletion of intracellular GSH would cause adduct formation targeting cellular proteins (25–28). A previous report showed that HepG2 cells which stably expressed human CYPs showed cytotoxicity by APAP, but normal HepG2 cells did not (29). H4IIE cells express no CYP enzymes (data not shown), thus APAP would not be metabolized to its toxic metabolite NAPQI and would not cause cytotoxicity.

We successfully produced a GSH-depleted rat model by means of adenovirus-mediated RNA interference technology in order to detect the drug-induced hepatotoxicity with more sensitivity than that provided by normal rats. A previous report described that infection of an adenovirus caused adenovirus-derived hepatotoxicity (30). Therefore, we validated the condition of adenovirus infection (Fig. 3). A significant increase of serum AST and ALT was observed at a high dose of AdGCSh-shRNA (8 $\times$ 10$^{11}$ pfu/ml/body), suggesting adenovirus-derived hepatotoxicity (Fig. 3C). On the other hand, doses up to 2 $\times$ 10$^{11}$ pfu/ml/body resulted in no hepatotoxicity and caused GSH depletion by 80% in rat liver. This GSH depletion level is the same as that in GCS light chain knock-out mice (7). Therefore, we determined that a single injection of AdGCSh-shRNA (2 $\times$ 10$^{11}$ pfu/ml/body) was the proper condition for testing the drug-induced hepatotoxicity in this study. Furthermore, the hepatic total P450 content was slightly increased in AdGCSh-shRNA injected rats.

The maximum depletion of GSH was obtained 14 days after infection in rats in the present study, but it was obtained in 5 days in mouse (13). Because there is no previous report of adenovirus-shRNA in rats, this is the first study showing the optimum experimental condition in vivo in rats. In regard to the knockdown effect, the GSH level was decreased at most by 50% in vitro and 80% in vivo. This result may be due to the different

**FIGURE 3.** Effects of adenovirus infection on hepatic GCSh mRNA (A), GSH level (B), ALT and AST (C) in rats. All experiments were performed 14 days after AdGCSh-shRNA or AdLuc-shRNA infection. Data represent the mean ± S.D. (n = 4 or 5). *, p < 0.05 and **, p < 0.01 compared with PBS-treated rats or AdGCSh-shRNA-infected rats.
GSH regulation mechanism, which remains to be elucidated in the future.

In previous APAP-induced hepatotoxicity studies, the rats were generally fasted for half or 1 day before drug administration (31–33). In the present study, in order to clarify the involvement of GSH depletion, the rats were not fasted before treatment. APAP-induced hepatotoxicity was observed at a single oral dose of 1000 mg/kg with fasting but was not observed without fasting (data not shown). Previous reports demonstrated that fasting caused a GSH decrease in liver (33, 34). Moreover, transcription of the CYP2E1 gene is potently activated by fasting (35). APAP is metabolized to NAPQI by CYP, mainly by CYP2E1, and thus fasting would cause an overestimation of the APAP-induced hepatotoxicity.

As an in vivo hepatotoxicity screening system, a single oral dose of APAP at 300 and 1,000 mg/kg to normal rats with fasting condition was reported by a Pfizer group (36), resulting in no increase of ALT and AST at 300 mg/kg after 24 h of p.o. administration, although a potent increase of ALT and AST in 1,000 mg/kg to normal rats. The same single oral administration of APAP (1,000 mg/kg) with fasting condition was also adopted by the National Toxicogenomics project in Japan as the screening system, resulting in significant increase of ALT and AST at 24 h after treatment (37). However, there is no report about the effect of fasting on APAP hepatotoxicity. In our experiments, without fasting treatment, no hepatotoxicity was observed by single oral administration of 1,000 mg/kg APAP in normal rat, as well as AdGCSh-shRNA-treated rat as shown in Fig. 4. Consequently, in our study, APAP-induced hepatotoxicity was potentiated only in rats with continuously depleted levels of GSH by AdGCSh-shRNA administration, but not by fasting. This indicated that APAP-induced hepatotoxicity would be potentiated only by the GSH depletion.

Infection of AdGCSh-shRNA caused a significant increase of APAP-glucuronide and decrease of APAP-sulfate in the plasma of the rats (Fig. 5), suggesting that GSH depletion caused the induction of UDP-glucuronosyltransferase activity. However, previous reports demonstrated that APAP-glucuronide was a substrate for multidrug resistance-associated protein 2 (MRP2) (38) and that the MRP2 expression level would change depending on the GSH concentration (39). Thus, biliary excretion of APAP-glucuronide would be decreased due to the down-regulation of MRP2 expression, and then the plasma concentration...
of APAP-glucuronide would be increased. The APAP-sulfate showed a tendency of decrease; however, the mechanism remains to be elucidated. The plasma concentrations of APAP-glucuronide would be increased. The APAP-sulfate

Knockdown Effects of γ-Glutamylcysteine Synthetase in Rat

FIGURE 6. Time-dependent changes of hepatic GSH level in rats infected with AdGCSh-shRNA. Rat liver was excised at 2, 3, 4, and 5 weeks after infection with AdGCSh-shRNA. Data represent the mean ± S.D. (n = 3 to 5). ***, p < 0.001 compared with the PBS-treated group.

induced hepatotoxicity test for drug candidates in preclinical drug development.

Acknowledgment—We thank Brent Bell for reviewing the manuscript.

REFERENCES

1. Reed, D. J. (1986) Biochem. Pharmacol. 35, 7–13
2. Lu, S. C. (1999) FASEB J. 13, 1169–1183
3. Meister, A., and Anderson, M. E. (1983) Annu. Rev. Biochem. 52, 711–760
4. Huang, C. S., Chang, L. S., Anderson, M. E., and Meister, A. (1993) J. Biol. Chem. 268, 19675–19680
5. Huang, C. S., Anderson, M. E., and Meister, A. (1993) J. Biol. Chem. 268, 20578–20583
6. Shi, Z. Z., Osei-Frimpong, J., Kala, G., Kala, S. V., Barrios, R. J., Habib, G. M., Lukin, D. J., Danney, C. M., Matzuk, M. M., and Lieberman, M. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5101–5106
7. Yang, Y., Dieter, M. Z., Chen, Y., Shertzer, H. G., Nebert, D. W., and Dalton, T. P. (2002) J. Biol. Chem. 277, 49446–49452
8. Zhou, Q., Renard, J. P., Le Fricc, G., Brochard, V., Beaujean, N., Cherifî, Y., Fraichard, A., and Cozzi, I. (2003) Science 302, 1179
9. Peng, Z. C. (2005) Hum. Gene Ther. 16, 1016–1027
10. Chu, R. L., Post, D. E., Khuri, F. R., and Van Meir, E. G. (2004) Clin. Cancer Res. 10, 5299–5312
11. Kruyt, F. A., and Curiel, D. T. (2002) Hum. Gene Ther. 13, 485–495
12. Meister, G., and Tuschi, T. (2004) Nature 431, 343–349
13. Xu, H., Wilcox, D., Nguyen, P., Voorbach, M., Suhar, T., Morgan, S. J., An, W. F., Ge, L., Green, J., and Wu, Z. (2006) Biochem. Biophys. Res. Commun. 349, 439–448
14. Dahlin, D. C., Miwa, G. T., Lu, A. Y., and Nelson, S. D. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 327–331
15. Moldeus, P., Anderson, B., Rahintula, A., and Berggren, M. (1982) Biochem. Pharmacol. 31, 1363–1368
16. Grover, P. L., and Sims, P. (1964) Biochem. J. 90, 603–606
17. Tietze, F. (1969) Anal. Biochem. 27, 502–522
18. Guengerich, F. P., Shimada, T., Yun, C. H., Yamazaki, H., Haney, K. D., Their, R., Coles, B., and Harris, T. M. (1994) Environ. Health Perspect. 102, Suppl. 49–53
19. Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974) J. Biol. Chem. 249, 7130–7139
20. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378
21. Kim, Y. C., and Lee, S. J. (1998) Biochemistry 37, 128, 53–61
22. Huang, K. C., Altinoz, M., Wosik, K., Larochelle, N., Koty, Z., Zhu, L., Holland, P. C., and Nalbantoglu, J. (2005) Int. J. Cancer 113, 738–745
23. Howie, D., Aria, P., and Prescott, L. F. (1977) J. Pharm. Pharmacol. 29, 235–237
24. Tone, Y., Kawamata, K., Murakami, T., Higashi, Y., and Yata, N. (1990) J. Pharmacobiokinetics 13, 327–333
25. Minn, D. J., and Kissing, P. T. (1979) Biochem. Pharmacol. 28, 3285–3290
26. Hinson, J. A., Pohl, L. R., Monks, T. J., and Gillette, J. R. (1981) Life Sci. 29, 107–116
27. Albano, E., Rundgren, M., Harvison, P. J., Nelson, S. D., and Moldeus, P. (1985) Mol. Pharmacol. 28, 306–311
28. van de Straat, R., Vromans, R. M. B., Bosman, P. de Vries, J., and Vermeulen, N. P. (1988) Chem. Biol. Interact. 64, 267–280
29. Yoshitomi, S., Ikemoto, K., Takahashi, J., Miki, H., Namba, M., and Asahi, S. (2001) Toxicol. In Vitro 15, 245–256
30. Callahan, S. M., Boquet, M. P., Ming, X., Brunner, L. J., and Croyele, M. A. (2006) J. Gene Med. 8, 566–576
31. Merrick, B. A., Bruno, M. E., Madenspach, J. H., Wetmore, B. A., Foley, J., Pieper, R., Zhao, M., Makusky, A. J., McGrath, A. M., and Zhou, J. (2006) J. Pharm. Pharmacol. 58, 792–802
32. Kim, Y. W., Kim, S. H., Lee, J. R., Lee, S. I., Kim, C. W., Kim, S. C., and Kim, S. G. (2006) Chem. Biol. Interact. 161, 125–138
33. Pessaye, D., Wanderschein, J. C., Cobert, B., Level, R., Degott, C., Batt, A. M., Martin, N., and Benhamou, J. P. (1980) Biochem. Pharmacol. 29,
Knockdown Effects of γ-Glutamylcysteine Synthetase in Rat

J. Biological Chemistry 282 (2007) 24003

J. Biological Chemistry 282 (2007) 24003

2219–2223
34. Jaeschke, H., and Wendel, A. (1985) Biochem. Pharmacol. 34, 1029–1033
35. Johansson, I., Lindros, K. O., Eriksson, H., and Ingelman-Sundberg, M. (1990) Biochem. Biophys. Res. Commun. 173, 331–338
36. Kikkawa, R., Fujikawa, M., Yamamoto, T., Hamada, Y., Yamada, H., and Horii, I. (2006) J. Toxicol. Sci. 31, 23–34
37. Morishita, K., Mizukawa, Y., Kasahara, T., Okuyama, M., Takashima, K., Toritsuka, N., Miyagishima, T., Nagao, T., and Urushidani, T. (2006) J. Toxicol. Sci. 31, 491–507
38. Xiong, H., Turner, K. C., Ward, E. S., Jansen, P. L., and Brouwer, K. L. (2000) J. Pharmacol. Exp. Ther. 295, 512–518
39. Sekine, S., Ito, K., and Horie, T. (2006) Free Radic. Biol. 40, 2166–2174
40. Hayder, H., Blanden, R. V., Korner, H., Rimington, D. S., Sedgwick, J. D., and Mullbacher, A. (1999) J. Immunol. 163, 1516–1520
41. Crettaz, J., Berraondo, P., Mauleon, I., Ochoa, L., Shankar, V., Barajas, M., van Rooijen, N., Kochanek, S., Qian, C., and Prieto, J. (2006) Hepatology 44, 623–632
42. Farkas, D., and Tannenbaum, S. R. (2005) Curr. Drug Metab. 6, 111–125
43. Henderson, C. J., Wolf, C. R., Kitteringham, N., Powell, H., and Park, B. K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12741–12745