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Disruption of redox homeostasis and induction of apoptosis by suppression of glutathione synthetase expression in a mammalian cell line

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Abstract

The stable HepG2 transfectants anti-sensing expression of the glutathione synthetase (GS) gene exhibited delayed cell growth and increased reactive oxygen species (ROS) level. After the treatment with hydrogen peroxide, the intracellular ROS level was much higher in the stable transfectants than in the vector control cells. However, the GSH levels decreased more significantly in the stable transfectants than in the vector control cells, in the presence of hydrogen peroxide. Hydrogen peroxide-induced apoptosis of the stable transfectants was notably higher than that of the vector control cells. The GS anti-sense RNAs rendered the HepG2 cells more sensitive to growth arrest caused by glucose deprivation. They also sensitized the HepG2 cells to cadmium chloride (Cd) and nitric oxide (NO)-generating sodium nitroprusside (SNP). In brief, the results confirm that GS plays an important role in the defense of the human hepatoma cells against oxidative stress by reducing apoptosis and maintaining redox homeostasis.

Keywords: *Glutathione, glutathione synthetase, reactive oxygen species, hydrogen peroxide, apoptosis, HepG2*

Abbreviations: *BSA, bovine serum albumin; BSO, L-buthionine-(S,R)-sulphoximine; DCFA-DA, 2',7'-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, foetal bovine serum; GCS, γ-glutamylcysteine synthetase; GS, glutathione synthetase; GSH, glutathione; hGSH, homoglutathione; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propodium iodide; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction.*

Introduction

Glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) is the most abundant intracellular non-protein thiol compound and is found in virtually all prokaryotic and eukaryotic cells in high concentrations (up to 10 mM). GSH participates in a variety of biological functions, such as cellular defense against oxidative stress, detoxification of xenobiotics and carcinogens, redox reactions, biosyntheses of DNA and leukotrienes, neurotransmission and neuromodulation [1]. One of the most crucial functions of GSH is that it is considered to act as an antioxidant against various kinds of oxidative stresses. Altered GSH homeostasis, in association with increased oxidative stress, is implicated in the pathogenesis of many diseases, including Alzheimer's disease and Parkinson's disease [2]. Increased levels of GSH are detected in proliferating hepatocytes and hepatocellular carcinoma [3] and are associated with drug-resistant tumour cell lines and tumour cells from patients with tumours resistant to drug therapy [4,5]. Depletion of cellular GSH underlies tissue damage that is caused by a variety of metals and other thiol-directed chemicals [6].

GSH is synthesized in the two sequential reactions catalysed by γ-glutamylcysteine synthetase (GCS; EC 6.3.2.2) and glutathione synthetase (GS; EC 6.3.2.3) in the presence of ATP as an energy source. GCS catalyses the formation of L-γ-glutamyl-L-cysteine from L-glutamate and L-cysteine, while GS adds a glycine moiety to this intermediate leading to GSH. GCS has been known to be a rate-limiting enzyme in the biosynthesis of GSH

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and its expression is regulated by a number of agents including oxidants, antioxidants, GSH-depleting compounds, heat shock, ionizing radiation, cytokines, hormones and drug-resistant tumours [7]. Although relatively little information is available on the regulation of GS, there are indications that modulation of its activity is also important for controlling intracellular GSH levels. The *Saccharomyces cerevisiae* GS gene is regulated by Yap1p in response to oxidative stress and heat shock [8]. The unique GS gene in the fission yeast *Schizosaccharomyces pombe* is regulated by nitric oxide (NO), L-buthionine-(*S,R*)-sulphoximine (BSO), a specific inhibitor of GCS, cadmium and carbon source limitation [9,10]. In the Leguminosae family, homoglutathione (hGSH; L-γ-glutamyl-L-cysteinyl-β-alanine) replaces GSH either completely or partially [11]. Homoglutathione synthetase is up-regulated in cowpea, a non-hGSH-producing species, during progressive drought stress that would be related to the drought tolerance of the cowpea cultivar used, while GS is mainly constitutively expressed [12].

The human GS gene, mapped to the long arm of chromosome 20 at band q11.2, spans over ~32 Kb and comprises 13 exons with intron sizes ranging from 102 bp to 6 Kb [13,14]. It was identified to encode a full-length GS of a 474 amino acid sequence. The GS promoter activity was significantly up-regulated by nuclear factor erythroid-related factor 1 (Nrf1) or Nrf2 [15]. The two nuclear factor erythroid 2 (NFE2) sites within the promoter region was suggested to play important roles in the basal expression of GS [15]. Since alternative splicing variants of GS mRNA were identified in human normal tissues, human cancer cell lines and mouse liver cells, it was further suggested that the regulation of GS would play an important role in controlling the development of GSH-based redox homeostasis [16,17].

Although lines of evidence have accumulated which suggest a more prominent role for GS than previously recognized, there still remain uncertainties about the regulatory roles of GS in the biosynthesis and functioning of GSH. In most experiments to down-regulate GSH biosynthesis in order to verify the roles of GSH, the specific inhibitors of GCS, such as BSO, have been chiefly used. Specific down-regulating techniques of GS have rarely been used. In the present work, we constructed the stably transfected anti-sense cell line using human GS anti-sense RNA to solely suppress the expression of the GS gene, which was used to assess the roles of GS in relation with GSH and to indirectly estimate replaceable capacity of L-γ-glutamyl-L-cysteine in the state of low GSH level.

Materials and methods

Cell culture

Human hepatocarcinoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (V/V) heat-inactivated foetal bovine serum (FBS; PAA Laboratories Inc., Canada), 100 μg/ml streptomycin and 100 units/ml penicillin in a humidified atmosphere in 5% CO_2 at 37°C. The HepG2 cells were grown in 6-well culture plates to 50–60% confluence before subjecting to various treatments.

Generation of the anti-sensing stable cell line

For down-regulating expression of the human GS gene, GS cDNA was inserted in the anti-sense orientation into the mammalian expression vector pLPCX. To construct the plasmid pLPCX/GS-AS, which produce the anti-sense RNA of the GS gene, the anti-sensing cDNA was amplified with two synthetic primers (primer 1, 5′-GTGGTGATCGATGCCCCATTCA-3′; primer 2, 5′-TCCTTCTCTCGAGCAATCAGTAGCAC-3′) which contained *Cla*I and *Xho*I restriction sites, respectively. The amplified DNA fragment was electro-eluted from 1% agarose gel and digested with *Cla*I and *Xho*I. The digested DNA fragments were ligated to pLPCX previously digested with *Cla*I and *Xho*I and the ligation mixture was transformed into the *E. coli* strain MV1184 using the calcium chloride procedure. The resultant recombinant plasmid pLPCX/GS-AS was confirmed by restriction mapping and nucleotide sequencing. The PCR condition used in amplifying the cDNA anti-sensing expression of the GS gene was 94°C (1 min), 57°C (1 min) and 72°C (1 min) for 30 cycles with the two PCR primers. Nucleotide sequencing was performed with automatic DNA sequencer in Cosmo Genetech Co. (Korea).

The anti-sense plasmid pLPCX/GS-AS was transfected into the HepG2 cells using the calcium phosphate method [18]. One day after transfection, the neomycin-resistant cells were selected. After 4 weeks of growth, neomycin-resistant colonies were isolated, expanded and analysed.

Enzyme assays

As previously described [19], the GS activity in cellular lysates was spectrophotometrically determined as follows. Reaction mixtures contained 100 mM Tris-HCl buffer (pH 8.0), 50 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA, 10 mM ATP, 2.5 mM DTT, 5 mM glycine and 5 mM L-γ-glutamyl-L-cysteine. Reactions were initiated by adding 50–150 μg protein in a final volume of 200 μl and incubated at 37°C. The 40 μl samples, taken every 5 min up to 20 min, were mixed with 60 μl sulphosalicylic acid and the acidified samples were centrifuged at 10 000 g for 5 min and the supernatant was used for the determination of GSH.

Caspase-3 activity in the cellular lysates was determined by using a colourimetric caspase-3 substrate,

acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD pNA; Calbiochem, Gibbstown, NJ). Caspase-3 activity was monitored with a change in the absorbance at 405 nm using a microplate reader, reflecting chromophore pNA. Its specific activity was represented as μmoles/min/mg protein.

Glutathione peroxidase (GPx) activity in the cellular lysates was determined using the GPx assay kit obtained from Cayman Chemical Co. (Ann Arbor, MI). Its specific activity was calculated as nmoles/min/mg protein.

For preparation of the cellular lysates for the enzyme assays, both adherent and non-adherent cells were harvested and pooled. The cell pellets were washed once with phosphate-buffered saline (PBS), resuspended in cell lysis buffer (50 mM HEPES, 10% sucrose, 0.1% Triton X-100; pH 7.5) and stored on ice for 30 min. Cell debris was discarded after centrifugation at 3000 g for 5 min.

Protein content in the cellular lysates was determined according to the procedure of Bradford [20] using bovine serum albumin (BSA) as a standard.

Viability assays

For trypan blue exclusion assay, cells were trypsinized and collected by centrifugation. After adding trypan blue dye (Sigma-Aldrich, St. Louis, MO), the number of dye-excluding cells was counted on a hemocytometer. Blue cells were counted as non-viable.

To assess the cellular survival in the presence of stressful agents, the cellular viability was determined using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay used to assess metabolic activity [21]. Trypsinized cells were incubated with 20 μl of 5 mg/ml MTT solution for 2 h. The cells were then lysed with isopropyl alcohol and the amount of formazan, produced from reduction of MTT by the mitochondria of living cells, was determined by the absorbance at 540 nm. The survived cells were relatively represented as % MTT reduction.

Apoptosis assay

Apoptosis was determined by cell cycle analysis. Adherent and non-adherent cells were collected, centrifuged for 10 min at 3000 g and resuspended in PBS followed by fixation in 70% ethanol (–20°C). The fixed cells were centrifuged and washed once with PBS followed by resuspending in PBS-EDTA containing 0.1% Triton X-100, 2 μg/ml propodium iodide (PI) and 2.5 μg/ml RNase A. The cells were analysed by flow cytometry (FACSCalibur, Beckton, Dickenson and Co., Franklin, NJ). The amount of cells in G2/M phase arrest and apoptosis ($subG_0/subG_1$) was calculated using the Cellquest software (Beckton, Dickenson and Co.).

Determination of total GSH

About 5×10^5 cells were obtained by scraping off the bottom of the dish with a cell scraper. Cell pellets were once washed with ice-cold PBS and resuspended in lysis buffer (50 mM HEPES, 10% sucrose, 0.1% Triton X-100; pH 7.5) for 30 min on ice. Supernatants were taken by centrifugation at 3000 g for 15 min. As previously described [22], the total GSH content in the supernatants was determined using an enzymatic recycling assay based on glutathione reductase. The reaction mixture contained 175 mM KH_2PO_4, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM 5,5'-dithio-(2-nitrobenzoic acid), 0.5 units/ml glutathione reductase and cellular lysates at 25°C. The absorbance at 412 nm was monitored using a microplate reader. The total GSH content was represented as ng/mg protein.

Determination of intracellular ROS

For analysis of intracellular ROS, the redox-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used as previously described [23]. Cells were incubated with 5 μM DCFH-DA for 30 min at 37°C. The harvested cells were immediately analysed by a flow cytometry. The ROS level was arbitrarily represented as DCF fluorescence.

Semi-quantitative RT-PCR

Total RNA was isolated from appropriate HepG2 cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 2 μg total RNA using M-MuLV reverse transcriptase. One twentieth of the synthesized first-strand cDNA was used as templates in PCR using primers GS-F (5′-GTGGTGATCGATGCCCCATTCA-3′), GS-R (5′-TCCTTCTCTCGAGCAATCAGTAGCAC-3′), GCS-F (5′-AAGGGACACCAGGACAGCCCTA-3′), GCS-R (5′-CTGCAGGCTTGGAATGTCACCT-3′), GAPDH-F (5′-AAGGTCGGAGTCAACGGATT-3′), GAPDH-R (5′-GCAGTGGGTCTCTCTCCT-3′). PCR was performed using *Ex Taq* polymerase (iNtRON Biotechnology, Seoul, Korea) as follows: denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min.

Statistical analysis

The results were expressed as mean ± SD. Statistical comparison between experimental groups was

performed by ANOVA test followed by the Tukey's multiple range tests. p-values less than 0.05 were considered to be significant.

Results

Hydrogen peroxide-induced apoptosis

The hydrogen peroxide-induced apoptosis of the HepG2 cells was verified through the measurement of cell death (Figure 1A caspase-3 activity (Figure 1B), which is a key apoptotic mediator of mammalian cells, PI incorporation (Figure 1C) and DNA fragmentation (Figure 1D). When the HepG2 cells were incubated with 0.5 mM hydrogen peroxide for up to 24 h, their cellular viabilities significantly dropped after 18 and 24 h compared to those of the untreated control (Figure 1A). Under the treatment with 0.5 mM hydrogen peroxide for 24 h, caspase-3 activity increased 5.4-fold in the treated cells than in the untreated cells (Figure 1B). Under the same treatment, the apoptotic cell death of the HepG2 cells, detected using PI staining, was notably increased (Figure 1C) and DNA fragmentation of the HepG2 cells also appeared to be significantly higher in the treated cells than in the untreated cells (Figure 1D). Taken together, these findings indicate that hydrogen peroxide gives rise to apoptosis of the HepG2 cells at the treatment of 0.5 mM hydrogen peroxide for 24 h.

Down-regulation of intracellular total GSH and GS activity upon apoptosis

During the apoptotic process caused by hydrogen peroxide, the total GSH level in the HepG2 cells gradually diminished up to 24 h-incubation (Figure 2A). At the 24 h-incubation, the total GSH level in the HepG2 cells was decreased to ~40% of that in the cells at the beginning of the incubation with hydrogen peroxide (Figure 2A). Contrary to this, the total GSH level was significantly increased in the extracellular culture medium of the HerG2 cells grown under the treatment with hydrogen peroxide for 24 h (Figure 2B). Under the same apoptotic condition, the GS activity dropped to 70% of that of the untreated cells (Figure 2C). These results imply that a diminishment in the total GSH level of the HepG2 cells partly results from down-regulation of the GS activity and GSH release to the extracellular medium during the hydrogen peroxide-induced apoptosis.

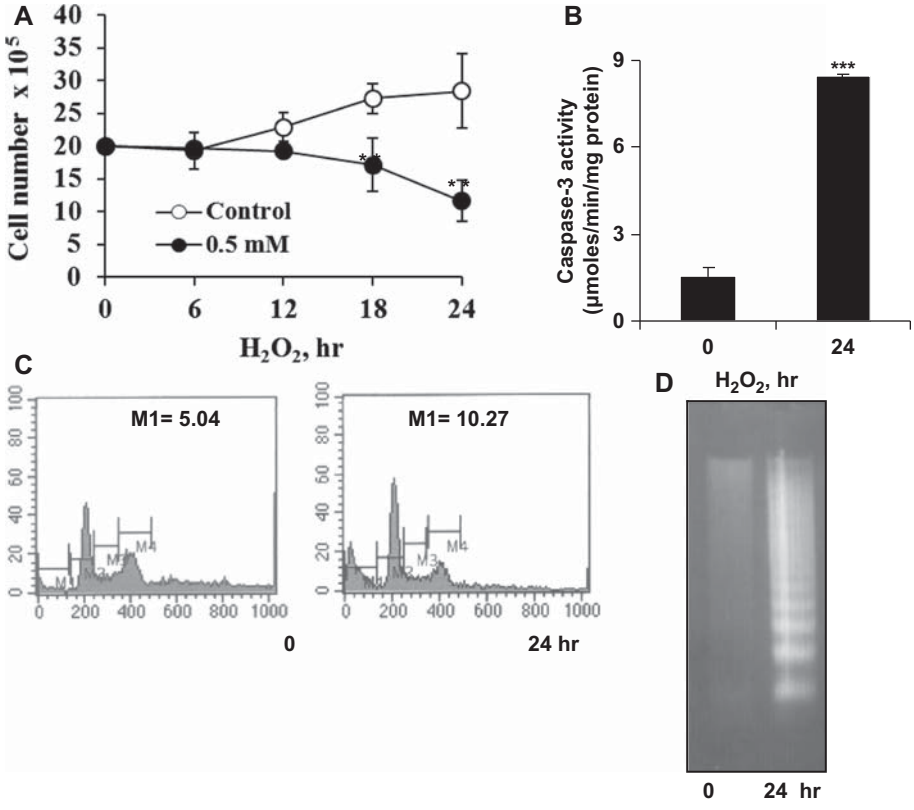

Figure 1. Hydrogen peroxide induces caspase-3-dependent cell death of the HepG2 cells. Cells were incubated with 0.5 mM hydrogen peroxide for varying time periods. Cellular proliferation was measured using the trypan blue exclusion assay (A). Caspase-3 activity in the HepG2 cells treated with hydrogen peroxide was represented as ΔA_{405}/min/mg protein (B). PI incorporation in HepG2 cells was detected after the treatment of hydrogen peroxide (0.5 mM for 24 h) (C). DNA fragmentation characteristics in HepG2 cells were also detected after hydrogen peroxide treatment (0.5 mM for 24 h) (D). $^{**}p < 0.01$; $^{***}p < 0.001$.

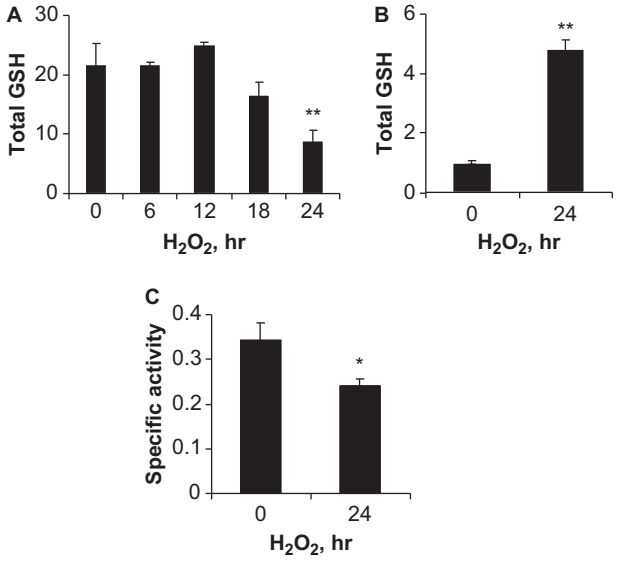

Figure 2. Effect of hydrogen peroxide exposure on intracellular GSH concentration (A), GSH release into the extracellular culture medium (B) and GS activity (C). Total intracellular GSH (A), GSH released into the culture media (B) and GS activity (C) were determined after the treatment with 0.5 mM hydrogen peroxide for varying time periods. GSH content is represented as ng/mg protein and GS activity is represented in μmoles GSH/min/mg protein. $^{*}p < 0.05$; $^{**}p < 0.01$.

Construction of the stable cell line down-regulating GS

The stable HepG2 cells anti-sensing expression of the GS gene was constructed as described in Materials and methods. Its construction was verified by decreased GS activity and mRNA levels and decreased total GSH level (Figure 3). As shown in Figure 3A, the GS activity of the GS-anti-sensing pLPCX/GS-AS cells was ~ 36% of that of the vector control cells (Figure 3A). This decrease was supported by the diminished GS mRNA levels in the GS-anti-sensing pLPCX/GS-AS cells (Figure 3B). Unexpectedly, the GCS mRNA levels in the transfected cells dropped to ~ 73% of those in the control cells (Figure 3C). As expected, the total GSH level in the GS-anti-sensing cells dropped to 30% of that in the vector control cells (Figure 3D). Collectively, the HepG2 stable cells anti-sensing expression of the GS gene was successfully prepared.

Characteristics of the GS-anti-sensing HepG2 stable cells

The GS-anti-sensing HepG2 cells were characterized in a few aspects. The cellular proliferation of the GS-anti-sensing HepG2 stable cells was significantly delayed compared to that of the vector control cells (Figure 4A). As shown in Figure 4A, the number of viable cells of the GS-anti-sensing HepG2 cells dropped to less than half of the number of the vector control cells after 4-days incubation. Although exogenous addition of 2.5 mM GSH was partly able to rescue the decreased proliferation of the GS-anti-sensing HepG2 cells, the enhancing effect of exogenous GSH was also detected upon the vector control cells (Figure 4A). In the absence of any stressful agents, the apoptotic percentage of the GS-anti-sensing HepG2 cells was ~ 2.1-fold higher than that of the vector control cells (Figure 4B). Concurrently, the caspase-3 activity was significantly higher in the GS-anti-sensing HepG2 cells than in the vector control cells (Figure 4C). The ROS level was ~ 1.9-fold higher in the GS-anti-sensing HepG2 cells than in the vector control cells (Figure 4D). Collectively, down-regulation of GS gives rise to significant enhancements in the

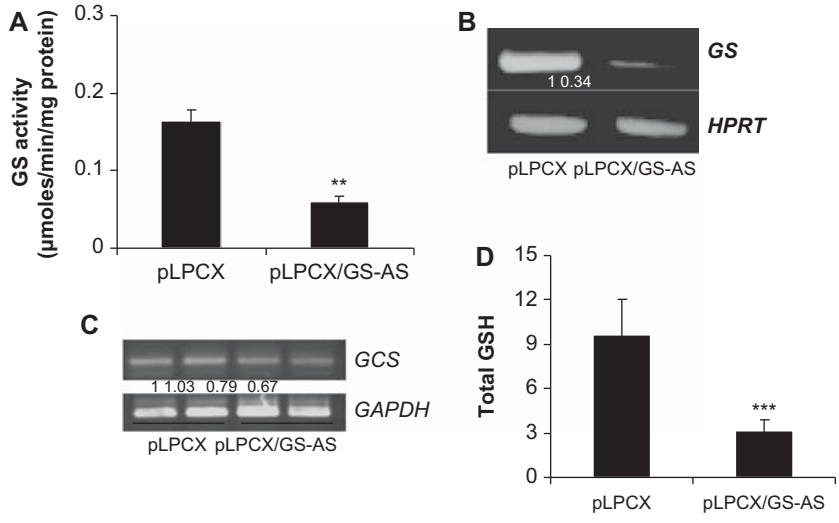

Figure 3. Characteristics of the HepG2 stable cells constitutively expressing the GS anti-sense RNA. GS activities in both the vector control cells and the GS-anti-sensing HepG2 cells were determined (A). GS mRNA (B), GCS mRNA (C) and total GSH (D) levels were also compared between both the vector control cells and the GS-anti-sensing HepG2 cells. The GS activity is represented in μmoles GSH/min/mg protein, while the GSH content is represented as ng/mg protein. The numbers below the GS pattern in (B) and below the GCS pattern in (C) show the relative band strengths. $^{**}p < 0.01$; $^{***}p < 0.001$.

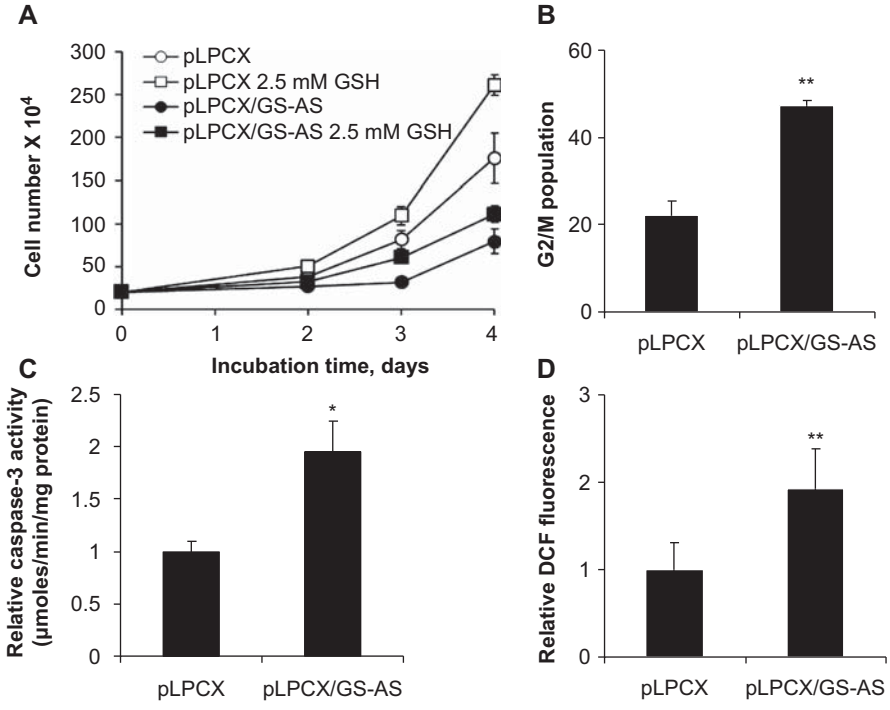

Figure 4. Comparisons of cellular proliferations, apoptosis and ROS levels of the vector control (pLPCX) and the GS-anti-sensing HepG2 (pLPCX/GS-AS) cells. Cells were grown in DMEM medium with or without 2.5 mM GSH and the viable cells were counted using the trypan blue exclusion assay (A). Apoptotic cells of both cell lines were detected using propidium iodide (PI) staining (B). Capase-3 activities were measured in both cell lines (C). ROS levels were also detected in both cell lines (D). The ROS content in D is represented in arbitrary units. $^{*}p < 0.05$; $^{**}p < 0.01$.

apoptotic percentage, caspase-3 activity and ROS level, but significant diminishment in the cellular proliferation in the HepG2 cells under normal growth conditions.

Enhancement of hydrogen peroxide-induced apoptosis by anti-sensing the GS gene

The apoptotic percentages of the GS-anti-sensing and the vector control cells by hydrogen peroxide were 1.9- and 2.5-fold those of the corresponding untreated HepG2 cells, respectively (Figure 5A). Under the treatment of hydrogen peroxide, the apoptotic percentage of the GS-anti-sensing HepG2 cells was significantly higher than that of the vector control cells (Figure 5A). Caspase-3, one of the effector caspases in the mitochondrial apoptotic pathway, is responsible for the cleavage of the key cellular proteins, which leads to the morphological changes observed in cells undergoing apoptosis. In the association of enhancement of hydrogen peroxide-induced apoptosis, caspase-3 activity in the GS-anti-sensing cells was found to be much higher than that in the vector control cells, under the incubation with hydrogen peroxide (Figure 5B). In brief, down-regulation of the GS gene markedly enhances both apoptosis and related caspase-3 activity in the HepG2 cells under hydrogen peroxide.

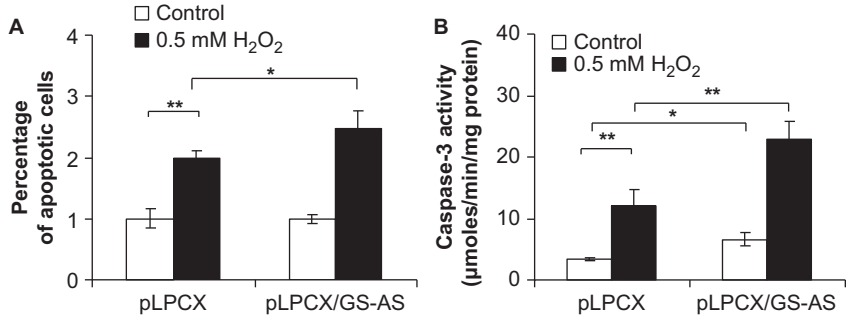

Figure 5. Differences in apoptosis (A) and caspase-3 activity (B) in the vector control cells and the GS-anti-sensing HepG2 cells exposed to 0.5 mM hydrogen peroxide for 24 h. The apoptotic cells of both cell lines were determined using propidium iodide (PI) staining (A). Percentages of apoptotic cells are relatively represented in (A). Caspase-3 activity is determined in the both cell lines and represented in μmoles/min/mg protein (B). $^{*}p < 0.05$; $^{**}p < 0.01$.

Anti-sensing the GS gene further enhances the ROS level but diminishes the total GSH level

Although ROS, at the physiological concentration required for normal cellular functions, are involved in intracellular signalling and redox regulation, those, at excessive concentrations, cause oxidative stress, threatening the integrity of various biomolecules and being involved in ageing [24]. Generation of ROS frequently triggers one of the representative pathways of apoptosis [25]. The ROS levels of the GS-anti-sensing and the vector control cells under the treatment of hydrogen peroxide were 2.1- and 2.7-fold higher than those of the corresponding untreated cells, respectively (Figure 6A). Under hydrogen peroxide, the ROS level in the GS-anti-sensing HepG2 cells was significantly higher than that in the vector control cells (Figure 6A). However, the total GSH level of the GS-anti-sensing was markedly lower than that of the vector control cells, under hydrogen peroxide (Figure 6B). The total GSH levels in the GS-anti-sensing HepG2 and the vector control cells under hydrogen peroxide appeared to be significantly lower than that in the corresponding untreated cells (Figure 6B). Collectively, down-regulation of the GS gene further decrease the total GSH level and sequentially enhances the ROS level in the HepG2 cells upon hydrogen peroxide-induced apoptosis.

Glutathione peroxidase (GPx) is one of the principal antioxidant enzymes which are predominantly responsible for detoxifying hydrogen peroxide and other lipid hydroperoxides at the expense of reduced GSH. It is also known to protect the hepatic cells from reactive oxygen metabolites and to be considered as a marker of redox status. GPx activity was identified to be ~ 3-fold higher in the human hepatoma HepG2 cells than in the human normal hepatic Chang cells [26]. Selenocysteine, present at the active site of GPx, has been known to regulate the GPx activity. As an example, the HepG2 cells were found to exhibit time-dependent decrease in GPx activity when incubated in selenium-free media and recover the GPx activity with the repletion of selenium [27]. In order to detect whether down-regulation of the GS gene could modulate the GPx activity in the HepG2 cells or not, the GPx activities were compared between the transfected cells and the control cells in the absence and presence of hydrogen peroxide. As shown in Figure 6C, there were no significant changes in the GPx activities between the transfected cells and the control cells in the absence of hydrogen peroxide. The GPx activity remained unchanged in the control cells under hydrogen peroxide, whereas it appeared to slightly increase in the transfected cells under hydrogen peroxide, when compared with the untreated corresponding cells, respectively (Figure 6C). This finding might indicate that down-regulation of the GS gene doesn't have significant influence on the GPx activity in the HepG2 cells.

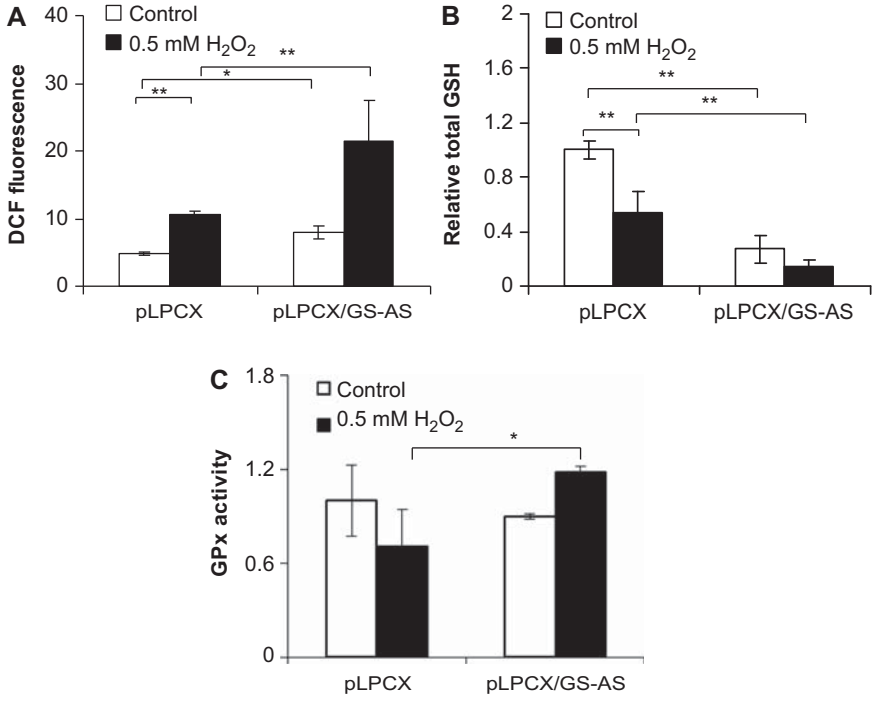

Figure 6. Differences in ROS (A), total GSH (B) and glutathione peroxidase (GPx) activity levels in the vector control cells and the GS-anti-sensing HepG2 cells exposed to 0.5 mM hydrogen peroxide for 24 h. ROS levels were detected in both cell lines and represented in arbitrary units (A). Total GSH levels in B and GPx activity in (C) were also determined in both cell lines and represented in relative values. $^{*}p < 0.05$; $^{**}p < 0.01$.

Suppressive effect of anti-sensing the GS gene on the cellular viabilities under glucose deprivation

Glucose deprivation increases generation of pro-oxidants and decreases scavenging of free radicals, leading to perturbations in cellular sensitivity to oxidative stress [28]. A decrease in cellular glucose level causes the transcriptional regulation of a number of genes that encode proteins involved in the unfolded protein response (UPR) or the endoplasmic reticulum (ER) stress response [29,30]. Glucose deprivation also induces the heme oxygenase-1gene by transcriptional activation via generation of ROS but not via a pathway dependent of the UPR [31,32]. Both the GS-anti-sensing HepG2 cells and the vector control cells were subjected to glucose-depleted media and their growths were compared using trypan blue exclusion assay. As shown in Figure 7A, the growth of the GS-anti-sensing HepG2 cells was completely arrested under glucose deprivation. However, the growth of the vector control cells were significantly delayed but not arrested under glucose deprivation (Figure 7A). After 4-day incubation, their cellular morphologies were observed through phase-contrast microscope. As shown in Figure 7B, the GS-anti-sensing HepG2 cells under glucose deprivation appeared to unstably attach to the culture plate, compared to the vector control cells. It is thought that, under glucose-limiting condition, the viable cells developed cytoplasmic bubbles and segments of their cell bodies were formed. In summary, down-regulation of the GS gene renders the HepG2 cells more sensitive to cytotoxicity caused by glucose deprivation.

Down-regulation of the GS gene diminishes cellular viabilities under nitric oxide and cadmium

In addition to ROS, reactive nitrogen species (RNS), produced in normal hepatocytes, are known to be important for normal physiologic processes, such as oxidative respiration, growth, regeneration, apoptosis and microsomal defense [33]. Oxidative stress, which occurred by exceeding the capacity of normal antioxidant system, causes significant damage to all types of liver cells through induction of inflammation, ischemia, fibrosis, necrosis and apoptosis or through malignant transformation by damaging lipids, proteins and/or DNA [33]. In the cadmium (Cd)-induced cell death in HepG2 cells, ROS, rapidly and transiently generated in the early stages, triggers apoptosis via Fas activation and subsequent caspase-8-dependent Bid cleavage [34]. N-acetylcysteine, used as an alternate means of boosting intracellular glutathione via elevated intracellular cysteine, is able to suppress Cd-induced apoptosis through the blocking of ROS generation as well as the catalase up-regulation [34]. Both the GS-anti-sensing HepG2 cells and the vector control cells were incubated with various concentrations of nitric oxide (NO)-generating sodium nitroprusside (SNP; 0, 0.25, 0.5 and 1 mM) or cadimium (Cd; 0, 8, 16 and 32 μM) for 24 h. As shown in Figure 8A, SNP was able to suppress the cellular viabilities of the GS-anti-sensing HepG2 cells in the concentration-dependent manner, which was detected using the MTT assay. In the treatment of cadmium, the similar suppression pattern could be obtained in the used concentration range (Figure 8B). Collectively, down-regulation of the GS gene also reduces the cellular viabilities of the HepG2 cells in the presence of nitric oxide and cadmium.

Discussion

GSH, a major antioxidant as well as a redox and cell signalling regulator, play important roles in cellular defense against oxidant aggression, redox regulation of protein thiols and maintenance of redox homeostasis that is critical for proper function of cellular processes, including apoptosis [35]. GSH was found to play a regulatory role in apoptosis in testicular cells and its depletion may be critical in pre-disposing

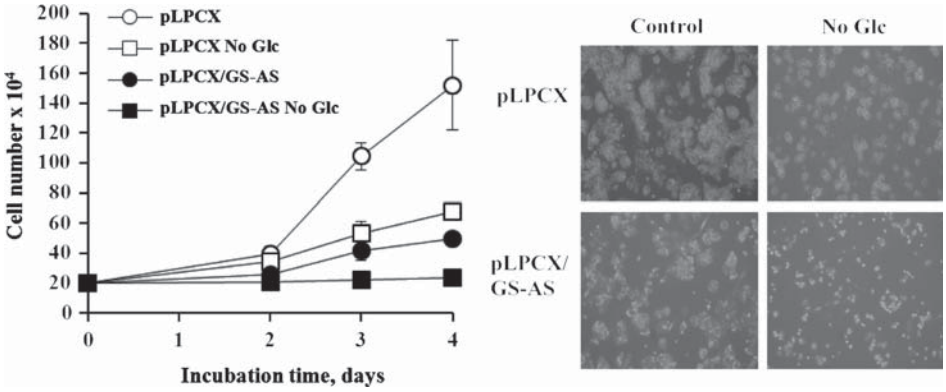

Figure 7. Cellular proliferations of the vector control cells and the GS-anti-sensing HepG2 cells under glucose deprivation. Cellular proliferations of both cell lines were determined using the trypan blue exclusion assay (A). After 4-day incubation, the changes in cellular morphologies of both cell lines were observed by phase-contrast microscopy (B).

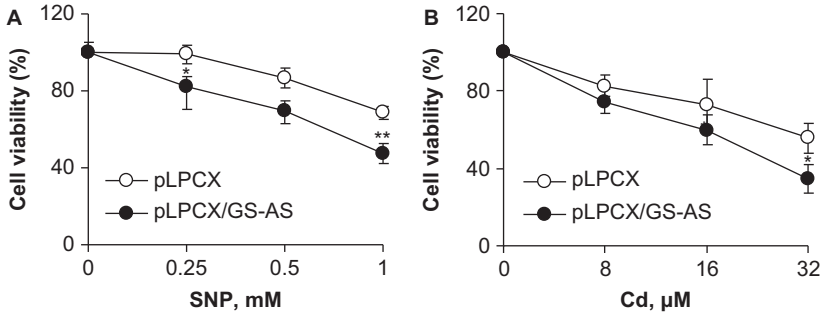

Figure 8. Differences in the cellular viabilities of the vector control cells and the GS-anti-sensing HepG2 cells in the presence of nitric oxide (NO) or cadmium (Cd). Cells were incubated for 24 h with varying concentrations of NO-generating sodium nitroprusside (SNP; 0, 0.25, 0.5 and 1 mM) (A) or Cd (0, 8, 16 and 32 μM) (B). The cellular viabilities were determined using MTT assay and represented in relative values in (A) and (B). $^{*}p < 0.05$; $^{**}p < 0.01$.

these cells to apoptotic cell death [36]. Over-expression of GCS, known as a rate limiting enzyme in the biosynthesis of GSH, in human granuloma cells augmented GSH synthesis and ameliorated various sequences associated with exposure to oxidative stress and irradiation and suppression of GSH synthesis reversed resistance to radiation [37]. Although a rate-limiting role of GCS has been well documented from various experiments, roles of GS, catalysing the last step in the biosynthesis of GSH, have not been sufficiently elucidated so far.

The important roles of GS under normal conditions as well as stressful conditions are predicted from a few findings. GS deficiency, a rare autosomal recessive inherited metabolic disorder, modulates the stability of GS, causing a compensated haemolytic anaemia in mildly affected patients, additionally gives rise to metabolic acidosis in moderately affected patients and further causes neurological defects and increased susceptibility to bacterial infections in severely affected patients [38]. Cultured fibroblasts from patients with GS deficiency have low levels of GSH, but instead accumulate L-γ-glutamyl-L-cysteine [39]. *S*-acetylglutathione, more stable in blood than GSH, was found to enhance intracellular GSH content in cultured fibroblasts from patients with GS deficiency, indicating that it may have implications for the treatment of patients with GS deficiency [40]. These findings propose that GS is required for normal physiological functions. In order to assess the physiological roles of GS, we constructed the GS-anti-sensing HepG2 cells, which was confirmed with significantly decreased GS activity and GS mRNA levels together with markedly lower GSH level. The GS-anti-sensing HepG2 cells appeared to be disadvantageous in normal conditions. They were more susceptible to apoptosis and less proliferative, probably due to the enhanced ROS levels. They were also identified to be unfavourable to cellular proliferation and more susceptible to apoptosis under stressful conditions, such as hydrogen peroxide, glucose deprivation, nitric oxide and Cd, which might be due to the enhanced accumulation of ROS. ROS are continuously generated by aerobic metabolism, during pathophysiological developments such as inflammatory and allergic diseases and by ionizing radiation [41]. ROS play a diverse role in different cellular processes ranging from apoptosis and necrosis to cellular proliferation and carcinogenesis [42]. These results strongly imply that L-γ-glutamyl-L-cysteine, estimated in the anti-sensing cells, is not sufficiently able to compensate for the decreased level of GSH in normal conditions and doesn't cope with stressful conditions. This estimation was further supported by the decreased GCS mRNA level in the anti-sensing cells. Although how the GCS mRNA level is diminished in the GS-anti-sensing cells currently remains unknown, one possibility is that down-regulation of GS in the anti-sensing cells would enhance the L-γ-glutamyl-L-cysteine level and subsequently decrease expression of the GCS gene in an indirect manner. On the contrary, cultured fibroblasts from patients with GS deficiency were found to contain higher levels of L-γ-glutamyl-L-cysteine than controls [39]. In the budding yeast *S. cerevisiae*, L-γ-glutamyl-L-cysteine was suggested to substitute for GSH under both normal and oxidative stress conditions due to its accumulation, although the growth of the yeast GS mutant on minimal media was relatively poor compared to the wild-type yeast [43]. Poor growth of the yeast mutant might imply the incomplete substitution of L-γ-glutamyl-L-cysteine under down-regulation of GS. It could further suggest selective substitution of L-γ-glutamyl-L-cysteine dependent on the growth conditions. Knockdown of GS expression by RNA interference in cultured Schneider's Drosophila S2 cells led to enhanced arsenite sensitivity, while GS RNAi applied to intact organisms dramatically reduced the concentrations of food-borne arsenite compatible with successful growth and development, which suggested that an optimally functioning two-step GSH biosynthetic pathway is required *in vivo* for a robust defense against arsenite [44]. In a plant cell, the over-expression of the *Escherichia coli* GS gene was able to accumulate significantly more cadmium and cause enhanced tolerance to cadmium than the

wild type, suggesting that GS is rate limiting for the biosynthesis of GSH [45].

There have been many findings on the cellular roles of GSH which were identified using inhibitors of GSH biosynthesis, especially BSO, a well-known specific inhibitor of GCS. Butenolide, 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone, one of the mycotoxins produced by *Fusarium* species, reduced viability of the HepG2 cells through rapid depletion of GSH and concomitant increase in intracellular ROS production and butenolide-induced GSH depletion was aggravated by the addition of BSO [46]. Depletion of GSH in the HepG2 cells with BSO significantly increased the susceptibility of the HepG2 cells to acrylamide- and trichloroethylene-induced cytotoxicity and DNA damage, suggesting oxidative DNA damage in the hepatoma cells was induced by intracellular ROS and depletion of GSH [47,48]. BSO was found to slightly enhance growth inhibition and death in gallic acid-treated lung cancer cells and also mildly increase ROS levels and GSH depletion in these cells, implying that gallic acid-induced lung cancer cells are related to GSH depletion as well as ROS level changes [49]. As shown in the above-mentioned findings, the cellular roles of GSH, verified using inhibitors of GSH biosynthesis, would be similar to those obtained with the stable GS-anti-sensing transfectant cells. However, based on the general characteristics of the stable transfectant cells, the GS-anti-sensing transfectant cells could be closer to genetically mutated GS-deficient cells. They could be useful for preserving sustained depletion of GSH without the exogenous application of the GSH biosynthetic inhibitors and for examining the probable role(s) of GS independent of GSH level. Perifosine, an Akt inhibitor inducing cytotoxicity via metabolic oxidative stress, was able to induce increases in oxidized glutathione (GSSG) in human head and neck cancer cells, which was reversed by simultaneous treatment with N-acetylcysteine, and BSO could sensitize the same cancer cells to perifosine-induced clonogenic killing as well as decreased total GSH and increased GSSG [50]. In plant cells, BSO has been known to inhibit the biosynthesis of reduced GSH, thereby switching the total GSH pool towards GSSG [51]. The erythrocyte systems for anti-oxidative protection fail to control the oxidative burst after burning, which is due to the decreased concentration of vitamin E and the reduced GSH and the increased GSSG [52]. Hydrogen peroxide was able to reduce cellular viability in the HepG2 cells over-expressing glutathione reductase via causing a marked enhancement in reduced and oxidized glutathione (GSH/GSSG) ratio [53]. Accordingly, it has been generally believed that down-regulation of GSH biosynthesis raise the GSSG level due to the limited GSH pool. Likewise, the GSH/GSSG ratio is estimated to be lower in the GS-anti-sensing transfectant cells than in the control cells. In other words, the relatively increased GSSG level in the transfectant cells will make the cells more sensitive to hydrogen peroxide-induced apoptosis of the HepG2 cells.

In conclusion, the results, obtained from this work, and a few other findings suggest that GS also plays fundamental and regulatory roles in the biosynthesis of GSH. Currently, the unknown roles of GS not via GSH remain elusive.

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Declaration of interest

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