Jpn. J. Cancer Res. **93**, 716–722, June 2002

Hammerhead Ribozyme against γ-Glutamylcysteine Synthetase Attenuates Resistance to Ionizing Radiation and Cisplatin in Human T98G Glioblastoma Cells

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Glioblastoma cells are highly malignant and show resistance to ionizing radiation, as well as anti-cancer drugs. This resistance to cancer therapy is often associated with a high concentration of glutathione (GSH). In this study, the effect of continuous down-regulation of γ-glutamylcysteine synthetase (γ-GCS) expression, a rate-limiting enzyme for GSH synthesis, on resistance to ionizing radiation and cisplatin (CDDP) was studied in T98G human glioblastoma cells. We constructed a hammerhead ribozyme against a γ-GCS heavy subunit (γ-GCSh) mRNA and transfected it into T98G cells. (1) The transfection of the ribozyme decreased the concentration of GSH and resulted in G1 cell cycle arrest of T98G cells. (2) The transfection of the ribozyme increased the cytotoxicity of ionizing radiation and CDDP in T98G cells. Thus, hammerhead ribozyme against γ-GCS is suggested to have potential as a cancer gene therapy to reduce the resistance of malignant cells to ionizing radiation and anti-cancer drugs.

Key words: γ-Glutamylcysteine synthetase — Hammerhead ribozyme — Glutathione — Cisplatin — Ionizing radiation

Resistance of malignant cells to radiotherapy and chemotherapy is a major problem in the treatment of cancers. Glioblastoma is one of the most malignant forms of neoplasm, and often shows resistance to radio- and chemotherapy.[1)] This resistance is due to direct factors, such as a low intrinsic radiation sensitivity, a high recovery capacity, an increased number of clonogens, and a high hypoxic fraction.[2)] Glutathione (γ-glutamylcysteinylglycine, GSH), participates in many biological processes, especially cellular defense against oxidative stress induced by reactive oxygen species (ROS) and anti-cancer drugs.[3–5)] GSH is synthesized via two ATP-requiring steps that are catalyzed by γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme for the synthesis of GSH, and GSH synthetase. γ-GCS is composed of a heavy subunit (γ-GCSh) that is catalytic, and a light subunit (γ-GCSl) that is regulatory.[6, 7)] We previously found that γ-GCS is over-expressed in T98G human glioblastoma cells concomitant with a high concentration of GSH.[8)] Since ionizing radiation produces ROS inside the cells, over-expression of γ-GCS was thought to be an important factor in the acquisition of T98G glioblastoma cell resistance to ionizing radiation. The over-expression of γ-GCS has also been reported in many human malignant cells resistant to cisplatin [*cis*-diamminedichloroplatinum (II)] (CDDP) and doxorubicin.[9–13)] Recently, we reported that the two subunits of γ-GCS are concomitantly expressed in response to CDDP in human cancer cells.[9)] We also reported that CDDP is transported in the form of a CDDP-GSH adduct[14)] and that GSH S-transferase π (GSTπ) detoxifies CDDP by forming an adduct with GSH, and is thus important for the efflux of CDDP.[15)] These findings suggest that a high concentration of GSH is a factor in malignant cell acquisition of resistance to anti-cancer drugs as well as ionizing radiation.

Concerning the expression of γ-GCS, Manna *et al.* reported that over-expression of γ-GCS heavy subunit suppresses tumor necrosis factor-induced apoptosis and the DNA-binding activity of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1),[16)] suggesting an important role for γ-GCS in regulating cell proliferation and death.

In this study, we developed a hammerhead ribozyme against γ-GCSh to constitutively suppress the *γ-GCSh* gene expression in T98G cells, and examined its effect on γ-GCSh and on the resistance to ionizing radiation and CDDP. Hammerhead ribozymes are derived from satellite RNA of tobacco ring spot virus, and are newly developed trans-acting RNA enzymes that modulate specific gene expression.[17)] The hammerhead ribozymes possess a catalytic core that cleaves the target RNA, and flanking regions that direct the ribozyme core to a specific target site.[18)] A previous report showed that hammerhead ribo-

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zymes against γ-GCS caused down-regulation of the intracellular GSH concentration in mouse islet cells.[19] Quite recently, we reported that hammerhead ribozymes against γ-GCSh not only down-regulate the levels of GSH, but also decrease expression of the ATP-binding cassette superfamily transporters, thereby effectively decreasing the efflux of anti-cancer drugs.[20]

Here we show that a decrease in the levels of GSH induced by anti-γ-GCSh ribozyme increased the cytotoxicity of ionizing radiation and CDDP in T98G human cancer cells.

MATERIALS AND METHODS

Materials Human glioblastoma cells (T98G) were purchased from the Health Sciences Research Resources Bank (Tokyo). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO_2 under 100% humidity.

Exposure to ionizing radiation For each experiment the cells were transferred to fresh medium with adjustment to a cell density of 1×10^6 cells/ml. The cells were preincubated in this fresh medium for 2 h at 37°C in T-75 flasks (Corning International, Corning, NY) prior to exposure to ionizing radiation. Cells were irradiated with 200 kV of X-rays (Toshiba EXS-300, Tokyo) at a fixed dose rate of 1.0 Gy per min at room temperature. Mock-irradiated control cells (0-Gy) were treated identically.

Estimation of GSH The concentration of intracellular GSH was estimated enzymatically as described by Beutler.[21]

Plasmid construction and transfection The anti-γ-GCSh ribozyme was cloned into the pH β plasmid using two synthetic single-stranded oligodeoxynucleotides, 5′-TCGACTGTATTGCTGATGAGTCCGTGAGGACGAAACTCGGAA-3′ and 5′-AGCTTTCCGAGTTTCGTCCTCACGGACTCATCAGCAATACAG-3′[19] with flanking *Sal*I and *Hin*dIII restriction sites. Subconfluent T98G cells were transfected with the pH β plasmid using Lipofectin Reagent (Gibco BRL, Life Technologies, Gaithersburg, MD). Stably transfected cells were selected for integration of the plasmid in growth media containing 500 μg/ml of G418 sulfate for 4 weeks. Selected G418-resistant colonies were grown and screened for expression of the ribozyme by reverse transcription (RT)-PCR assay.[19] The ribozyme-transfected cells, in which the GSH concentration was decreased by approximately 20%, were further selected and used in these experiments. Disabled hammerhead ribozyme was synthesized by PCR with the following primers: 5′-TCGACTGTATTGCTAATGAGTCCGTGAGGACGAAACTCGGAA-3′ and 5′-AGCTTTCCGAGTTTCGTCCTCACGGACTCATTAGCAATACAG-3′. The disabled ribozyme contained a single base exchange (G to A) in the catalytic core compared with the original ribozyme.[19] Fig. 1(A) shows a model of the anti-γ-GCS ribozyme. Fig. 1(B) shows the results of PCR to detect the presence of the ribozyme in the cells. The ribozyme insert sequence was analyzed using the dsDNA Cycle

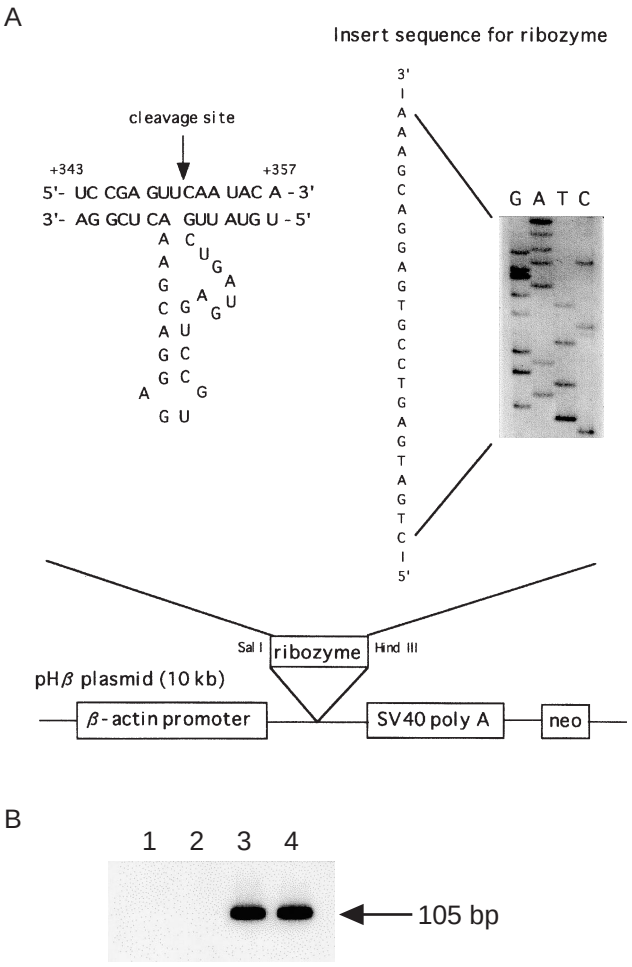

Fig. 1. Construction and transfection of anti-γ-GCSh ribozyme. Anti-γ-GCSh hammerhead ribozyme was constructed. The anti-γ-GCSh ribozyme was cloned into the pH β plasmid using two synthetic single-stranded oligodeoxynucleotides with flanking *Sal*I and *Hin*dIII restriction sites. These primers were used for screening cell lines to detect the presence of pHβ/anti-γ-GCSh ribozyme. The ribozyme insert sequence was analyzed using a dsDNA Cycle Sequencing System. The sequence primer used was 5′-GTCTGGATCCCTCGAAGC-3′. Subconfluent T98G cells were transfected with the pH β plasmid using Lipofectin Reagent. Stably transfected cells were selected for integration of the plasmid in growth media containing 500 μg/ml of G418 sulfate for 4 weeks. (A) A model of anti-γ-GCSh hammerhead ribozyme. (B) Results of RT-PCR to detect the presence of the ribozyme in the cells. PCR primers to detect ribozyme expression were pH β-PCR-1,5′-AGCACAGAGCCTCGCCTTT-3′ and pH β-PCR-2,5′-GTCTGGATCCCTCGAAGC-3′. Lane 1, parent cells; lane 2, vector only; lane 3, ribozyme transfectant, #1; lane 4, ribozyme transfectant, #2.

Sequencing System (Gibco BRL). The sequence primer used was 5′-GTCTGGATCCCTCGAAGC-3′. PCR primers to detect ribozyme expression were pH β-PCR-1,5′-AGCACAGAGCCTCGCCTTT-3′ and pH β-PCR-2,5′-GTCTGGATCCCTCGAAGC-3′. The following experiments were performed using the ribozyme transfectant #1.

RT-PCR assay RT-PCR was performed according to a commercial protocol (Perkin-Elmer Cetus, Norwalk, CT), and was used to detect the ribozyme and *γ-GCSh* gene expression using Superscript II (Gibco BRL) and *Taq* DNA polymerase (Perkin-Elmer Cetus) as described previously.[22] Briefly, the specific PCR products were size-fractionated by horizontal agarose gel electrophoresis, and stained with ethidium bromide. The authentic sense primer for γ-GCSh was 5′-CCAGTTCCTGCACATCTACCACG-3′, and the antisense primer was 5′-GTCGCTGGG-GAGTGATTTCTGC-3′.

Cytotoxicity assay The sensitivity of the cells to ionizing radiation and CDDP was assayed by tetrazolium salt assay (MTT) as described.[14, 23] Cells (1500) were placed in 100 μl of medium/well in 96-well plates. One to 7 days after treatment with various doses of ionizing radiation, or 72 h after treatment with various concentrations of CDDP, the cells were incubated for 4 h at 37°C with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (652 μg/ml). The cells were lysed with 100 μl of 20% SDS/50% N,N-dimethylformamide (pH 4.7) in each well. After an overnight incubation at 37°C, the absorbance at 570 nm was measured. Wells without cells served as blanks.

Cell cycle analysis Cell cycle distribution was analyzed flow-cytometrically. Cells were harvested by trypsinization, washed with ice-cold PBS, resuspended in cold PBS, and fixed by adding absolute ethanol while vortexing to a final concentration of 70%. After overnight refrigeration at −20°C and subsequent rehydration in PBS for 30 min at 4°C, the cell nuclei were stained for 30 min in the dark with 50 μg/ml propidium iodide (Sigma Aldrich, St. Louis, MO) containing 125 units/ml protease-free RNase (Calbiochem-Novabiochem. Co., Darmstadt, Germany), both diluted in PBS. Cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Mansfield, MA). DNA histograms were modeled off-line using Modifit-LT software (Verity, Topsham, ME).

Statistical analysis Data are presented as the mean±SD. Differences were examined using Student's two-tailed *t* test, or otherwise with a two-factor factorial ANOVA test. The criterion of significance was taken as $P<0.05$ for Student's *t* test and $P<0.01$ for the two-factor factorial ANOVA test.

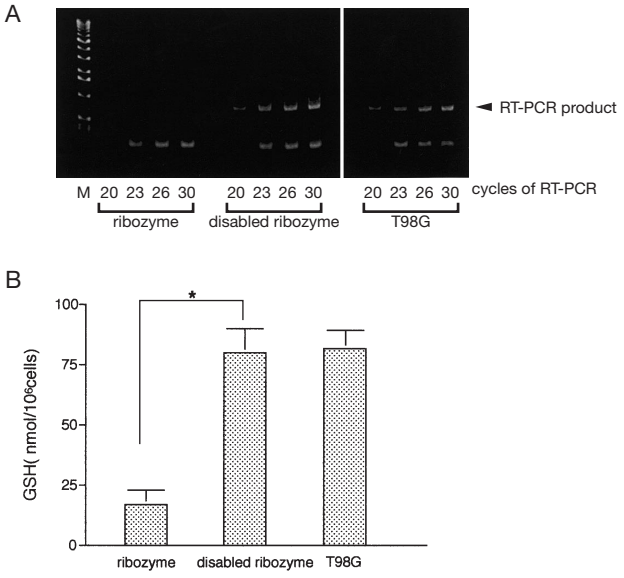

Fig. 2. The effect of anti-γ-GCSh ribozyme on the level of GSH. The effect of anti-γ-GCSh ribozyme was studied on the expression of γ-GCSh mRNA using RT-PCR (A). The specific PCR products were size-fractionated by horizontal agarose gel electrophoresis, and stained with ethidium bromide. The arrow indicates the position of the γ-GCSh mRNA product (822 bp). The intracellular concentration of GSH was studied in T98G cells (B). Values are expressed as nmol GSH/10^6 cells. The data are the mean±SD of three independent analyses. * $P<0.05$ vs. +disabled ribozyme.

RESULTS

Suppression of GSH levels by the ribozyme Fig. 2 shows the characteristics of T98G cells transfected with hammerhead ribozyme against γ-GCSh. The anti-γ-GCSh ribozyme down-regulated the expression of γ-GCSh mRNA in T98G-transfected cells (Fig. 2A). The transfection resulted in a significant decrease in the level of GSH of approximately 26% in cloned cells ($P<0.05$ vs. the cells transfected with disabled ribozyme). The concentration of GSH was 81.2±5.0 nmol/10^6 cells in T98G cells, 80.0±5.1 nmol/10^6 cells in the cells transfected with disabled ribozyme, and 21.5±3.1 nmol/10^6 cells in the cells transfected with anti-γ-GCSh ribozyme (Fig. 2B).

Proliferation rate and sensitivity to ionizing radiation The effect of transfection with anti-γ-GCSh ribozyme on the proliferation rate of T98G cells and on the sensitivity to ionizing radiation was studied. The cells were treated with ionizing radiation and the cytotoxicity was estimated by MTT assay. Fig. 3 shows the findings of MTT assay. The proliferation of T98G cells was expressed in terms of absorbance at 570 nm. (A) During culture, cells proliferated approximately 20-fold by the 7th day in the cases of control cells and the cells transfected with disabled ribozyme. The cells transfected with anti-γ-GCSh ribozyme showed suppressed proliferation (only 10-fold on the 7th day). Two grays of ionizing radiation had no apparent

effect on cell survival. On the 7th day, 10-Gy radiation decreased the survival of these cells. (B) The data in (A) were analyzed using a two-factor factorial ANOVA test. On the 7th day, the survival rate of anti-γ-GCSh ribozymetransfected cells exposed to 10-Gy radiation was significantly less than that of disabled ribozyme-transfected cells ($P<0.01$).

By cell cycle analysis, as shown in Fig. 4, it was found that transfection with anti-γ-GCSh ribozyme increased the percentage of G0/G1 phase (75.7%) and decreased the percentage of cells in S phase (15.1%) compared with disabled ribozyme (59.5% of G0/G1 phase, and 29.2% of S phase), suggesting that transfection with anti-γ-GCSh ribozyme causes G1 cell cycle arrest.

Sensitivitiy to CDDP The effect of different concentrations of CDDP on cell survival was studied. The cells were treated with various concentrations of CDDP for 72 h and the cytotoxicity was estimated by MTT assay (Fig.

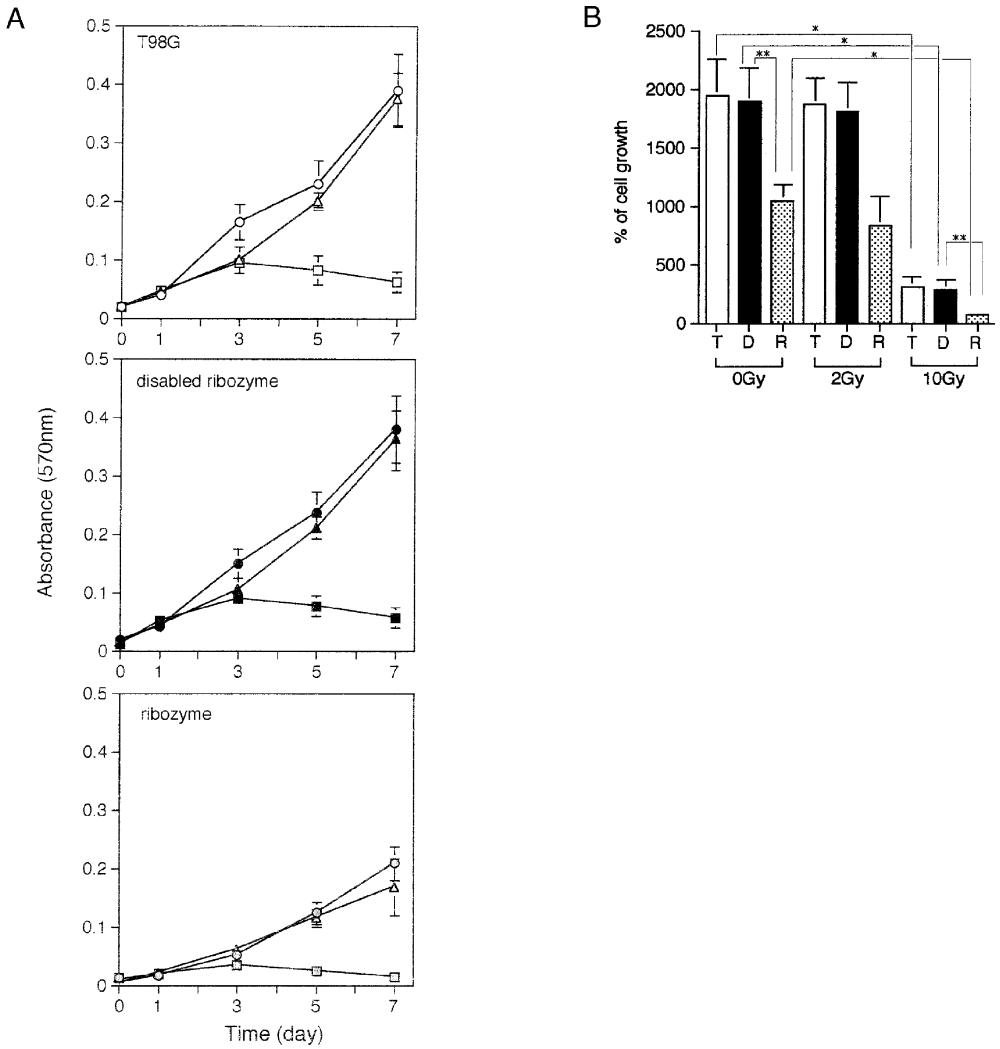

Fig. 3. The sensitivity of T98G cells to ionizing radiation. The sensitivity of T98G cells to ionizing radiation was examined by MTT assay. Cells (1500) were placed in 100 μl medium/well in 96-well plates. Up to 7 days after treatment with 2 or 10 Gy of radiation, the cells were incubated for 4 h at 37°C with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (652 μg/ml) and lysed as described in "Materials and Methods." (A) The absorbance at 570 nm was measured for control (upper panel), +disabled ribozyme (middle panel) and +anti-γ-GCSh ribozyme (lower panel). Open circle, control/0 Gy; open triangle, control/2 Gy; open square, control/10 Gy; closed circle, disabled ribozyme/0 Gy; closed triangle, disabled ribozyme/2 Gy; closed square, disabled ribozyme/10 Gy; slashed circle, ribozyme/0 Gy; slashed triangle, ribozyme/2 Gy; slashed square, ribozyme/10 Gy. (B) Cell survival (%) was shown using the same data, and comparing the cell numbers on day 0 and on the 7th day. T, T98G cells; D, disabled ribozyme; R, ribozyme. The data are ±SD of three independent analyses. * $P<0.01$ vs. each cell at 0 Gy; ** $P<0.01$ vs. +disabled ribozyme.

5). The number of survivors (%) among the control cells as well as the cells transfected with disabled ribozyme decreased gradually, depending on the concentration of CDDP. As in the case of the data for ionizing radiation, the cell number increased by 7-fold at 3 days of pre-incubation, but this decreased to only 5-fold after treatment with 25 μM CDDP for 3 days. In contrast, the cell number of T98G cells transfected with anti-γ-GCSh ribozyme showed an increase of only 2.5-fold at 3 days of pre-incubation, and this was decreased to 25% by the treatment with 25 μM CDDP for 3 days.

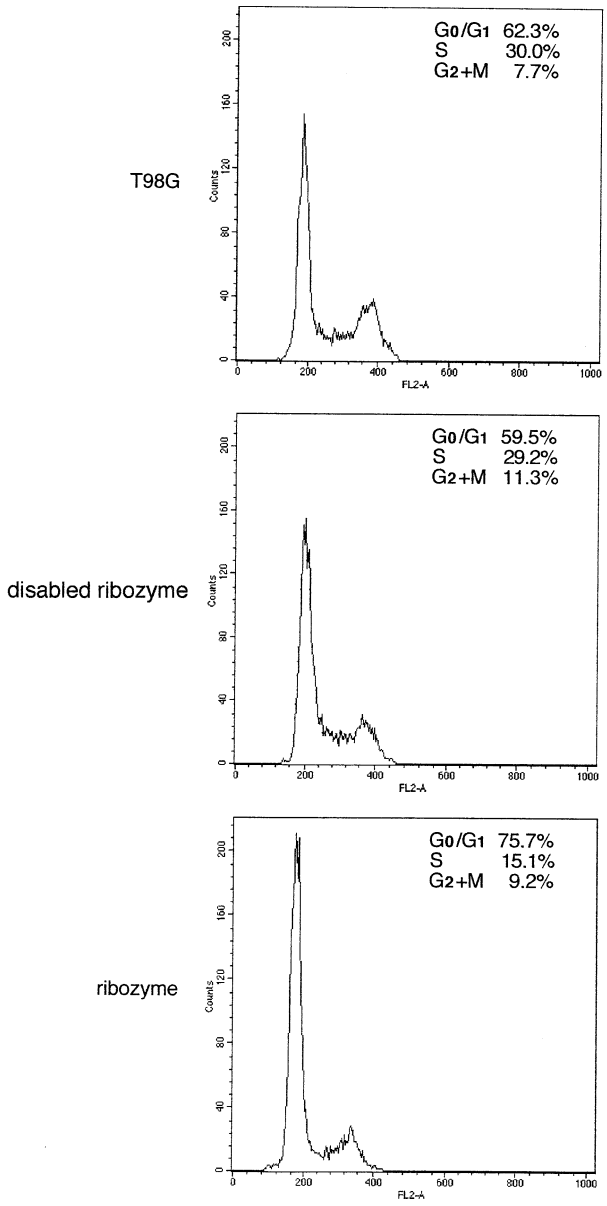

Fig. 4. Flow cytometric analysis of cell cycle. The effect of anti-γ-GCSh ribozyme on the cell cycle was analyzed using a FACScan flow cytometer. Cells in the steady state were harvested and employed for the cell cycle analysis. The proportion of cells in each stage was calculated using Modifit-LT software. Upper plot, T98G; middle plot, T98G cells transfected with disabled ribozyme; lower plot, T98G cells transfected with anti-γ-GCSh ribozyme.

DISCUSSION

In this study, we clarified the effects of continuous suppression of GSH synthesis on the resistance of T98G human glioblastoma cells to ionizing radiation and CDDP. We transfected anti-γ-GCSh ribozyme into T98G cells to effectively and consistently decrease the levels of GSH (Fig. 2). The decrease in GSH levels was associated with a decrease in the cell survival after exposure to ionizing radiation (Fig. 3) and CDDP (Fig. 5) in T98G cells. CDDP metabolism consists of various processes, such as binding of CDDP to cellular DNA to cause damage, detoxification of CDDP by coupling with GSH to form adducts and ATP-dependent efflux of CDDP-GSH adducts through the multidrug resistance related protein II.[15, 24] According to reports on the over-expression of γ-GCS in CDDP-resis-

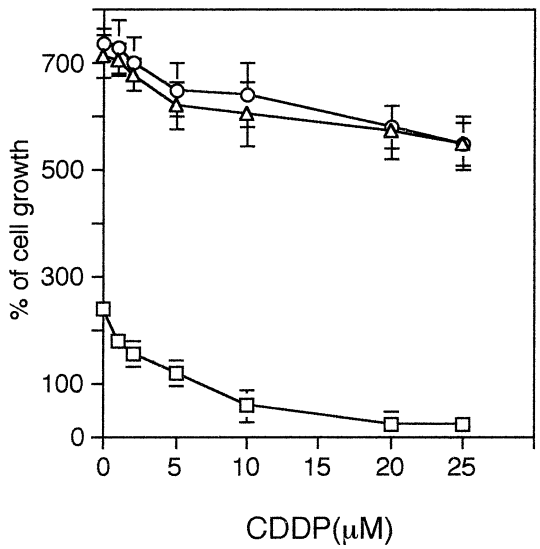

Fig. 5. Cytotoxicity of CDDP. The effect of anti-γ-GCSh ribozyme on the cytotoxicity of CDDP was examined by MTT assay. Seventy-two hours after treatment with various concentrations of CDDP, the cells were incubated for 4 h at 37°C with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (652 μg/ml). The cells were lysed with 100 μl of 20% SDS/50% N,N-dimethylformamide (pH 4.7) in each well. After overnight incubation at 37°C, the absorbance at 570 nm was measured. The data were expressed as the number of survivors (%), with those at day 0 as 100%. Wells without cells served as blanks. Data are the mean±SD of three independent analyses.

tant cancer cells,[9–12, 25] an increase in GSH synthesis is closely related to resistance of cancer cells to CDDP. Our findings show that GSH plays an important role in CDDP resistance and ribozyme-mediated specific modulation of γ-GCSh expression causes an increase in the cytotoxicity of CDDP to cancer cells.

In the present study, transfection of cells with anti-γ-GCSh ribozyme resulted in G1 cell cycle arrest and suppression of cell growth (Fig. 4). Depletion of GSH using buthionine sulfoximine (BSO), a specific inhibitor of GSH synthesis, is known to inhibit cell growth by arresting the cell cycle at G1.[26, 27] The data in this study are in good agreement with these reports.

Previously, we reported that depletion of GSH by exposure to BSO enhanced the ionizing radiation-dependent induction of γ-GCSh mediated by NF-κB.[8] It was thought that the treatment with BSO caused an increase in the NF-κB-DNA binding activity suppressed by GSH. A role for GSH in the redox regulation of transcriptional factors and intracellular signal transduction pathways has been suggested.[28] In such cases, cysteine residues may be modified by GSH at the phosphorylation site of transcriptional factors or protein kinases. We reported the importance of GSH in regulating the phosphorylation of IκB and the NF-κB-mediated expression of NF-κB subunits by tumor necrosis factor-α,[29] and in Fas-mediated apoptosis.[30] Similarly, it has been reported that an increase in the intracellular GSH concentration induced by the transfection of γ-GCSh caused down-regulation of cytokine-induced activation of NF-κB and AP-1.[16] Klatt *et al*. reported that the DNA binding activity of c-Jun is down-regulated by GSH through its reversible S-glutathionylation and protein disulfide formation.[31] Cell growth arrest by the transfection of anti-γ-GCSh ribozyme observed in the present study suggests that a decrease in GSH level negatively attenuates intracellular factors for cell growth. However, the mechanism of regulation of cell growth by GSH remains to be clarified.

In the present study, sensitivity of ionizing radiation was attenuated by the transfection of T98G cells with anti-γ-GCS ribozyme (Fig. 3). This suggests a role of GSH in the protection of cells against damage by ionizing radiation. According to other reports, radiosensitivity is determined by GSH[32] and other anti-oxidants such as superoxide dismutase and GSH S-transferase.[33] The contributions of other anti-oxidants to the sensitivity to ionizing radiation should be further studied.

The findings suggest that the acquisition of resistance to ionizing radiation and CDDP by cancer cells, which leads to difficulty in anti-cancer chemotherapy, can be attenuated to some extent using the anti-γ-GCSh hammerhead ribozyme.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

(Received September 21, 2001/Revised March 15, 2002/Accepted March 22, 2002)

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