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Retrovirus-mediated transduction of a short hairpin RNA gene for *GRP78* fails to downregulate GRP78 expression but leads to cisplatin sensitization in HeLa cells

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Abstract. Glucose-regulated protein 78 (GRP78) is expressed abundantly in various types of cancer cells and is believed to contribute to chemotherapeutic resistance. In this study, we investigated the effect of a continuous approach for the expression of a short hairpin RNA (shRNA) targeted to *GRP78* with retrovirus transduction on the sensitivity to the anticancer drugs VP-16 and cisplatin. The reduction of GRP78 expression failed, and the expression of *GRP94* and *P5* chaperon mRNA increased; this increase was associated with a mild activation of the unfolded protein response in HeLa cells, which were stably transduced with *GRP78* shRNA gene. The transduced cells exhibited similar sensitivity to VP-16-induced cell death when compared to control *GFP* shRNA gene-transduced cells. However, sensitivity to cisplatin-induced cell death was higher in *GRP78* shRNA gene-transduced cells compared to control cells. These results demonstrate that the continuous or prolonged approach targeting GRP78 confers sensitization of HeLa cells to cisplatin independently of the down-regulation of GRP78 expression. The role of the unfolded protein response in sensitization to cisplatin is discussed.

Introduction

Cancer cells in poorly vascularized solid tumors are often subjected to stressful microenvironments, including hypoxia, low pH, and the deprivation of glucose and other nutrients (1). These microenvironments disrupt protein folding in the endoplasmic reticulum (ER) (2,3). The accumulation of unfolded proteins in the ER activates the unfolded protein response (UPR) pathways and enhances the expression of ER chaperone proteins, including glucose-regulated protein (GRP) 78 and GRP94 (4-6). There are a number of studies stating that overexpression of GRP78 and GRP94 frequently occurs in various types of cancer, including prostate, breast, lung, esophageal, gastric, colon, brain and liver cancers (7-14).

GRP78 associates with nascent proteins, facilitates protein folding, prevents intermediates from aggregating, and targets misfolded proteins for proteasome degradation (15). GRP94 is thought to facilitate the assembly or oligomerization of folding intermediates in the ER (16). Both GRP78 and GRP94 also bind Ca^{2+}, help to immobilize Ca^{2+}, and maintain ER calcium homeostasis (4). With these properties, induction of GRP78 and GRP94 is involved in the protection of tumor cells from death under stressful microenvironmental conditions. Furthermore, overexpression of GRP78 and 94 may confer chemotherapeutic resistance to solid tumors (5,17). Knockdown of GRP78 or GRP94 expression has been shown to sensitize cancer cells to chemotherapeutic agents and ionizing radiation (13,18-23). Additionally, tumor cells derived from *GRP78* heterozygous mice grew more slowly and showed enhanced apoptosis compared with tumor cells from *GRP78* wild-type mice (24). Therefore, targeting GRP78/GRP94 expression or activity may be a promising anticancer strategy.

There is a possibility that the reduction of GRP78 expression increases the amount of misfolded or aggregated proteins, activates the UPR signaling pathway and enhances the expression of UPR-induced genes, including ER chaperone proteins, because GRP78 itself serves as a negative modulator of the UPR signaling pathways by binding to three ER stress sensor proteins: activating transcription factor 6, inositol-requiring 1 protein (Ire1p) and protein kinase RNA-activated-like ER kinase (PERK) (25).

We previously observed enhanced expression of *GRP94*, ER-localized DnaJ homologue 4 *(ERdj4)* and *P5* chaperone mRNA, as well as activation of the *GRP78* promoter, when the expression of GRP78 was suppressed by a small interfering RNA (siRNA) in HeLa cells (26). This finding suggests that the approach of prolonging suppression of GRP78 expression

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may fail to sensitize cells to anticancer drugs. On the contrary, it might confer chemotherapeutic resistance. To search for the possibility that such unintended consequences might occur under *GRP78*-targeted anticancer therapy, we used a retrovirus vector containing an shRNA-expressing gene targeted to *GRP78*. Transduction of an shRNA gene enabled us to suppress the expression of the target gene specifically and stably (27,28). Retrovirus-mediated transduction is widely used to introduce genetic material efficiently and stably into the genome of any dividing cell type (29). HeLa cells were infected with the *GRP78* shRNA vector and the stably transduced cells were selected. We then analyzed the expression of the UPR-activated genes, including *GRP78* and *GRP94*, and sensitivity to two representative anticancer drugs, VP-16 and cisplatin, in the stable *GRP78* shRNA gene-transduced cells.

Materials and methods

Plasmid construction and siRNA. The retrovirus vector pSUPER.retro (27), containing the H1-RNA promoter, was obtained from OligoEngine (Seattle, WA, USA). The pSUPER.retro vector was digested with *Bgl*II and *Hin*dIII and the following pairs of annealed oligos were ligated into the vector to create pSUPER.retro-shGRP78, pSUPER.retro-shGRP94 and pSUPER.retro-shGFP, respectively: 5'-gat ccc ccA GTG TTG GAA GAT TCT GAT ttc aag aga ATC AGA ATC TTC CAA CAC Ttt ttt gga aa-3' and 5'-agc ttt tcc aaa aaA GTG TTG GAA GAT TCT GAT TCT GAT Ttc tct tga aAT CAG AAT CTT CCA ACA CTg gg-3'; 5'-gat ccc ccG AAG AAG CAT CTG ATT ACC ttc aag aga GGT AAT CAG ATG CTT CTT Ctt ttt gga aa-3' and 5'-agc ttt tcc aaa aaG AAG AAG CAT CTG ATT ACC tct ctt gaa GGT AAT CAG ATG CTT CTT Cgg g-3'; and 5'-gat ccc ccG CAA GCT GAC CCT GAA GTT Ctt caa gag aGA ACT TCA GGG TCA GCT TGC ttt ttg gaa a-3' and 5'-agc ttt tcc aaa aaG CAA GCT GAC CCT GAA GTT Ctc tct tga aGA ACT TCA GGG TCA GCT TGC ggg-3'. The 19 nt *GRP78* and *GRP94* target sequences and the 20 nt *GFP* target sequences are indicated in capitals in the oligonucleotide sequences. The target sequences for *GRP78* and *GRP94* were reported previously (26). The predicted sequences of the short hairpin transcripts of *GRP78* shRNA, *GRP94* shRNA and *GFP* shRNA are shown in Fig. 1A.

Adenovirus vector constructs were generated by excising shRNA-expressing cassette fragments from the retrovirus vectors with *Eco*RI and *Hin*dIII and blunt-ended with Klenow enzyme. These fragments were ligated into the *Bam*HI- and *Xba*I-digested, and blunt-ended pENTR-U6 entry vector (Invitrogen, Carlsbad, CA, USA), yielding pENTR-shGRP78, pENTR-shGRP94 and pENTR-shGFP. Each shRNA-expressing cassette sequence in the pENTR vectors was transferred to an adenovirus vector, pAd/BLOCK-iT™-DEST, using Gateway® LR Clonase™ Enzyme mix (Invitrogen), yielding pAd/shGRP78, pAd/shGRP94 and pAd/shGFP.

Cell culture, transfection and viral infection. The cervical tumor cell line, HeLa S3, was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The packaging cells for adenovirus production, 293A, were purchased from Invitrogen. Phoenix-Ampho packaging cells for retrovirus production (ATCC catalogue number SD 3443) were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa cells were cultured in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% (v/v) calf serum (Invitrogen). Phoenix and 293A packaging cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical) supplemented with 0.1 mM MEM non-essential amino acids (Invitrogen), 60 μg/ml kanamycin (Sigma, St. Louis, MO, USA) and 10% (v/v) fetal bovine serum (Roche Diagnostics, Mannheim, Germany). All cell lines were grown at 37˚C in a humidified atmosphere containing 5% CO_2 and subcultured when confluence was reached.

Amphotropic retroviral supernatants were produced by transfection of each of the pSUPER.retro shRNA-expressing vectors into Phoenix-Ampho packaging cells using Lipofectamine™ 2000 reagent (Invitrogen). At 24 h post-transfection, the culture medium was refreshed, and the cells were incubated for a further 24 h. The culture medium was then filtered through a 0.45 μm filter, and each viral supernatant (Ret/shGRP78, Ret/shGRP94 and Ret/GFP) was used for infection of cells with polybrene solution (4 mg/ml). HeLa cells were infected for 5 h and allowed to recover for 48 h in fresh medium. The infected cells were cultured in the presence of 0.8 μg/ml puromycin for 8 days to remove uninfected cells. The stably infected cells were then used in experiments.

High titer adenoviral stocks ranging from 2-5x10^8 pfu/ml were produced by transfection of each of the pAd/shRNA-expressing vectors into 293A packaging cells according to the manufacturer's instructions for the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen). HeLa cells were infected with the each of the adenoviral stocks (Ad/shGRP78, Ad/shGRP94 and Ad/shGFP) at an MOI of 50 for 24 h. The infected cells were replaced with fresh medium, cultured for a further 24 h and used for Western blot analysis.

Western blot analysis. Western blot analysis was carried out as described previously (26). Whole cell protein samples were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Detection of GRP78, GRP94, the cleaved form of caspase-3 and ß-actin proteins was performed with mouse anti-GRP78 (Assay Design, Ann Arbor, MI, USA), rat anti-GRP94 (Assay Design), rabbit anti-cleaved caspase-3 (Trevigen, Gaithersburg, MD, USA) and mouse anti-ß-actin (MP Biomedicals Inc., Aurora, OH, USA) monoclonal antibodies, respectively. The antigen-antibody complexes were detected by horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, Buckinghamshire, UK), anti-rabbit IgG (GE Healthcare) or anti-rat IgG (Assay Design) and visualized using the ECL system (GE Healthcare) according to the manufacturer's instructions.

RT-PCR. Isolation of total RNA, first-strand cDNA synthesis and RT-PCR analysis was carried out as described previously (26). The following sets of primers were used: 5'-GTT CTT CAA TGG CAA GGA ACC ATC-3' and 5'-CCA TCC TTT

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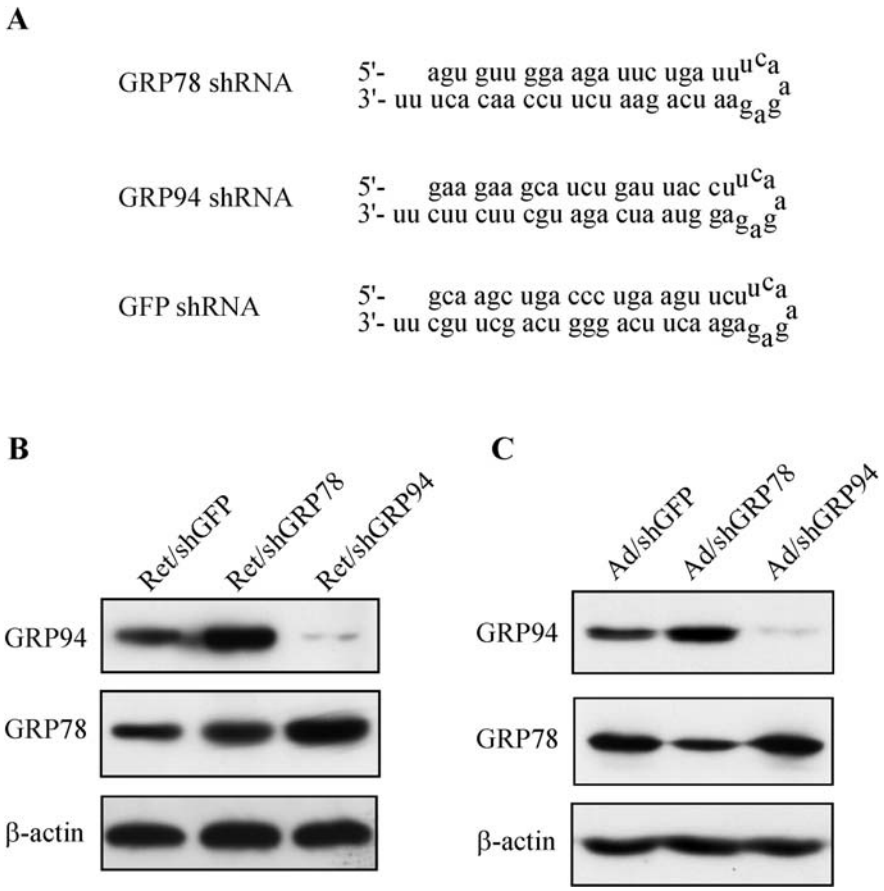

Figure 1. Effect of *GRP78* shRNA gene transduction on GRP78 and GRP94 protein levels in HeLa cells. (A) Sequences of the predicted shRNA specific to *GRP78, GRP94* and *GFP* (control) mRNA. (B) Stable transduction of the *GRP78* shRNA gene by a retrovirus vector failed to suppress the expression of GRP78 protein but increased the expression of the GRP94 protein. Cell lysates prepared from the surviving population of the transduced cells were analyzed by Western blot analysis. The amount of actin was used as a loading control. Ret/shGFP, Ret/shGRP78, and Ret/shGRP94 represent the retrovirus vector-transduced cells, respectively. (C) Transient expression of *GRP78* shRNA with an adenovirus vector suppressed the expression of GRP78 protein and increased the expression of GRP94. Cell lysates prepared 2 days after infection with adenovirus were analyzed by Western blot analysis. Ad/shGFP, Ad/shGRP78, and Ad/ shGRP94 represent the adenovirus vector-infected cells, respectively.

CGA TTT CTT CAG GTG-3' for *GRP78*; 5'-AAG AAC CTG CTG CAT GTC ACA GAC-3' and 5'-ATG GGC TCC TCA ACA GTT TCA GTC-3' for *GRP94*; 5'-TTC CAG ACT GAT CCA ACT GCA GAG-3' and 5'-AGC TGT GCC ACT TTC CTT TCA TTC-3' for *CHOP*; 5'-TCG GAG GGT GCA GGA TAT TAG AAA-3' and 5'-GGA TCC AGT GTT TTG GTT TTG ACC-3' for *ERdj4*; 5'-GAA AGG CGA GTC TCC TGT GGA TTA-3' and 5'-AGA GCT CCC TGA GAA ACT CGT TGA-3' for *P5*; 5'-GAC CTC AAC TAC ATG GTC TAC ATG-3' and 5'-TGT CGC TGT TGA AGT CAG AGG AGA C-3' for glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*; and 5'-CCT TGT AGT TGA GAA CCA GG-3' and 5'-GGG GCT TGG TAT ATA TGT GG-3' for X-box binding protein 1 *(XBP1)*. The PCR products were visualized after electrophoresis on 1.5% (w/v) agarose gels by ethidium bromide staining. To detect the spliced form of *XBP1* cDNA, samples were electrophoresed on 4% polyacrylamide gels to separate the spliced form from the unspliced form.

Determination of drug sensitivity. Cell viability after treatment with VP-16 or cisplatin was determined by a colony-formation assay. Cells were seeded onto 60-mm dishes at a density of 200-300 cells per dish. The next day, VP-16 (0-40 μM) or cisplatin (0-20 μM) were added to the cell cultures. One hour after incubation, drug-containing media were replaced with fresh MEM containing 10% calf serum, and the cells were cultured for 2 weeks to form colonies. Colonies comprising >50 cells were counted after staining with 0.1% (w/v) methylene blue tetrahydrate. The survival fraction of each drug concentration was calculated as the ratio of colony numbers in the drug-treated samples to those in mock-treated samples.

Results

Expression of GRP78 is not suppressed in GRP78 shRNA gene-transduced HeLa cells. We isolated HeLa cells transduced with the *GRP78* shRNA gene by retrovirus infection with Ret/shGRP78. We also isolated *GRP94* and *GFP* shRNA gene-transduced HeLa cells by infection with Ret/shGRP94 and Ret/GFP, respectively, for comparison. After removal of the uninfected and infected but inactivated fraction of cells by puromycin selection, the presence of retroviral DNA within the cellular genome was confirmed by PCR (data not shown). Total cell lysates were prepared from polyclonal pools of the transduced cells, and the expression of GRP78 protein was determined by Western blot analysis

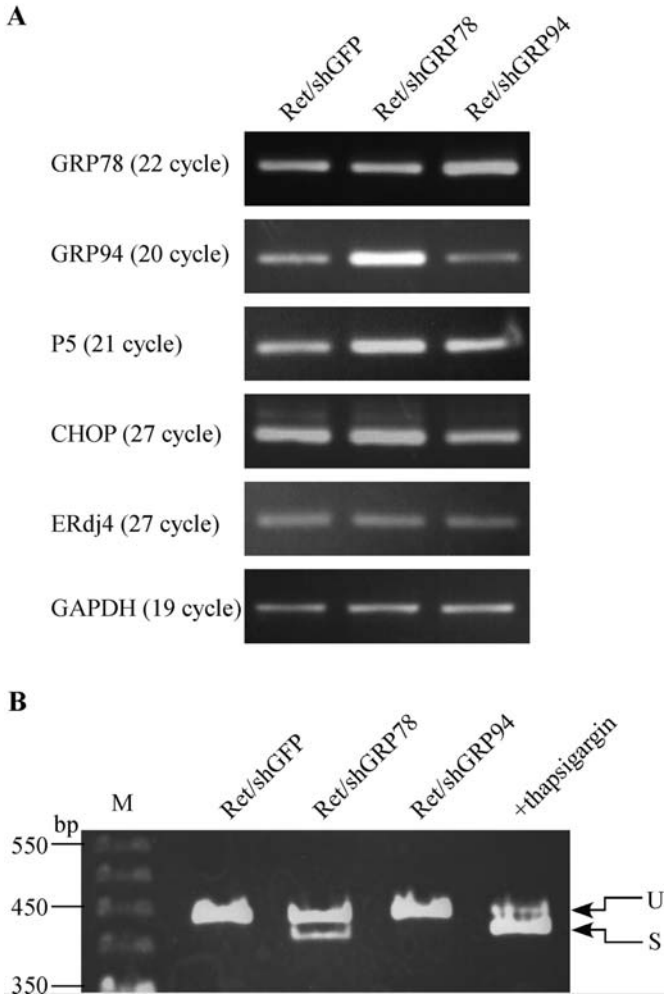

Figure 2. mRNA expression of *GRP78, GRP94* and UPR-induced genes in Ret/shGRP78-, Ret/shGRP94- and control Ret/shGFP-transduced HeLa cells. (A) The mRNA levels of *GRP78, GRP94, P5, CHOP* and *ERdj4* were analyzed by RT-PCR. The PCR cycles indicated in brackets were predetermined to allow semiquantitative comparisons among cDNAs developed from identical reverse transcriptase reactions. The reference gene, GAPDH, was amplified as an internal control. (B) Stable expression of *GRP78* shRNA partially activates the Ire1p-XBP1 pathway. RT-PCR analysis of *XBP1* mRNA was performed as previously described. The PCR products were separated on a 4% polyacrylamide gel and visualized by ethidium bromide staining. The positions of the unspliced (442 bp) and spliced (416 bp) products are shown as U and S, respectively. RNA samples isolated from thapsigargin-treated (1 μM for 8 h) cells were used as a positive control for activation of the Ire1p-XBP1 pathway.

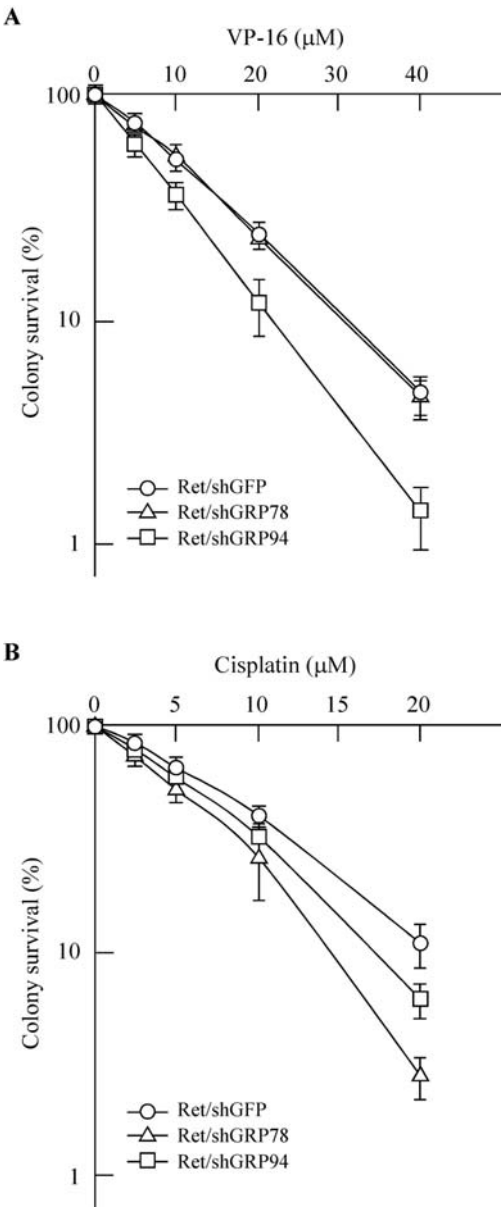

Figure 3. Comparison of sensitivity to VP-16 (A) and cisplatin (B) in Ret/shGRP78-, Ret/shGRP94- and control Ret/shGFP-transduced HeLa cells by a colony survival assay. Cells were exposed to VP-16 or cisplatin for 1 h at the different concentrations indicated. Values represent the percentage of colony numbers relative to those of mock-treated cells. Values are the mean ± SD of three independent experiments.

(Fig. 1B). Contrary to our expectations, the amount of GRP78 protein was not reduced, but rather appeared to have slightly increased in Ret/shGRP78-transduced cells. Additionally, the amount of GRP94 had increased in the Ret/shGRP78-transduced cells. On the other hand, a marked knockdown of GRP94 protein was observed in Ret/shGRP94-transduced cells, although the expression of the GRP78 protein appeared to have increased.

To determine whether the *GRP78* shRNA can inhibit the expression of the target protein, the gene cassette was inserted into an adenovirus vector and used for transient expression of *GRP78* shRNA. Adenovirus vectors containing *GRP94* or the *GFP* shRNA gene were also used as controls. HeLa cells express high levels of coxsackie-adenovirus-receptor and showed highly-efficient adenovirus-vector-mediated transient transgene expression (30,31). Two days after infection with each shRNA-expressing adenovirus vector at a MOI of 50, the expression of the targeted protein had decreased in both Ad/shGRP78- and Ad/shGRP94-infected cells, as expected (Fig. 1C). This result demonstrates that the *GRP78* shRNA designed by us can actually inhibit expression of the target protein when transiently introduced. The expression of the GRP94 protein also increased with Ad/shGRP78 infection, similar to when the cells were transfected with *GRP78* siRNA (26). These results suggest that stable transduction of the *GRP78* shRNA gene with the retrovirus vector is not capable of suppressing GRP78

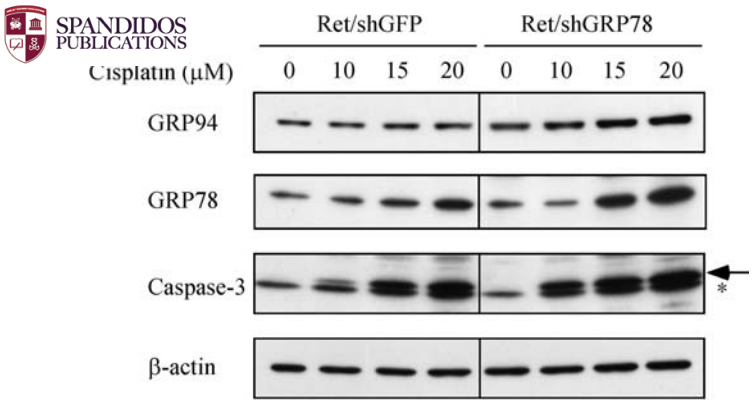

Figure 4. Activation of caspase-3 by cisplatin is enhanced in Ret/shGRP78-transduced HeLa cells. Cells were exposed to cisplatin for 1 h at the different concentrations indicated, cultured for a further 23 h in normal culture media, and then analyzed by immunoblotting using anti-GRP94, anti-GRP78, anti-cleaved form of caspase-3 and anti-actin antibodies. The amount of actin was used as a loading control. Bands representing the cleaved form of caspase-3 are indicated by an arrow. The asterisk indicates non-specific bands.

expression in HeLa cells, even if the gene can suppress the expression of the target protein when it is transiently introduced.

Expression of UPR-induced genes increases in GRP78 shRNA gene-transduced HeLa cells. One possible explanation for the lack of suppression of GRP78 in Ret/shGRP78-transduced cells may be the activation of the UPR, as upregulation of UPR-induced genes and activation of UPR-dependent transcription was observed when HeLa cells were transfected with a *GRP78* siRNA (26). Next, we analyzed the expression of three representative UPR-induced mRNAs, in addition to *GRP78* and *GRP94*, by RT-PCR analysis in Ret/shGRP78-transduced cells (Fig. 2A). The levels of *GRP94* and *P5* mRNA increased more than two-fold, whereas the levels of *CHOP* and *ERdj4* mRNA did not increase significantly. Reduction of *GRP78* mRNA expression was not observed in Ret/shGRP78-transduced cells. The mRNA levels of *P5, CHOP* and *ERdj4* did not increase in Ret/shGRP94-transduced cells.

We previously reported that the spliced form of *XBP1* mRNA, which increased after Ire1p activation and produces the active form of the XBP1 protein necessary for the induction of UPR-induced genes, increased in *GRP78* siRNA-transfected HeLa cells (26). To ascertain that this pathway is also activated in Ret/shGRP78-transduced cells, we analyzed the amount of the spliced form of *XBP1* mRNA by RT-PCR (Fig. 2B). As a positive control for UPR activation, thapsigargin-treated HeLa cells were also used. The amount of spliced mRNA (Fig. 2B) increased in Ret/shGRP78-transduced cells, although the extent of the increase was smaller than in thapsigargin-treated cells.

These results suggest that the UPR is somewhat activated and that UPR-induced genes, including GRP78 itself, are moderately upregulated in Ret/shGRP78-transduced cells. It should be noted that the increased expression of *GRP78* mRNA, as well as GRP78 protein, was observed in Ret/shGRP94-transduced cells (Fig. 1B and 2A). This increase by the suppression of GRP94 may occur independently of UPR activation, as expression of other UPR-induced genes was not upregulated and the spliced form of *XBP1* mRNA did not increase in the Ret/shGRP94-transduced cells.

Sensitization to cisplatin by transduction of the GRP78 shRNA gene without suppression of GRP78 expression. Prolonged *GRP78* shRNA expression could not maintain GRP78 knockdown because the levels of GRP78 protein in Ret/shGRP78-transduced cells was similar to that in control Ret/shGFP-transduced cells. It is possible that continuous treatment for suppression of GRP78 function may activate the UPR and result in an adverse overexpression of GRP78, allowing the treated cancer cells to survive and maintain ER-homeostasis. Such an adaptation to suppression might also contribute to the resistance of cells to chemotherapy and radiotherapy.

To determine the effect of UPR activation on Ret/shGRP78-transduced cells and their sensitivity to anticancer drugs, we treated the cells with various concentrations of VP-16 and cisplatin, which are clinically and widely used as anticancer drugs and whose sensitivities were reported to depend on the expression levels of GRP78 and GRP94 (13,19,22,23). Sensitivity to VP-16 was similar between Ret/shGRP78- and control Ret/shGFP-transduced cells (Fig. 3A). However, surprisingly, sensitivity to cisplatin was higher in Ret/shGRP78-transduced cells than in Ret/shGFP-transduced cells regardless of similar GRP78 protein levels in both cells (Fig. 3B). Colony survival data for Ret/shGRP94-transduced cells confirmed that knockdown of GRP94 increased sensitivity to VP-16 as previously reported (Fig. 3A) (18). Knockdown of GRP94 also sensitized HeLa cells to cisplatin although to a lesser extent than with *GRP78* shRNA gene transduction (Fig. 3B).

To further confirm cisplatin sensitization by *GRP78* shRNA expression without GRP78 knockdown, the amount of the active form of caspase-3 was analyzed by Western blot analysis (Fig. 4). Compared with Ret/shGFP-transduced cells, the levels of active caspase-3 were higher in Ret/shGRP78-transduced cells when treated with various concentrations of cisplatin for 24 h. In addition, induction of both GRP78 and GRP94 by cisplatin was higher in Ret/shGRP78-transduced cells than in control Ret/shGFP cells, implying increased sensitivity to cisplatin in the Ret/shGRP78-transduced cells. These results suggest that *GRP78* shRNA expression sensitized HeLa cells to cisplatin not via suppression of GRP78 but through mild activation of the UPR.

Discussion

A number of studies have reported that the downregulation of GRP78 expression with siRNA or antisense DNA sensitized cancer cells to chemotherapeutic agents, including VP-16 and cisplatin (13,19,22,23). The reports suggested that GRP78 itself contributed to the resistance to those agents and could be a potential target for enhancing chemosensitivity of cancer cells. However, we previously reported that knockdown of GRP78 activated the UPR (26). Since the UPR is involved not only in apoptosis but also cell survival (6), the possibility that GRP78-targeted anticancer therapies might

induce unfavorable events cannot be excluded, especially if the treatment is performed continuously or repeatedly.

In this study, we stably transduced HeLa cells with a *GRP78* shRNA-expressing gene and found that this continuous approach for GRP78 knockdown was not effective, even if the same *GRP78* shRNA gene can actually reduce the expression of GRP78 when transiently transduced with an adenovirus vector system (Fig. 1). Moreover, the expression of *GRP94* and *P5* mRNAs increased and was accompanied by UPR activation in the Ret/shGRP78-transduced cells (Fig. 2). The reason why the expression of GRP78 did not decrease in the transduced cells is unclear. One possible explanation is that only the population of cells that were adapted for cell growth remained after puromycin selection, while the cells with suppressed GRP78 expression died due to strong UPR activation. This outcome is similar to what is observed in cells transfected with *GRP78* siRNA (26). If so, the approach for GRP78 knockdown or inactivation should not be used continuously or repeatedly because it may no longer sensitize the cells to anticancer drugs. Indeed, sensitivity to VP-16-induced cell death in Ret/shGRP78-transduced cells was similar to that in control Ret/shGFP-transduced cells (Fig. 3A).

Nevertheless, sensitivity to cisplatin was still higher in Ret/shGRP78-transduced cells when compared with control Ret/shGFP-transduced cells (Figs. 3B and 4). This could be because suppression of GRP78 expression is not involved in the sensitization to cisplatin by shRNA expression or siRNA treatment targeted to *GRP78*, but activation of the UPR is involved. Disruption of GRP78 metabolism by the expression of *GRP78* shRNA may be involved in the activation of the UPR in Ret/shGRP78-transduced cells, although the apparent protein levels of GRP78 in the transduced cells were similar to those in control Ret/shGFP-transduced cells. This idea is further supported by several studies that reported higher sensitivity to cisplatin-induced cell death following upregulation of GRP78 with 2-deoxyglucose or 6-amino-nicotineamide treatment in human colon cancer cells, human ovarian cancer cells and Chinese hamster V79 cells (32-34). In contrast, induction of ER chaperone proteins, including GRP78, by treatment with 2-deoxyglucose or A23817 conferred resistance of V79 and SK-MES-1 human lung cancer cells to doxorubicin, vincristine and VP-16 (35-37).

In addition, celecoxib, a non-steroidal anti-inflammatory drug that activates the UPR, potentiated the anticancer effect of cisplatin in vulvar cancer cells independently of cyclo-oxygenase (38,39). Considering all of the various published studies, the possibility that the continuous but mild activation of the UPR by the expression of *GRP78* shRNA can sensitize cancer cells to cisplatin is conceivable. Cisplatin has the unique ability to activate apoptosis via both DNA damage-dependent and -independent pathways, and the ER is likely its non-nuclear target (40). Induction of apoptosis by VP-16 requires nuclear events; cisplatin induced the activation of caspase-3 in enucleated cells (cytoplasts), but VP-16 did not (40). Therefore, the ability to activate two pathways for apoptosis may be involved in sensitization to cisplatin when the UPR is activated.

In summary, retroviral-mediated stable transduction of a *GRP78* shRNA gene failed to suppress the expression of GRP78 in HeLa cells. Upregulation of *GRP94* and *P5* mRNAs with a mild activation of UPR was observed in the transduced cells. However, sensitivity to cisplatin-induced cell death was higher in Ret/shGRP78-transduced cells than in control cells, suggesting that the activation of UPR is involved in sensitization. These results may offer a new chemotherapeutic approach using cisplatin.

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