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Stress Chaperone GRP78/BiP Confers Chemoresistance to Tumor-Associated Endothelial Cells

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Abstract

The tumor vasculature is essential for tumor growth and survival, and is a key target for anticancer therapy. Glioblastoma multiforme, the most malignant form of brain tumor, is highly vascular and contains abnormal vessels, unlike blood vessels in normal brain. Previously, we demonstrated that primary cultures of human brain endothelial cells, derived from blood vessels of malignant glioma tissues (TuBEC), are physiologically and functionally different from endothelial cells derived from non-malignant brain tissues (BEC) and are substantially more resistant to apoptosis. Resistance of TuBEC to a wide range of current anticancer drugs has significant clinical consequences as it represents a major obstacle towards eradication of residual brain tumor. We report here that the endoplasmic reticulum chaperone GRP78/BiP is generally highly elevated in the vasculature derived from human glioma specimens, both *in situ* in tissue and *in vitro* in primary cell cultures, as compared to minimal GRP78 expression in normal brain tissues and blood vessels. Interestingly, TuBEC constitutively overexpress GRP78 without concomitant induction of other major UPR targets. Resistance of TuBEC to chemotherapeutic agents such as CPT-11, etoposide and temozolomide (TMZ) can be overcome by knockdown of GRP78 using siRNA or chemical inhibition of its catalytic site. Conversely, overexpression of GRP78 in BEC rendered these cells resistant to drug treatments. Our findings provide the proof-of-principle that targeting GRP78 will sensitize the tumor vasculature to chemotherapeutic drugs, thus enhancing the efficacy of these drugs in combination therapy for glioma treatment.

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Keywords

glioma; vasculature; endothelial cells; GRP78; chemosensitization

Introduction

Tumor growth and survival is dependent on the supply of nutrients and oxygen provided by blood vessels within the cancer; thus eliminating the tumor vasculature is a key target for anticancer therapy (1). Glioblastoma multiforme, the most malignant form of brain tumor, is highly vascular and contains abnormal vessels, unlike blood vessels found in normal brain (2). We previously demonstrated that primary cultures of human brain endothelial cells derived from blood vessels of malignant glioma tissues (TuBEC) are physiologically and functionally different from endothelial cells derived from non-malignant brain tissues (BEC). TuBEC demonstrate consistent resistance to cell death with treatment of a wide range of chemotherapeutic agents (3,4). This chemoresistance of TuBEC has significant clinical consequences representing a major obstacle towards eradicating residual brain tumor, and possibly causing disease recurrence.

The unfolded protein response (UPR) is an evolutionarily conserved mechanism which activates both pro-apoptotic and survival pathway to allow eukaryotic cells to adapt to endoplasmic reticulum (ER) stress (5). A major UPR protective response is the induction of the ER chaperone protein GRP78/BIP, which is required for the proper folding and assembly of membrane and secretory proteins (5,6). GRP78 is upregulated under stress conditions such as glucose deprivation, hypoxia, or the presence of toxic agents (5,7). Overexpression of GRP78 is prominent in a wide variety of tumors, and protects tumor cells against ER stress as well as a range of cancer therapeutic agents (8,9). The potential mechanisms responsible for this protection include: preventing protein misfolding, binding of ER Ca^{2+} , and blocking activation of caspases and pro-apoptotic proteins associated with the ER (9-13). Downregulating GRP78 has been shown to reverse these cytoprotective effects (9). Despite extensive studies on tumor cells, the expression and function of GRP78 in the tumor vasculature, an integral component of the cancer, has not been reported.

In this study, we showed that GRP78 is generally highly elevated in the tumor vasculature, both *in situ* in tissue and *in vitro* in primary cell cultures, in contrast to the minimal expression in normal brain. Knockdown of GRP78 by siRNA significantly sensitized TuBEC to a variety of chemotherapeutic agents, whereas upregulation of GRP78 in BEC renders these cells drug resistant. Recently, it was discovered that the green tea component (-)-epigallocatechin gallate (EGCG) blocks the ATPase domain of GRP78 and suppresses its anti-apoptotic property (14). We showed here that EGCG mimicked siRNA against GRP78 in sensitizing TuBEC to chemotherapeutic agents, providing proof-of-principle that small molecules targeting GRP78 will enhance the efficacy of chemotherapeutic drugs by eliminating the chemoresistant tumor vasculature.

Results

GRP78 Expression is Generally Highly Elevated in Human Tumor-Associated Brain Endothelial Cells

To study the expression and function of GRP78 protein in the glioma vasculature, purified human primary cultures of TuBEC were examined; BEC served as the control. Due to the heterogeneity inherent in human specimens, 10 specimens from BEC and TuBEC each were examined by immunostaining with anti-GRP78 antibody. Representative staining of two different TuBEC patient samples and two different BEC specimens are shown in Fig. 1A. The

intensity of GRP78 staining for all 20 specimens were evaluated; the results are summarized graphically, and exhibit significant differences ($p < 0.001$) in intensity (Fig. 1B). Thus, the staining results demonstrate that despite some variations among the individual cells, TuBEC specimens show strong positive staining for GRP78, as compared to the faintly positive cells observed in BEC samples. To quantitate differences in GRP78 protein levels, Western blots were performed on TuBEC specimens from two patients and two BEC specimens (Fig. 1C). These results demonstrate that GRP78 protein expression in TuBEC is 3- to 4-fold higher compared to BEC.

To determine whether this *in vitro* observation is valid *in situ*, in the vasculature of tumor tissues, frozen sections of gliomas were immunostained for either GRP78 (red) or the endothelial cell marker CD31 (green), with DAPI (blue) staining the nuclei (Fig. 1D). The results showed that GRP78 was intensely positive in glioma specimens; and the vasculature was prominently labeled with CD31. The merged images validated that GRP78 was highly expressed in both the tumor vasculature and glioma cells. By contrast, normal brain tissues exhibited CD31 (green) positive blood vessels, but minimal staining for GRP78 (red) (Fig. 1D). Merged images confirmed negligible GRP78 expression in normal brain and vasculature. These data demonstrate that GRP78 is preferably expressed in the tumor vasculature and tumor cells compared to normal brain.

TuBEC Constitutively Overexpress GRP78 without Concomitant Induction of Other Major UPR Targets

Having observed that GRP78 is elevated in TuBEC, we investigated whether this is the consequence of the constitutive activation of the UPR. Two different TuBEC and BEC samples were analyzed for expression of proteins identifying the activated UPR: ATF4 and CHOP and the XBP-1 (spliced variant) (Fig. 2A). BEC treated with thapsigargin [30 nM] for 24 h served as the positive control for ER stress and UPR activation. Our results showed that ATF4 expression was absent in TuBEC, though slightly expressed in BEC; moreover, CHOP and XBP-1 (spliced variant) (S) were not detected in either TuBEC or BEC. Quantitative analysis of the Western blots showed the differential expression profile of these UPR components in TuBEC and BEC (Fig. 2B), highlighting the unique overexpression of GRP78 in TuBEC in contrast to the lack of induction of other major UPR targets.

GRP78 Regulates Chemoresistance in TuBEC

The constitutive overexpression of GRP78 in TuBEC suggested that this protein may confer chemoresistance to these cells. To test this, the sensitivities of TuBEC and BEC to chemotherapeutic agents were compared. Cells were treated with the topoisomerase II inhibitor, etoposide [1-50 μ M] or DMSO (vehicle) [0.1%] for 72 h, and analyzed for cytotoxicity using the MTT assay. At 50 μ M etoposide, over 60% of BEC died, whereas no significant cell death was observed with TuBEC (Fig. 3A). These results are not a reflection of cell proliferation, since the rate of TuBEC replication is low (3). Similar results were obtained using the Cell Death Elisa Assay (data not shown). Thus, TuBEC were highly chemoresistant compared to BEC.

To determine the effects of reducing GRP78 expression, TuBEC were infected with lentivirus expressing control siRNA (siCtrlA) or siRNA specifically targeted against human GRP78 (siGRP78A). Five days post-infection, cell preparations were analyzed for GRP78 protein expression using immunostaining. As shown in Fig. 3B, GRP78 protein was reduced by siGRP78A; no significant change in GRP78 expression was observed with siCtrlA as compared to uninfected TuBEC (Fig. 1A). GRP78 protein levels remained low for at least 3 passages (21 days) (data not shown). If GRP78 confers drug resistance to TuBEC, then knockdown of this protein should overcome resistance. To test this, TuBEC cultures were infected with lentivirus

expressing siGRP78A or siCtrlA. Five days post infection, cultures were left untreated (media), or treated with CPT-11 [100 μ M], etoposide (Eto) [50 μ M] or temozolomide (TMZ) [300 μ M], for another 7 days, then analyzed for cytotoxicity. We observed that while untreated TuBEC were relatively resistant to these drugs (<10% cell death), TuBEC infected with siGRP78A exhibited a significant increase ($p<0.01$) in cytotoxicity with each drug tested (Fig. 3C); significance was determined by comparing TuBEC infected with siGRP78 to TuBEC infected with siControl. It was noted that endothelial cells infected with siControl and treated with CPT-11 and etoposide exhibited significantly more cell death than media alone ($p<0.05$); this increase is likely due to the effects of lentiviral infection in these sensitive cells and therefore of little biological significance. Interestingly, infection with siGRP78A alone, without drug treatment, did not increase cell death as compared to infection with control siRNA, suggesting that decreased GRP78 does not induce spontaneous cytotoxicity within this treatment period.

GRP78 siRNA Induces Caspase-Dependent Cell Death in TuBEC

To eliminate potential “off target” effects of siRNA to GRP78, cytotoxicity measurements were confirmed in TuBEC infected with a second siRNA targeted against human GRP78 (siGRP78B); another control siRNA (siCtrlB) was also used (Fig. 4). The ability of siGRP78B to suppress GRP78 expression in TuBEC was confirmed by immunostaining (Fig. 4A); siCtrlB did not reduce staining, similar to what was observed previously with siCtrlA. To determine whether the observed cell death induced by reduced GRP78 was caspase-dependent, cells were treated with CPT-11, Eto or TMZ in the absence or presence of the caspase inhibitor Q-VDOPH [10 μ M] (C.I.) for 7 days. Caspase inhibition blocked cell death with all three drugs (Fig. 4B). Significance to this and all subsequent experiments was determined by comparing TuBEC infected with siGRP78 to TuBEC infected with siControl. To confirm that GRP78 knockdown induced apoptotic cell death, the TUNEL assay was performed with uninfected TuBEC or TuBEC infected with lentivirus siGRP78B or siCtrlA, and treated with Eto [50 μ M] (Fig. 4C). These data demonstrated that TuBEC, with reduced GRP78, became sensitive to apoptotic cell death upon drug treatment.

Inhibition of GRP78 Activity by EGCG Enhances Chemosensitivity in TuBEC

To target GRP78 using a small molecule approach, TuBEC were incubated with EGCG alone or in combination with TMZ, CPT-11, and Eto; cytotoxicity was measured after 7 days (Fig. 4D). Treatment with TMZ, CPT-11, Eto or EGCG alone did not cause TuBEC cell death; however, combining EGCG and TMZ (35%, $p=0.003$), EGCG and CPT-11 (46%, $p=0.005$) or EGCG and Eto (49%, $p=0.001$) caused significant cell death. Significance was calculated as EGCG alone compared to EGCG with drug. These data imply that small molecule inhibitors such as EGCG, capable of inhibiting GRP78 activity, can be used in combination therapy to increase TuBEC chemosensitivity.

Overexpression of GRP78 Causes Drug Resistance in Normal Endothelial Cells

To determine whether GRP78 overexpression is a key contributing factor to drug resistance in endothelial cells, BEC, which normally express low levels of GRP78 and are sensitive to chemotherapeutic agents, were left uninfected, or infected with lentivirus expressing either green fluorescence protein (GFP) or GRP78. After 5 days, BEC infected with the latter exhibited an overexpression of GRP78 (Fig. 5A). BEC were then treated with Eto [50 μ M] (Fig. 5B) or CPT-11 [100 μ M] (Fig. 5C), for 5 or 7 days, and tested for cytotoxicity. After 7 days, GRP78 overexpression in BEC provided significant protection against both Eto ($p=0.008$) and CPT-11 ($p=0.046$). Cell death data for BEC infected with GRP78 were compared with BEC infected with GFP. To determine whether EGCG can reverse the resistance acquired by BEC infected with GRP78, these cells were treated with EGCG [40 μ M] and

CPT-11 [100 μ M] for 7 days. As shown in Fig. 5D, in cells overexpressing GRP78, treatment with EGCG significantly ($p < 0.007$) increased sensitivity of these cells to the drug. It is noted that BEC are intrinsically resistant to TMZ; therefore protection from cell death by GRP78 could not be assessed with this agent (data not shown).

Discussion

We previously demonstrated that TuBEC are more chemoresistant to drugs compared to BEC (3), although the mechanism of chemoresistance is unknown. Uncovering novel mechanisms for this chemoresistant nature is critical for eradicating residual tumor, which remains a major challenge in cancer therapy. GRP78 has been reported to suppress stress-induced apoptosis through multiple mechanisms (9-13). Further, there is considerable evidence that GRP78 confers drug resistance in a variety of human tumors, including gliomas (8,9,13). The data presented here demonstrate that the glioma vasculature constitutively overexpresses GRP78, and this overexpression confers chemoresistance to these tumor-associated endothelial cells.

Induction of GRP78 in cells is often indicative of ER stress and the activation of the UPR (5). To determine whether this is the case in TuBEC, these cells were analyzed for specific proteins identifying UPR activation, such as the induction of CHOP and ATF4, which are downstream targets of the PERK pathway, and the formation of the XBP-1 spliced variant, which results from activation of the IRE-1 pathway (15). Our studies revealed that these other major UPR targets are not induced in TuBEC. Thus, the constitutive overexpression of GRP78 in TuBEC raises the interesting possibility that novel mechanisms independent of the UPR selective for GRP78 induction, occur in TuBEC. This could involve genetic as well as epigenetic changes as part of the adaptive mechanisms of TuBEC to survive in the tumor microenvironment. On the other hand, GRP78 overexpression is known to suppress UPR signaling pathways (16,17). Thus, it is also possible that TuBEC are subject to ER stress, however, the elevated levels of cytoprotective GRP78 in these cells suppresses the induction of the other branches of the UPR. Future studies will address these interesting issues.

What is the consequence of GRP78 overexpression of TuBEC in clinical settings? We demonstrate in this study that reducing GRP78 caused TuBEC sensitization to TMZ, CPT-11 and etoposide, drugs used in treating gliomas and other tumors (18). These agents have different mechanisms of action but are predominantly directed against proliferating cells. Etoposide is a topoisomerase II inhibitor, which also disrupts mitochondrial activity (19). Previously, it has been demonstrated that etoposide induces the activation of caspase 7 in Chinese hamster ovary cells and overexpression of GRP78, which co-localizes with caspase 7 in the perinuclear region, suppresses this activation (10). In TuBEC cells, we also observed that GRP78 co-localized with caspase 7 and this could explain, in part, the protective effect of GRP78 against etoposide in these cells (data not shown). TMZ acts as a DNA methylating agent (20). CPT-11, irinotecan, inhibits the religation step of topoisomerase I-mediated DNA cleavages (21). TuBEC cultures have a low proliferation rate, which is not increased with lentiviral infection (data not shown). Our studies show that drugs, which normally act on replicating cells, will also cause cytotoxicity in these low-proliferating TuBEC when the cytoprotective effects of GRP78 are reduced. These data suggest that GRP78 is an effective target for sensitizing both proliferating and non-proliferating cells to drugs.

The direct role of GRP78 in tumor formation, progression and angiogenesis has been demonstrated in xenograft models in syngeneic mice and in transgene-induced tumor developed in the heterozygous *Grp78* mice, which express reduced levels of GRP78 (22,23). These *in vivo* data showed that reducing GRP78 protein expression resulted in inhibition of tumor cell proliferation, and an increase in tumor cell apoptosis, as well as diminished microvessel density. This implies that drugs that target GRP78 expression and/or activity could

complement conventional cancer therapy to eliminate residual tumor. Recently, several compounds have been discovered to be GRP78 antagonists (8); they have anticancer activity and work in synergy with chemotherapeutic drugs to reduce tumor growth (14,24). As proof-of-principle, we demonstrated that EGCG, which binds to the ATP binding domain of GRP78 and thereby blocks its function, was effective in chemosensitizing TuBEC. Of concern was that combination treatment, especially with TMZ, drug of choice for glioma treatment, may have deleterious effects on the normal vasculature; however, confluent cultures of normal endothelial cells proved to be relatively insensitive to the affects of TMZ and EGCG together (data not shown). In parallel studies, we have also observed that siRNA against GRP78 as well as EGCG, enhanced the sensitivity of glioma cells to chemotherapeutic agents used in this study (13). Collectively, these results show that decreasing GRP78 protein expression or blocking its activity would significantly enhance both TuBEC and glioma susceptibility to currently available chemotherapeutic agents, thereby eliminating both the tumor and its supplying vasculature.

GRP78 is predominantly expressed as an intracellular protein residing in the ER lumen; however, several experimental approaches indicate that GRP78 is also expressed on the tumor cell surface, and that normal endothelial cells when stimulated with growth factors, enhance cell surface GRP78 expression (8,25-27). Interestingly, Kringle 5, a ligand binding to surface GRP78 can block endothelial cell migration and mediate cell death (26,28). These studies predict that in the abnormally stressful tumor environment, tumor-associated endothelial cells are likely to express GRP78 on the cell surface, and this protein may regulate cell death (26); our studies are consistent with this notion although localization of GRP78 in tumor endothelial cells remains to be established. Identifying mechanisms of drug resistance in TuBEC is essential for appropriate, long-term therapy with the goal of targeting the tumor vasculature as well as tumor cells. Our discovery of the relationship between GRP78 and TuBEC chemoresistance will open new directions in anti-vascular therapies for gliomas and other cancers.

Materials and Methods

Cell Culture

Endothelial cells (EC) were isolated from normal human brain tissue or human glioma tissue as previously described (3,4). These specimens were not obtained from the same patient. Tissues were obtained in accordance with University of Southern California Institutional Review Board guidelines. EC were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 100 ng/ml endothelial cell growth supplement (Upstate Biotechnologies, Rochester, NY), 2 mM L-glutamine (GIBCO), 10 mM HEPES (GIBCO), 24 mM sodium bicarbonate (GIBCO), 300 U heparin USP (Sigma-Aldrich, St. Louis, MO), 1% penicillin/streptomycin (GIBCO) and 10% fetal calf serum (FCS) (Omega Scientific, Tarzana, CA). Purity of endothelial cells was analyzed by immunostaining for the following specific endothelial cell markers: CD31/PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA), von Willebrand Factor (DAKO, Carpinteria, CA), and CD105/endoglin (Santa Cruz Biotechnology); cells were greater than 98% positive for these markers. Cells were also analyzed for the astrocyte/glioma marker, glial fibrillary acidic protein (GFAP) (DAKO), and the macrophage/microglia marker, CD11b (Immunotech, Villepinte, France) to rule out other cell types; endothelial cell cultures used were negative for both these proteins. All experiments were performed on subconfluent (60-80%) BEC and TuBEC cultures, and on cells between passages 4 to 6 only.

Immunostaining

Cytoentrifuge cell preparations and cryostat tissue sections were stained as previously described (3,4). Briefly, cell preparations were fixed in acetone; tissue sections were fixed in 4% paraformaldehyde. Specimens were then stained with rabbit polyclonal anti-GRP78 antibody (1:100) (H129, Santa Cruz Biotechnology) for 16 h, followed by the secondary biotinylated goat anti-rabbit antibody (1:400) (Vector Laboratories, Burlingame, CA). Subsequently the samples were treated with the ABC (avidin biotin peroxidase complex) (Vector Laboratories), followed by aminoethyl carbazol (AEC) substrate kit (Vector Laboratories), which provided the red precipitate at the site of the antigen. Samples were counterstained with hematoxylin, the blue nuclear stain. The red precipitate identifies positive staining. Staining controls included using isotype-matched serum in place of the primary antibody and staining known negative cell preparations. For double immunofluorescence, tissues were incubated with both rabbit anti-GRP78 and mouse monoclonal anti-human CD31/PECAM-1 antibodies (1:100) (R&D Systems, Minnesota, MN) for 16 h, and subsequently stained with Texas-red labeled anti-rabbit antibody and fluorescein-green labeled anti-mouse antibody (Vector Laboratories). Nuclei were identified using DAPI (blue)-containing mounting medium (Vector Laboratories). Staining controls included isotype-matched serum.

Western Blot Analysis

EC cells were lysed in buffer containing 20 mM Tris Base, 300 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, and protease inhibitor cocktail at 1:100 dilution (Sigma-Aldrich). About 50 to 80 µg of protein lysate were subjected to 10% SDS polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes. The primary antibodies used for Western blots were: rabbit anti-GRP78 (H129) (1:500); monoclonal anti-β-actin (1:5000); rabbit anti-GAPDH (FL-335) (1:5000); monoclonal anti-CHOP (1:500), rabbit anti-ATF4 (1:1000) and rabbit XBP-1 (M-186) (1:1000), all purchased from Santa Cruz Biotechnology. Protein bands were detected either by chemiluminescence using the SuperSignal™ substrate (Pierce, Rockford, IL) and analyzed using a Phosphorimager (Hope Micro-max, Freedom Imaging, Anaheim, CA), or quantitated by densitometry (Quantitation one-4.2.1, BIO-RAD, Hercules, CA).

MTT Cell Viability Assay

Cells were plated in triplicate (3×10^3 cells/well; 100 µl/well) into 96-well plates coated with 1% gelatin, and treated with: etoposide (Calbiochem, La Jolla, CA), temozolomide (TMZ) (Schering-Plough, Kenilworth, NJ), or CPT-11 (Pharmacia, New York, NY). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the manufacturer's instructions (Sigma-Aldrich). Percent cell viability was calculated relative to vehicle-treated controls.

Cell Death ELISA

EC were treated with drugs or the caspase inhibitor Q-VDOPH (Calbiochem), or EGCG (Sigma-Aldrich), then assayed using the Cell Death Detection ELISA Plus kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions. Percent death was calculated based on 100% positive cell death control.

Apoptosis Assay

The ApopTag® *In Situ* Apoptosis Detection Kit (Chemicon International, Inc., Temecula, CA) was performed according to manufacturer's protocol.

Lentiviral Construct

The sequences of the siRNA against human GRP78 are: siGRP78A: 5'-GGAGCGCAUUGAUACUAGAUU-3'; siGRP78B: 5'-AAGAAAAGCUGGGAGGUAAC-3'. The sequences of control siRNA are: siControl A: 5'-GGAGAAGAAUAGCAACGGUAA-3'; siControl B: 5'-AAGGUGGUUGUUUUGUUCUU-3'. Their subcloning into lentiviral constructs has been previously described (29). For construction of lentivirus expressing GRP78, full length human GRP78 was prepared by reverse transcription of total HEK293T RNA followed by a 2-step PCR amplification and subcloning into the BamHI/XhoI sites of pcDNA3 (Invitrogen) to yield pcDNA3-hGRP78. Non-replicating lentiviral vectors co-expressing bicistronic human GRP78 and EGFP linked via the EMCV IRES were produced using the pLenti6/V5-D-TOPO and ViraPower Lentivirus Expression system supplied by Invitrogen (Carlsbad, CA). Initially, EGFP (Clontech, Mountain View, CA) was inserted into the CMV driven expression cassette by TOPO cloning. After the viability of this construct was established, the parent construct was modified. The human GRP78 gene digested from pcDNA3/h78 using Xba I and Xho I was ligated into pLenti6/EGFP between the CMV promoter and the EGFP gene. Next, an insert encoding the EMCV IRES, was generated by PCR from pIRES-EGFP (Clontech, Mountain View, CA) and subcloned into the Xho I/Age I sites of pLenti6/huGRP78 EGFP. The IRES sequence was inserted between the human GRP78 gene and the EGFP allowing hGRP78 expression to be monitored by EGFP fluorescence. The manufacturer's manual was followed for TOPO cloning and production of viral particles. For infection, 10^4 cells were plated in 6-well dishes and infected with lentivirus at titers of 5×10^6 units/ml (TU/ml). Infected cells were monitored for green fluorescent protein under the fluorescent microscope. Cells were used when cultures were 100% GFP positive.

Statistical Analysis

Values are presented as mean and SEM. Statistical significance was evaluated using the Student's two-tailed t-test, with $P < 0.05$ considered significant.

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FIGURE 1.

Overexpression of GRP78 protein in tumor-associated brain endothelial cells (TuBEC) and tumor vasculature. **A.** Cytocentrifuge cell preparations of primary cultures from two different specimens of TuBEC (upper panel) and two different specimens of control brain endothelial cells (BEC) (lower panel) were immunostained with anti-GRP78 antibody. The red precipitate designates positive staining; hematoxylin was used as the nuclear stain. **B.** Intensity of immunostaining from 20 specimens was quantitated: 0 = no staining; 4 = intense staining; the star (*) represents significance between groups $p < 0.001$. **C.** Two representative samples of TuBEC and BEC were subjected to Western blot analysis. The relative GRP78 protein expression levels, after normalization to β -actin level, were plotted below. **D.** Cryostat sections of glioma tissues (upper panel) or normal brain tissues (lower panels) were stained with anti-GRP78 antibody (red), anti-CD31 antibody (green) and DAPI (blue) nuclear staining; the images were merged in the last panel. The arrows designate blood vessels. Bar = 100 microns.

FIGURE 2.

TuBEC constitutively overexpress GRP78 without concomitant induction of other major UPR targets. **A.** Western blot analysis of two different specimens of TuBEC and BEC for proteins induced by the UPR: ATF4, CHOP and spliced-variant (S) of XBP-1; GRP78 expression was also analyzed. BEC stimulated with 30 nM of thapsigargin for 24 h served as the positive control. **B.** The relative levels of the proteins, normalization to respective GAPDH levels which served as loading control, were plotted.

FIGURE 3.

Chemoresistance is reversed in TuBEC with reduced GRP78 protein. **A.** TuBEC and BEC were exposed to 1, 10, 50 μ M etoposide, and vehicle control (DMSO) for 72 h, then examined for cell viability using the MTT assay. Vehicle treatment served as 100% viable control. Significance ($p < 0.05$) is calculated by comparing drug treated (50 μ M) cells and vehicle control treated cells. **B.** TuBEC were infected with control siRNA (siCtrlA) or siRNA specifically targeted against human GRP78 (siGRP78A); 5 days post-infection, cells were stained with anti-GRP78 antibody. Red precipitate identified positive staining; hematoxylin stained nuclei blue; 200X magnification. **C.** TuBEC were infected with lentivirus siCtrlA or lentivirus siGRP78A constructs, or left uninfected. Five days post-infection, cells were treated with control media, CPT-11 [100 μ M], Eto [50 μ M] or TMZ [300 μ M] for another 7 days. At the conclusion of the experiments, cells were analyzed using the Cell Death Elisa Assay. The star (*) represents $p < 0.05$; comparisons were made between TuBEC/siGRP78 and TuBEC/siControl groups for each drug treatment.

FIGURE 4.

TuBEC with reduced GRP78 are susceptible to caspase-dependent apoptotic cell death when treated with chemotherapeutic agents. **A.** A second lentivirus containing siRNA targeted against human GRP78 (siGRP78B) also reduced GRP78 expression in TuBEC. Cells were infected with siCtrlB or siGRP78B, and analyzed for GRP78 staining after infection; TuBEC infected with control siRNA (siCtrlB) did not affect GRP78 expression. Red color denotes positive staining. 400X magnification. **B.** Uninfected TuBEC or cultures infected with siCtrlB or siGRP78B were treated with media or drugs (CPT-11 [100 μ M], Eto [50 μ M] or TMZ [300 μ M]) alone, or incubated with the caspase inhibitor (Q-VDOPH) (C.I.) [10 μ M] for 7 days. Cultures were then analyzed using the Cell Death Elisa Assay. The star (*) represents $p < 0.05$; comparisons were made between TuBEC/siGRP78 and TuBEC/siControl groups for each drug treatment. **C.** TuBEC were uninfected or infected with lentivirus expressing siCtrlA or lentivirus expressing siGRP78B. After 5 days, cells were treated with etoposide (Eto) [50 μ M] for 7 days and stained using the TUNEL assay. Apoptotic death was calculated as percent positive in drug treated compared to cells incubated in media alone. Statistical comparisons were made between TuBEC/siGRP78 and TuBEC/siControl groups; the star (*) represents $p < 0.05$. **D.** TuBEC were treated with EGCG (20 μ M) alone or in combination with TMZ (300 μ M), CPT-11 (100 μ M), or etoposide (50 μ M), then incubated with drugs for 7 days. Media

containing DMSO, served as the vehicle control. Statistical comparisons were made between cells treated with EGCG + drugs and EGCG alone; the star (*) represents $p < 0.05$.

FIGURE 5.

Overexpression of GRP78 in BEC promotes chemoresistance. **A.** BEC were uninfected (UI), infected with lentivirus expressing either the control green fluorescence protein (GFP) or GRP78. Five days post-infection cells were harvested for Western blot analysis, and probed for GRP78 and GAPDH. **B.** BEC, uninfected or infected with lentivirus GFP or GRP78, were treated with Eto [50 μ M] for 0, 5 or 7 days. At the termination of the experiments, cell death was analyzed using the Cell Death Elisa Assay. Percent death was calculated based on total cell death control. Statistical comparisons were made between BEC/GRP78 and BEC/GFP on day 7; the star (*) represents $p < 0.05$. **C.** BEC, uninfected or infected with lentivirus GFP or GRP78, were treated with CPT-11 [100 μ M] for 0, 5 or 7 days. At the termination of the

experiments, cell death was analyzed using the Cell Death Elisa Assay. Statistical comparisons were made between BEC/GRP78 and BEC/GFP on day 7; the star (*) represents $p < 0.05$. **D.** BEC, uninfected or infected with lentivirus GFP or GRP78, were either untreated, treated with EGCG [40 μM] alone, CPT-11 [100 μM] alone, or EGCG [40 μM] plus CPT-11 [100 μM] for 7 days. At the termination of the experiments, cell death was analyzed using the Cell Death Elisa Assay. Statistical comparisons were made between BEC/GRP78 treated with CPT-11 and BEC/GRP78 treated with CPT-11 and EGCG; the star (*) represents $p < 0.05$.