Research Article

Vascular Targeting and Antiangiogenesis Agents Induce Drug Resistance Effector GRP78 within the Tumor Microenvironment

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Abstract

Therapeutic targeting of the tumor vasculature that destroys preexisting blood vessels of the tumor and antiangiogenesis therapy capitalize on the requirement of tumor cells on an intact vascular supply for oxygen and nutrients for growth, expansion and metastasis to the distal organs. Whereas these classes of agents show promise in delaying tumor progression, they also create glucose and oxygen deprivation conditions within the tumor that could trigger unintended prosurvival responses. The glucose-regulated protein GRP78, a major endoplasmic reticulum chaperone, is inducible by severe glucose depletion, anoxia, and acidosis. Here we report that in a xenograft model of human breast cancer, treatment with the vascular targeting agent, combretastatin A4P, or the antiangiogenic agent, contortrostatin, promotes transcriptional activation of the Grp78 promoter and elevation of GRP78 protein in surviving tumor cells. We further show that GRP78 is overexpressed in a panel of human breast cancer cells that has developed resistance to a variety of drug treatment regimens. Suppression of GRP78 through the use of lentiviral vector expressing small interfering RNA sensitizes human breast cancer cells to etoposide-mediated cell death. Our studies imply that antivascular and antiangiogenesis therapy that results in severe glucose and oxygen deprivation will induce GRP78 expression that could lead to drug resistance. (Cancer Res 2005; 65(13): 5785-91)

Introduction

The tumor vasculature provides a new and attractive target for cancer therapy because of the reliance of most tumor cells on an intact vascular supply for their growth and survival (1). This concept is also based on the premise that normal endothelial cells may be more amendable to therapeutic intervention than tumor cells, many of which harbor adaptive mutations rendering them insensitive to anticancer drugs (2). Therapeutic targeting of the tumor vasculature includes antivascular approaches that occlude or destroy preexisting blood vessels in solid tumor and antiangiogenic approaches that target the formation of new blood vessel development. Antivascular agents cause shutdown in the blood supply to the tumor that kills tumor cells by depriving them of oxygen and nutrients. Among the different types of vascular targeting agents, the small-molecule combretastatin A4P (CA4P) represents the lead compound in a group of novel tubulin depolymerizing agents that cause rapid cell shape changes in proliferating immature endothelial cells, whereas quiescent mature endothelial cells are resistant to these changes (3). Because the proportion of proliferating endothelial cells is much higher in tumor than normal blood vessels, CA4P has a selective effect on tumor vasculature, destroying in particular the central, poorly perfused hypoxic regions of tumors. This selectivity also depends on a short *in vivo* exposure of the drug and on its rapidly reversible effects on the cytoskeleton.

Angiogenesis is not only required for tumor growth and expansion but is also critical for the tumor to metastasize to the distal organs through new angiogenic vasculature (4). However, because the tumor cell and not the endothelial cell is the final target of antiangiogenic therapy, an agent that can target both cell types could offer an advantage. Contortrostatin, a novel dimeric disintegrin isolated from the venom of the southern copperhead snake (*Agkistrodon contortrix contortrix*), represents a novel form of an antiangiogenic agent. Contortrostatin possesses unique anti-angiogenic and antitumor properties (5). Contortrostatin is not a cytotoxic protein but rather possesses cytostatic activity in its actions against tumor and endothelial cells. Contortrostatin interacts with high affinity on several integrins displayed on both cancer cells and newly growing vascular endothelial cells. As such, contortrostatin blocks cancer cell adhesion and migration; in addition, it inhibits tumor-induced angiogenesis by blocking critical angiogenic pathways in endothelial cells mediated by integrins $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ (6).

Whereas both vascular targeting agents and angiogenesis inhibitors show promise in delaying tumor progression, these classes of agents that act through depriving tumors of oxygen and nutrients can have unintended consequences that affect the long-term clinical outcome. Thus far, a large number of studies have focused on the effect of tumor hypoxia on tumor progression and metastasis (7–9). However, because most cancers are already hypoxic, the shift from normoxia to hypoxia that activates cancer into aggressive behavior *in vitro* may not be a clinically relevant problem. It has been proposed that effective therapy should aim at shifting the tumors into anoxia, a more severe form of oxygen deprivation (10). Severe oxygen depletion as a result of highly efficient antivascular or angiogenesis therapy also leads to depletion of glucose and other nutrients. Whereas in principle this will starve the cancer cells to death, these same adverse conditions are potent inducers of a specific class of stress proteins called the glucose-regulated proteins (GRP; refs. 11–13). This glucose-regulated response is part of an evolutionarily conserved adaptive mechanism for cell survival when cells are subjected to

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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environmental or physiologic stress conditions that target the endoplasmic reticulum (ER; refs. 14, 15).

GRP78, the most abundant and well-characterized GRP, is a major stress-inducible chaperone localized to the ER (16). Through its calcium-binding properties and ability to block caspase activation, GRP78 protects cells against ER stress and topoisomerase inhibitors (17–19). The transcriptional activation of Grp78 is mediated by the endoplasmic reticulum stress response element (ERSE), which is regulated by a multitude of transcription factors (20). Interestingly, in contrast to other hypoxia-inducible genes, transcriptional activation of Grp78 only occurs under severe oxygen deprivation conditions and is independent of HIF, the hypoxia-inducible factor (20–22). Here, using human breast cancer as a model system, we show that both CA4P and contortrostatin promote transcriptional induction of Grp78, leading to accumulation of high levels of GRP78 protein in viable tumor tissues surviving the drug treatment. Our studies further reveal that this induction occurs only in the tumor microenvironment, but not in tissue culture, suggesting that the destruction of the tumor vasculature by CA4P and contortrostatin could be a shared mechanism for Grp78 induction. We further show that GRP78 is overexpressed in a panel of human breast cancer cells that has developed drug resistance. Through the use of lentiviral vector expressing small interfering (siRNA) specifically targeting the Grp78 transcript, we provide evidence that GRP78 confers resistance to etoposide (VP-16) in human breast cancer cells. Our results imply that vascular targeting agents as well as antiangiogenesis therapy could lead to GRP78 induction in residual tumor cells, thus resulting in drug resistance.

Materials and Methods

Cell lines and culture conditions. The human embryonic kidney cell line 293T and the human breast cancer cell line MDA-MB-435 (gift of Dr. Janet Price, M.D. Anderson Cancer Center) have been described (23, 24). The cells were cultured at 37°C with 5% CO_2 in high-glucose DMEM containing 4.5 mg/mL glucose supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 1% penicillin-streptomycin-neomycin antibiotics. The establishment of the stable MDA-MD-435 cell line transduced with the retroviral vector G1NaGrpTK with the Grp78 promoter driving the expression of HSVTK has been described (23, 25). The transduced cells were selected and maintained in DMEM containing 1.5 mg/mL G418. The panel of parental and drug resistant human breast adenocarcinoma MCF-7 cell lines with characterized drug resistance properties has been described (26).

Combretastatin A-4 treatment conditions. CA4P (OXiGENE, Inc., Waltham, MA) was dissolved in 0.9% saline with a few drops of Na_2CO_3 to obtain a 20 mg/mL stock solution (27). For tissue culture treatment, various doses of the drug were added to the cells for 24 hours before harvest for Western blot analysis. For xenograft treatment, 4×10^6 MDA-MB-435 cells were resuspended in 0.1 mL PBS and injected s.c. in the mammary fat pad area of an 8-week-old, female BALB/c *nu/nu* mouse (Simonsen Laboratories, Inc., Gilroy, CA), with $n = 4$ for control and experimental groups. Tumors were measured with a caliper daily. The volume of the tumor was calculated from the formula: volume (cm^3) = $(width)^2 \times (length) \times 0.5$ (23). After tumor cell inoculation, when objectively measurable xenografts were confirmed, mice were randomly assigned to treatment groups. At the indicated time points, when volume of the tumor reached about 0.5 cm^3, the mice in the experimental group were treated with peritoneal injections of CA4P at a dosage of 200 mg/kg of body weight, once per day for four consecutive days. The mice in the control group were injected with 0.9% saline.

Contortrostatin treatment conditions. Venom of *A. contortrix contortrix* was purchased from Miami Serpentarium (Punta Gorda, FL). Contortrostatin was purified according to an established protocol. It was dissolved in PBS and added to the tissue culture cells at dosages indicated for 24 hours. For xenograft treatment, purified contortrostatin was formulated into liposomes as described previously (6). Orthotopic, xenograft breast cancer models were established by implantation of 5×10^5 MDA-MB-435 cells in the mammary fat pad of nude mice as previously described (6). Contortrostatin is given as a liposomal formulation via i.v. administration twice weekly (105 μg/dose) over a period of 8 weeks. At the conclusion of the tumor growth cycle, sections were taken from the MDA-MB-435 tumors and analyzed for antiangiogenic activity of contortrostatin by surgical removal of the tumor followed by fixing and immunostaining as described previously (6).

Immunohistochemical staining. Immunostaining was done on paraffin-embedded MDA-MB-435 tumor sections using the Vectastain elite avidin-biotin complex kit (Vector Laboratories, Burlingame, CA; ref. 23). The primary antibodies were rabbit polyclonal anti-GRP78 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; at 1:100 dilution), rabbit polyclonal anti-HSVTK antibody (obtained from Dr. W. Summers, Yale University School of Medicine; at 1:200 dilution), and rabbit polyclonal anti-CD31 antibody (Sigma-Aldrich, St. Louis, MO; at 1:100 dilution). The sections were lightly counterstained with H&E.

Transfection with small interfering RNA oligomers and transduction with lentiviral vectors expressing small interfering RNAs. The siRNA against Grp78 is 5′-AAGGTTACCCATGCAGTTGTT-3′ and the control siRNA sequence is 5′-AAGGTGGTTGTTTTGTTCACT-3′. The former matches only to human Grp78 and the latter does not match to any sequence in the human genome. 293T and MDA-MB-435 cells were transfected with increasing concentrations of siGrp78 oligomers using siPORT Amine Kit (Ambion, Inc., Austin, TX). Transfection protocol is provided by the kit. The siRNAs were subcloned into a nonreplicating lentiviral vector using the pLenti6/V5-D-TOPO and ViralPower Lentivirus Expression system (Invitrogen, Carlsbad, CA) with modifications to generate a U6-driven siRNA and EGFP expression (Supplementary Information 1). The titers of the Lenti6/cPPT U6 siGRP78-GFP and Lenti6/cPPT U6 control siRNA-GFP were about 6×10^6 TU/mL. For transduction, MDA-MB-435 cells were seeded in a 6-well plate (2×10^5 cells per well), incubated at 37°C overnight. After aspirating the medium, the cells were incubated with 1 mL viral supernatant (Lenti6/cPPT U6 siGRP78-GFP or Lenti6/cPPT U6 control siRNA-GFP) and 6 μg polybrene per well for 8 hours at 37°C. The viral supernatant was aspirated and the cells were washed with PBS and 2 mL cell culture medium was added to each well. The transduced cells were incubated for 96 hours at 37°C before survival assays and Western blot analysis.

Western blots. For tumor samples, the tissue was frozen in liquid N_2 and homogenized. Protocols for Western blot analysis with total cell lysates have been described (23). The primary antibodies were rabbit anti-GRP78 (Santa Cruz Biotechnology), mouse anti-β-actin (Sigma-Aldrich), rabbit anti-HSVTK, rabbit anti-caspase-9 (BD Biosciences, PharMingen, San Diego, CA), rabbit anti-p21 and rabbit anti-histidine-tag (Santa Cruz Biotechnology), goat anti-HSP70 (Santa Cruz Biotechnology), rat anti-GRP94 and mouse anti-KDEL (StressGen, Victoria, Canada). The experiments were repeated two to four times.

Cell death assays. Etoposide (Calbiochem, La Jolla, CA) was dissolved in DMSO to obtain a 50 mmol/L working solution. The cells were treated with 25 μmol/L VP-16 for 6 hours and harvested after recovering for 12 hours. Cell death was assessed by the trypan blue exclusion assay. Briefly, 20 μL of 0.4% trypan blue (Sigma-Aldrich) was added into 20 μL of harvested cells resuspended in media. After 5 minutes of equilibration, the cells were counted under the microscope. The experiments were done in triplicates.

Results

Cambretastatin A-4P induces the expression of the prosurvival GRP78 protein in human breast tumor. To investigate the effectiveness of CA4P to promote regression of sizable tumors, human breast tumor MDA-MB-435 xenografts were established in BALB/c *nu/nu* mice. When the tumors reached a volume of about 0.5 cm^3, CA4P was given for four consecutive days. We observed that whereas CA4P treatment was able to arrest the growth of the

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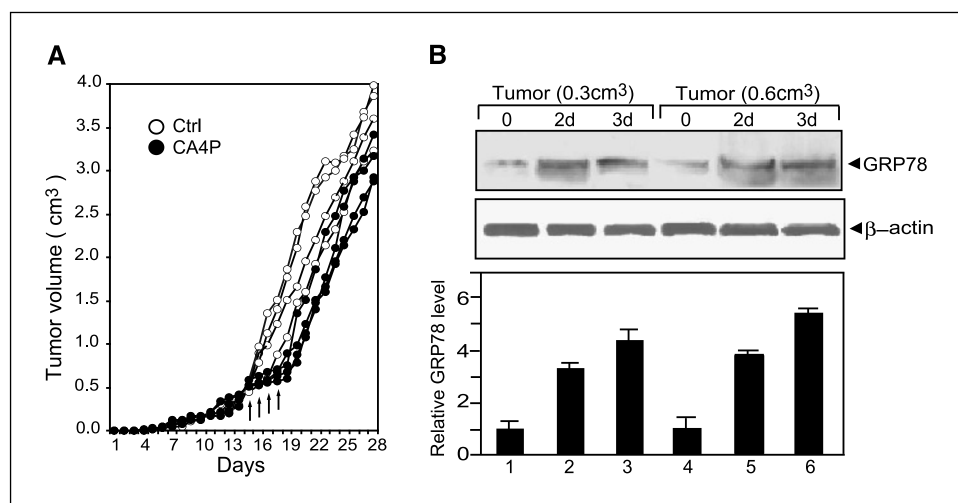

Figure 1. CA4P induces endogenous GRP78 level in xenografted human breast cancer model. *A,* MDA-MB-435 cells stably transduced with G1NaGrpTK were injected s.c. into the left flank area of BALB/c *nu/nu* mice (n = 4 for each group). At the indicated times, CA4P (*vertical arrows*) was given. The tumor volume was monitored daily. *B,* tumors reaching the size of 0.3 or 0.6 cm^3 were treated for 0, 2, or 3 days with CA4P. Equal amounts of protein extracts from tumors were subjected to Western blot analysis with antibodies against GRP78 and β-actin. The intensity of the protein bands were quantitated by densitometry and normalized against that of β-actin serving as internal loading control. The relative levels of GRP78 under each condition were plotted below the autoradiograms. *Bars,* SD.

xenografts immediately following the treatment, upon withdrawal of the drug, tumor growth resumed rapidly at a rate similar to the nontreated control (Fig. 1*A*). To examine the possible role of GRP78 in the survival response of tumors subjected to CA4P treatment, we analyzed the expression level of GRP78 by Western blot in the tumors. Both small (0.3 cm^3) and large tumors (0.6 cm^3) were tested and the level of β-actin served as the loading control. Upon CA4P treatment, the level of GRP78 protein increased for both tumor types, reaching a 4- and 6-fold increase respectively for the small and large tumors (Fig. 1*B*). Because GRP78 is a stable protein with a >48-hour half-life, this suggests that CA4P can substantially up-regulate GRP78 expression above its endogenous level in the tumor.

Cambretastatin A-4P activates the Grp78 promoter in human breast tumor xenografts. The promoter region of the rat *Grp78* gene contains three tandem copies of the ERSE that mediate stress-inducible activation of the Grp78 promoter. Previously, we have constructed a retroviral vector G1NaGrpTK where the Grp78 promoter containing the ERSEs drives the expression of the suicide gene *HSVTK* (Fig. 2*A*). Using a MDA-MB-435 cell line stably transduced with G1NaGrpTK, we investigate whether CA4P activates the Grp78 promoter within the tumor microenvironment by following the kinetics of induction of the HSVTK expression upon CA4P treatment. In the first analysis, Western blots were done with xenografts derived from the MDA-MB-435 cells transduced with G1NaGrpTK, with β-actin serving as loading control. Our results showed that with both small and big tumors, a detectable increase in the level of the reporter gene product HSVTK was observed after 2 days of CA4P treatment, and a substantial increase in HSVTK was evident within 3 days (Fig. 2*B*). These results show that the Grp78 promoter activity can be highly activated by CA4P in the tumor microenvironment.

To visualize the induction of HSVTK within the tumor, paraffin sections of the xenografts derived from the larger tumors were stained with H&E or with the anti-HSVTK antibody. Confirming that the CA4P treatment protocol is effective, necrotic regions devoid of tumor cells were detectable after 2 days of CA4P treatment, and by 3 days, substantial necrotic regions were evident throughout the tumor (Fig. 3*A-C*). In agreement with the Western blot results, brown staining depicting HSVTK staining, was detectable after day 2 and the intensity of staining was substantially increased after 3 days of CA4P treatment (Fig. 3*D-F*). A higher magnification of the HSVTK staining further showed that the majority of the staining was within the cytosolic compartment of the tumor cells as expected (Fig. 3*F*, *inset*). These results confirm that cytotoxic CA4P treatment of the tumor cells increases Grp78 promoter activity in human breast cancer xenografts. This activation of the Grp78 promoter could be a direct or indirect effect, directly due to stress from a cytotoxic effect of CA4P, or indirectly through stress resulting from hypoxia due to its antiangiogenesis activity.

Cambretastatin A-4P is not an inducer of GRP78 under tissue culture conditions. It has been well established that Grp78 transcription can be activated by a variety of pharmaceutical

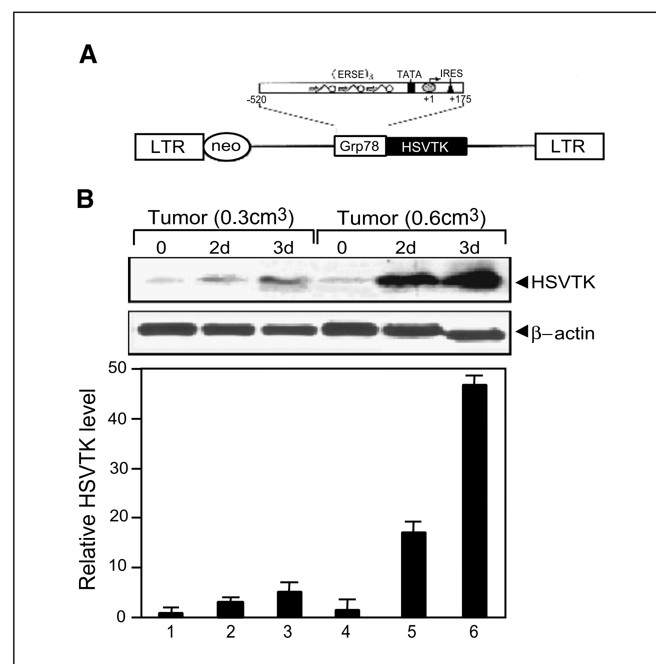

Figure 2. CA4P enhances Grp78 promoter-driven HSVTK reporter gene expression in MDA-MB-435 xenografts. *A,* schematic drawing of the retroviral vector G1NaGrpTK. LTR refers to the long terminal repeat of murine leukemia virus; *neo,* neomycin phosphotransferase gene; *IRES,* internal ribosome entry site; *HSVTK,* herpes simplex virus thymidine kinase gene. *B,* tumors reaching the size of 0.3 or 0.6 cm^3 were treated for 0, 2, or 3 days with CA4P. Equal amounts of protein extracts from tumors were subjected to Western blot analysis done with antibody against HSVTK and β-actin. The relative levels of HSVTK under each condition, after normalization against β-actin, were plotted below the autoradiograms. *Bars,* SD.

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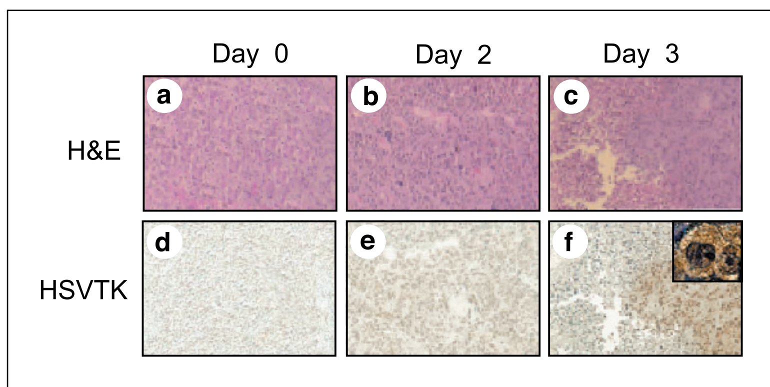

Figure 3. Immunohistochemical staining of HSVTK in tumor sections following CA4P treatment. *Top,* H&E staining of the paraffin-embedded tumor sections of MDA-MB-435 cells stably transduced with G1NaGrpTK. *Bottom,* the same sections were stained with anti-HSVTK antibody. Brown staining depicts HSVTK staining. Low magnification (*A-F*). *F,* high magnification of tumor cells expressing a high level of HSVTK in the cytosol (*inset*). Duration of CA4P treatment (in days, *top*).

agents that perturb cellular homeostasis. One classic inducer of GRP78 is tunicamycin which blocks NH_2-linked protein glycosylation, resulting in accumulation of underglycosylated proteins in the ER. To resolve whether the induction of GRP78 by CA4P is direct or indirect, we tested whether CA4P is able to induce GRP78 in MDA-MB-435 cells under tissue culture conditions. As shown in Fig. 4, treatment of MDA-MB-435 cells with tunicamycin resulted in strong induction of GRP78; in contrast, CA4P in all the doses being tested had minimal effect on the basal level of GRP78. The CA4P treatment was effective, because caspase-9 was activated in a manner dependent on the CA4P dosage, as evidenced by its proteolytic cleavage and p21 level was elevated (Fig. 4). These results show that CA4P cannot induce GRP78 under tissue culture conditions, suggesting that the induction of GRP78 by CA4P in xenograft models is not mediated by the cytotoxicity of the drug; rather, it is likely due to disruption of tumor vasculature and the resulting hypoxia or anoxia.

Contortrostatin inhibits tumor angiogenesis and induces GRP78 expression. To test whether the induction of GRP78 can be extended to agents that disrupt development of new blood vessels within the tumor, we used contortrostatin. Mice bearing MDA-MB-435 xenografts were either injected with PBS or contortrostatin, and paraffin-fixed tumor sections were subjected to immunohistochemical staining. CD31 staining showed that the contortrostatin treatment was effective because the blood vessel density as depicted in brown was substantially reduced in tumors following contortrostatin treatment compared with PBS treatment (Fig. 5*A, a* and *b*). Consistent with the results observed with CA4P, contortrostatin treatment resulted in increase in GRP78 protein expression level compared with the PBS treatment (Fig. 5*A, c* and *d*). Higher magnification further showed that GRP78 overexpression is primarily perinuclear/cytosolic (Fig. 5*A, e* and *f*). As in the case with CA4P, the induction of GRP78 by contortrostatin occurs in the context of the tumor microenvironment but not under tissue culture conditions (Fig. 5*B*).

Drug-resistant human breast cancer cell lines overexpress GRP78. One consequence of the induction of GRP78 by antiangiogenesis inhibitors is that GRP78 overexpression in tumors could

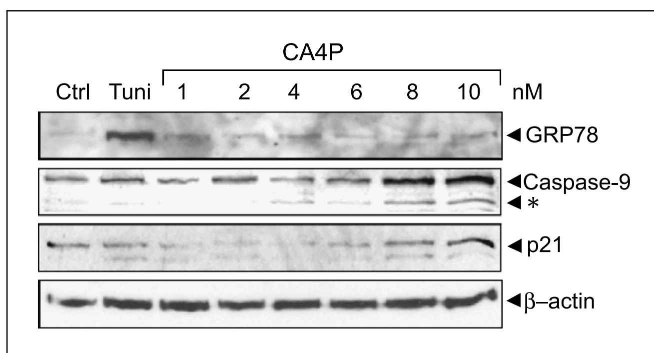

Figure 4. CA4P is not a stress inducer of GRP78 under tissue culture conditions. MDA-MB-435 cells were either nontreated (*Ctrl*), treated for 16 hours with tunicamycin (*Tuni*) at 1.5 μg/mL, or CA4P at the concentration (nmol/L, *top*). Equal amounts of cell lysates were subjected to Western blot analysis with antibodies against GRP78, caspase-9, p21, and β-actin. *, the cleaved, activated form of caspase 9.

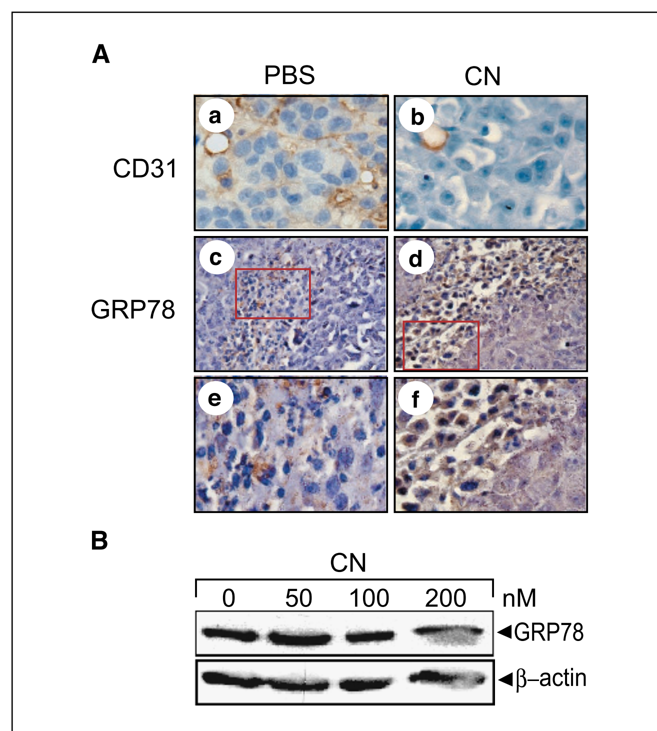

Figure 5. Contortrostatin (*CN*) induces GRP78 expression in MDA-MB-435 xenografts but not in tissue culture. *A,* mice bearing MDA-MD-435 xenografts were treated with either PBS (*a*, *c*, and *e*) or contortrostatin (*b, d*, and *f*). Paraffin-embedded tumor sections were stained with either anti-CD31 antibody (*a* and *b*) or anti-GRP78 antibody (*c-f*) and counterstained with H&E. Brown staining depicts CD31 and GRP78 and the nuclei were stained in blue. *a* and *b,* high magnification; *c* and *d,* middle magnification; *e* and *f,* high magnification of the boxed areas in (*c*) and (*d*). *B,* MDA-MB-435 cells were treated with contortrostatin at the concentration (nmol/L, *top*). Equal amounts of cell lysates were subjected to Western blot analysis with antibodies against GRP78 and β-actin.

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lead to drug resistance. To test this, a panel of human MCF-7 cell lines with characterized drug resistance properties was used (26). The resistant sublines were generated by stepwise increases in the concentration of the selecting drugs and maintenance at the selecting concentrations until stable lines were established. The cells were cultured in drug-free medium and protein lysates were prepared and subjected to Western blot analysis for GRP78 protein level with β-actin serving as a loading control (Fig. 6). In comparison with the parental MCF-7 cell line, an elevated level of endogenous GRP78 was detected in all the resistant sublines with varying magnitude. The most prominent induction of GRP78 was detected in sublines resistant to the topoisomerase II inhibitors doxorubicin (AdVp and Ad75) and VP-16. The induction of GRP78 by AdVP and VP-16 was independently confirmed by microarray analysis of drug resistant MCF-7 cells compared with parental cells.[5] Considering our previous finding that specific overexpression of ectopic GRP78 in Chinese hamster ovary cells and human bladder carcinoma cell lines confers resistance to doxorubicin and VP-16 in colony survival assays (19), these results further suggest that up-regulation of GRP78 is a mechanism of resistance available to cells undergoing the chronic stress of drug selection.

Suppression of GRP78 by small interfering RNA sensitizes human breast cancer cells to etoposide. To investigate the role of GRP78 in drug resistance in human breast cancer cells, we designed control siRNA and siRNA specific for human Grp78. Upon transient transfection into 293T cells, the siRNA against Grp78 efficiently suppressed the endogenous level of GRP78 and had no effect on GRP94, another ER chaperone protein coregulated with GRP78, or on HSP70, a cytosolic chaperone that shares 50% protein sequence homology with GRP78 (Fig. 7*A*). This shows that the effect of the siRNA is specific for GRP78. Next, we tested the suppression of GRP78 expression in the human breast cancer MDA-MB-435 cells by siRNA. As shown in Fig. 7*B*, the endogenous GRP78 protein level was suppressed in a dosage-dependent manner (Fig. 7*B*). To stably express the siRNA in the MDA-MB-435 cells, we constructed lentiviral vectors with the U6 promoter driving the expression of either a siRNA against human Grp78 or a negative control of random siRNA sequence. The vectors efficiently infected these human cells (about 80-90%) as evidenced by GFP expression. The MDA-MB-435 cells were transduced with the optimal MOI and were either nontreated or treated with VP-16. Under both conditions, the level of GRP78 was suppressed in cells transduced with the siGRP78 vector compared with the control vector (Fig. 7*C*). We note that MDA-MD-435 cells contain a mutated *p53* suppressor gene and are relatively more resistant to VP-16 treatment than other cell lines. Suppression of GRP78 protein levels by siRNA resulted in a higher sensitivity to VP-16-induced cell death compared with cells subjected to control siRNA treatment (Fig. 7*D*).

Discussion

Vascular targeting agents and antiangiogenic inhibitors represent a promising novel form of cancer therapy that has been the topic of intensive investigation and drug development. Recent clinical trials integrating the anti–vascular endothelial growth factor-A-humanized monoclonal antibody, bevacizumab, with chemotherapy show significantly increased response rates and delay in tumor progression (28, 29). Phase I trials with the antitubulin and vascular

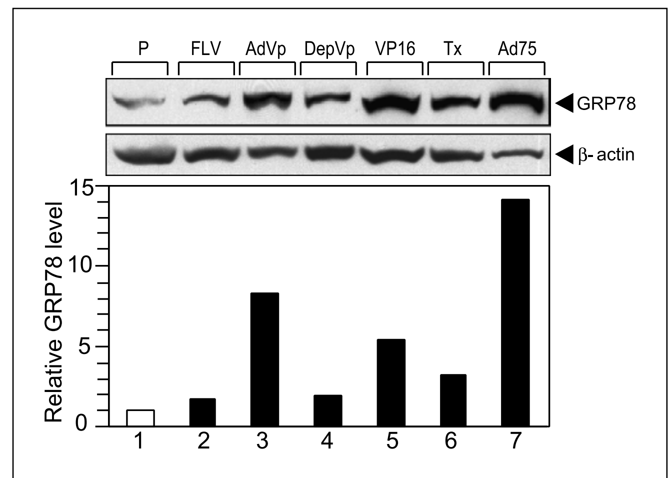

Figure 6. GRP78 overexpression in drug resistant MCF-7 human breast cancer cell lines. Equal amounts of cell lysates from the parental MCF-7 (*P*) and the resistant cells selected with various drug treatment regimens (*top*) were subjected to Western blot analysis with antibodies against GRP78 and β-actin. The FVL subline was selected in 1,000 nmol/L flavopiridol, AdVp in 100 ng/mL doxorubicin in the presence of 5 μg/mL verapamil, DepVp in 200 ng depsipeptide in the presence of 2.5 μg/mL verapamil, VP-16 in 4 μmol/L VP-16 (*Etop*), Tx in 200 ng/mL paclitaxel, and Ad75 in 75 ng/mL doxorubicin. The intensity of the protein bands was quantitated by densitometry and normalized against that of β-actin serving as internal loading control. The relative level of GRP78 in each subline was normalized against the parental line set as 1 and plotted below the autoradiograms.

targeting agent CA4P have established that significant tumor perfusion changes could be achieved; combination studies of CA4P with established therapies are in progress (30).

Despite this promise, it has been argued that although vascular targeting and antiangiogenic therapy target genetically stable endothelial cells in the tumor vasculature, genetic alterations and adaptive mechanisms acquired by the cancer cells in response to this type of therapy could lead to resistance. For example, tumors bearing a mutation in the *p53* tumor suppressor gene displayed a diminished rate of apoptosis under hypoxic conditions and were less responsive to antiangiogenic therapy (31). There are also a large number of studies suggesting that in theory, hypoxia could lead to more aggressive tumor behavior. For example, it has been reported that hypoxia promotes invasive growth of tumor cells by activating the hepatocyte growth factor, resulting in c-met activation, motility, and invasion (8). Furthermore, HIF transcriptionally induces the chemokine receptor CXCR4 that facilitates metastasis (32). However, the clinical relevance of these hypoxic responses is unclear because most tumors are inherently hypoxic; thus, it has been proposed that effective therapy has to shift the tumor from hypoxia to anoxia (10).

Mechanisms for cell survival under anoxia differ from the hypoxic response in that they involve HIF-independent pathways (33). Here we propose that one such pathway is the activation of the antiapoptotic protein GRP78. In tissue culture systems, transcriptional induction of GRP78 requires a glucose concentration of ≤1 mmol/L (11), and for oxygen deprivation, ≤0.02% (12). The Grp78 promoter does not contain any HIF binding site and ectopic expression of HIF has no effect on the Grp78 promoter activity (ref. 34; data not shown). Interestingly, one transcription factor that can activate Grp78 outside the ERSE is ATF4 (35), which is induced by anoxia (33). The first indication that the Grp78 promoter is activated through inhibition of angiogenesis is provided by the strong induction of the Grp78 promoter in the tumor environment by photodynamic therapy (23). Photodynamic

[5] T. Litman, personal communication.

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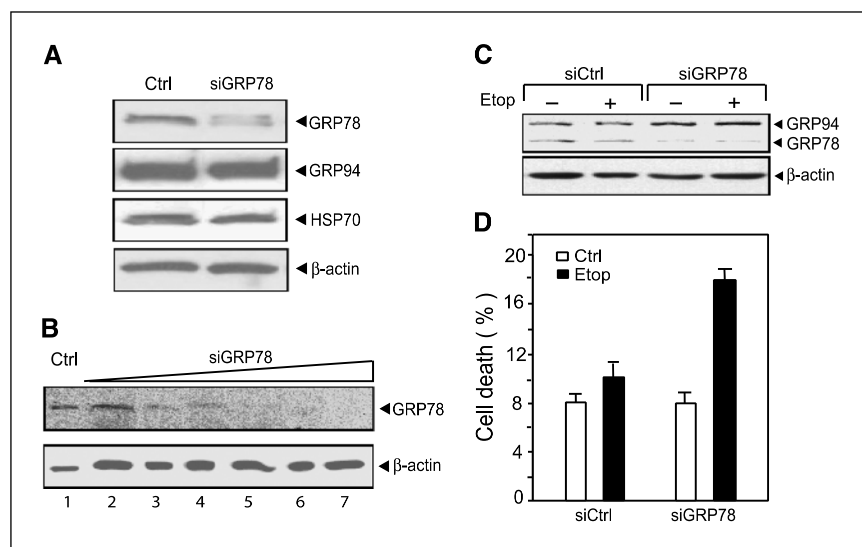

Figure 7. Specific suppression of GRP78 by siRNA increases VP-16-mediated cell death. *A,* 293T cells were mock transfected or transfected with 200 nmol/L of siGrp78 oligomers. After 72 hours, cell lysates were prepared and subjected to Western blot analysis with antibodies against GRP78, GRP94, HSP70, and β-actin. *B,* MDA-MB-435 cells were transfected with increasing amounts of siGRP78 oligomers (*lanes 1-7* at 0, 10, 20, 40, 80, 160, and 320 nmol/L, respectively). The levels of GRP78 and β-actin were determined by Western blot. *C,* MDA-MB-435 cells were transduced with lentiviral vectors expressing either control siRNA or siGRP78. After 96 hours, the infected cells were either nontreated or treated with 20 μmol/L etoposide for 6 hours. Following a 14-hour recovery period, Western blot was done to determine the level of GRP78, GRP94, and β-actin. *D,* the percentage of cell death was determined for the transduced cells that were either nontreated or treated with etoposide. *Bars,* SD.

therapy, which causes oxidative stress *in vitro* can also cause rupture of tumor vasculature *in vivo* (36). Here we discover that in contrast to photodynamic therapy that can induce the Grp78 promoter both *in vitro* and *in vivo*, neither CA4P that targets the tubulin structure of endothelial cells nor contortrostatin, a disintegrin, is a stress inducer of GRP78 *in vitro*. Nonetheless, in the context of the tumor, both can lead to strong induction of the Grp78 promoter and elevation of the GRP78 protein level, suggesting that the two agents are likely to act through a common mechanism of nutrient and oxygen deprivation resulting from destruction of tumor vasculature.

The protective effect of GRP78 against apoptosis caused by disturbance of ER stress through the use of pharmacologic agents, such as tunicamycin or thapsigargin, has been well established using both overexpression and antisense approaches (16). Despite studies correlating ER stress conditions that induce GRP78 with VP-16 and camptothecin resistance in a variety of human cell lines derived from different solid tumor types (37, 38), direct evidence that GRP78 overexpression contributes to the development of drug resistance in human cancer cells is just emerging. Here, we constructed a lentiviral vector expressing siRNAs specifically targeting Grp78. The viral vector was able to transduce efficiently human breast cancer lines including the model system MDA-MB-435. Compared with control vectors, the level of endogenous GRP78 was suppressed and the cells become more sensitized to VP-16 treatment. Etoposide induces cell death primarily through the p53 pathway, but there are also p53-independent pathways that are not well understood (39–41). The MDA-MB-435 cells are more resistant to VP-16-induced cell death compared with primary embryo fibroblast cell lines (data not shown). This is likely due to the mutated p53 status and other genetic mutations and adaptive mechanisms acquired by these cells against drug-induced cell death (42). Although the magnitude is in the range of 2-fold, it is remarkable that by down-regulating a single variable, GRP78, sensitization can already be observed. These results are in agreement with our previous studies showing that overexpression of GRP78 in both Chinese hamster ovary and a human bladder cancer cell line results in drug resistance using clonogenic survival assays and annexin labeling apoptotic assays (19).

We further show here that human breast cancer cells that have developed drug resistance, in particular those against VP-16 and doxorubicin, exhibit elevated levels of GRP78. This observation was made by Western blot analysis of cell lysates and confirmed in microarray analysis which provides additional evidence that Grp78 is activated at the transcript level in cells that develop drug resistance. It is interesting to note that the selected cell lines contain previously defined mechanisms of drug resistance, including the overexpression of P-glycoprotein and MRP1, the multidrug resistance–associated protein (26). The observation that such cell lines could shelter additional mechanisms of resistance will be of no surprise to the oncologist well acquainted with the multifactorial nature of drug resistance. Indeed, the ability of a cancer cell to up-regulate a survival pathway may enhance its ability to acquire a second and more powerful mechanism of resistance. These results are in agreement in previous studies that GRP78 is overexpressed in fibroblasts subjected to long exposure of VP-16 (43). The expression of GRP78 has been examined in human breast ductal carcinoma and its overexpression is observed in most of the more aggressive estrogen receptor–negative tumors (44). Synthetic chimeric peptides targeted against GRP78 can suppress tumor growth in xenograft and isogenic mouse models of breast and prostate cancer (45). Preliminary analysis of GRP78 in a series of primary and recurrent breast, prostate, and lung cancer samples suggest a correlation between GRP78 overexpression, recurrence, and drug resistance (46).[6] Collectively, these studies imply that GRP78 is a general contributor to tumor growth and drug resistance in human cancer.

Whereas mechanisms for the antiapoptotic effect of GRP78 are still under investigation, recent studies reveal that the ER contributes significantly to apoptosis, both by sensitizing the mitochondria to a variety of extrinsic death stimuli and by initiating cell death signals of its own (47–49). GRP78 is able to inhibit activation of caspases both *in vitro* and *in vivo* (18, 19, 50). Whereas the majority of GRP78 resides in the ER lumen, a subfraction of GRP78 exists as an ER transmembrane protein, rendering it possible to interact with cytosolic components of the apoptotic pathways (19). It is interesting to note that the activation of caspase-7 by VP-16 is blocked by GRP78 overexpression, presumably mediated by the

[6] Unpublished results.

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chaperone function of GRP78 in maintaining procaspase-7 in an inactive complex. Mutation of the ATPase domain of GRP78 abolishes this protective function (19). It is also possible that GRP78 in its capacity as a chaperone protein can interact and modulate the function of other apoptotic signaling molecules at the ER. As a calcium binding protein in the ER, GRP78 may also serve as a buffer against calcium efflux from the ER that could trigger the major mitochondrial apoptotic program. Collectively, our results suggest that glucose deprivation and anoxia, the ultimate goal of vascular targeting and antiangiogenesis therapy, will lead to induction of GRP78 in surviving residual cancer cells and may result in drug resistance. These results also suggest that therapy targeting the tumor vasculature may need to be coupled with therapy targeted towards the protective function of GRP78.

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