Ligation of cancer cell surface GRP78 with antibodies directed against its COOH-terminal domain up-regulates p53 activity and promotes apoptosis

Uma Kant Misra, Yvonne Mowery, Steven Kaczowka, and Salvatore Vincent Pizzo

Department of Pathology, Duke University Medical Center, Durham, North Carolina

Abstract

Binding of activated α₂-macroglobulin to GRP78 on the surface of human prostate cancer cells promotes proliferation by activating signaling cascades. Autoantibodies directed against the activated α₂-macroglobulin binding site in the NH₂-terminal domain of GRP78 are receptor agonists, and their presence in the sera of cancer patients is a poor prognostic indicator. We now show that antibodies directed against the GRP78 COOH-terminal domain inhibit [³H]thymidine uptake and cellular proliferation while promoting apoptosis as measured by DNA fragmentation, Annexin V assay, and clonogenic assay. These antibodies are receptor antagonists blocking autophosphorylation and activation of GRP78. Using 1-LN and DU145 prostate cancer cell lines and A375 melanoma cells, which express GRP78 on their cell surface, we show that antibodies directed against the COOH-terminal domain of GRP78 up-regulate the tumor suppressor protein p53. By contrast, antibody directed against the NH₂-terminal domain of GRP78 shows negligible effects on p53 expression. PC-3 prostate cancer cells, which do not express GRP78 on their cell surface, we show that antibodies directed against the NH₂-terminal domain of GRP78 up-regulate the tumor suppressor protein p53. By contrast, antibody directed against the NH₂-terminal domain of GRP78 shows negligible effects on p53 expression. PC-3 prostate cancer cells, which do not express GRP78 on their cell surface, are refractory to the effects of anti-GRP78 antibodies directed against either the COOH- or NH₂-terminal domains. However, overexpression of GRP78 in PC-3 cells causes translocation of GRP78 to the cell surface and promotes apoptosis when these cells are treated with antibody directed against its COOH-terminal domain. Silencing GRP78 or p53 expression by RNA interference significantly blocked the increase in p53 induced by antibodies. Antibodies directed against the COOH-terminal domain may play a therapeutic role in cancer patients whose tumors trigger the production of autoantibodies directed against the NH₂-terminal domain of GRP78. [Mol Cancer Ther 2009; 8(5):1350–62]

Introduction

The unfolded protein response consists of the signaling network responding to various stresses placed on cells. As a part of unfolded protein response, expression of chaperones, including GRP78 (glucose-regulated protein of 78 kDa), is up-regulated (1). GRP78 is often overexpressed in cancers where it mediates tumor growth by enhancing cell proliferation, protecting against apoptosis, and promoting tumor angiogenesis (1–4). Its up-regulation, therefore, is associated with an aggressive malignant phenotype and a poor prognosis. GRP78 exerts an antiapoptotic role by alleviating the accumulation of unfolded proteins in the estrogen receptor (ER); by regulating ER Ca²⁺ storage, thus reducing mitochondrial Ca²⁺ overload; and by binding to and inactivating caspase-12. Xenograft studies show that down-regulation of GRP78 inhibits tumor growth (1, 4). Partial suppression of GRP78 expression in heterozygous knockout mice extends the latency period and significantly inhibits tumor growth and tumor angiogenesis (3).

A small pool of newly synthesized GRP78 may translocate to the cell surface in association with MTJ-1 and G proteins, including Gs and Gaq11 (5–7). GRP78 lacks a transmembrane domain, unlike MTJ-1, and both its termini are on the external side of the membrane (6). Presumably, the G proteins interact with the cytoplasmic domain of MTJ-1 (6, 7). Cell surface–associated GRP78 is seen in several malignant tumors, such as prostate and melanoma (8). Melanoma frequency is predicted to increase with changes in global climate, whereas prostate cancer is the most commonly diagnosed malignancy in men (9). Whereas the plasma proteinase inhibitor α₂-macroglobulin (α₂M) does not bind to cells, the activated form, α₂M*, binds with extremely high affinity (Kₐ, 50–100 pmol/L; refs. 10, 11). α₂M* ligation of cell surface–associated GRP78 in 1-LN human prostate cancer cells triggers cell proliferation and prevents apoptosis as a result of activating several signaling pathways (10–12). 1-LN cancer cells are derived from the less metastatic PC-3 line, which does not express GRP78 on its cell surface and does not respond to α₂M* treatment (10–13).

Based on these in vitro observations, we proposed that up-regulation of cell surface GRP78 is part of a more aggressive phenotype in prostate cancer (10, 11, 13). Support for this hypothesis is derived from studies of human prostate cancer and melanoma patients (8, 14, 15). The sera of these patients contain high titers of an autoantibody directed against an epitope that is identical to the α₂M* binding site.
located in the NH2-terminal domain of GRP78 (8). Clinically, occurrence of these antibodies correlates with a more metastatic phenotype and poor prognosis. This antibody shows agonist properties such as α2M* and is proproliferative and antiapoptotic (8). However, antibodies directed against GRP78 may directly target or enhance sensitivity of tumor cells to chemotherapeutic agents (4, 16, 17). These effects may depend on down-regulation of its functions as a cell surface receptor by these antibodies, in contrast to the agonist-like properties of autoantibodies in the sera of cancer patients.

The p53 tumor suppressor protein is a crucial protector against cancer by eliminating cells with mutagenic alterations or by blocking cell cycle progression (18–21). The activity of p53 depends not only on its level but also on specific posttranslational modifications induced by different stress-induced signaling pathways (18–21). In normal, un-stressed cells, p53 activity is very low, but stress causes p53 activation through a series of posttranslational modifications and it then binds to specific DNA sequences. p53 controls the expression of genes associated with apoptotic pathways and it responds to death stimuli by rapid stabilization and activation.

In the present study, we show down-regulation of GRP78 activation by antibodies directed against its COOH-terminal domain. They are antagonistic to α2M* binding to cell surface-associated GRP78 in 1-LN and DU145 prostate cancer cells as well as A375 melanoma cells. These antibodies block receptor autophosphorylation, inhibit DNA synthesis, and induce apoptosis as measured by DNA fragmentation, Annexin V assay, and clonogenic assay in 1-LN cells. Silencing p53 or GRP78 expression by RNA interference (RNAi) abrogated GRP78-induced up-regulation of p53. Antibodies directed against the NH2-terminal domain of GRP78 show minimal effect on p53 levels in these cells. Further, neither anti-GRP78 antibody preparation affected p53 levels in PC-3 cells, which do not express GRP78 on the cell surface. However, overexpression of GRP78 in PC-3 caused translocation of GRP78 to the cell surface and anti-GRP78 antibody directed against the COOH-terminal domain induced increases in p53 protein and apoptosis. These studies show that depending on the domain of GRP78 that is bound by antibodies, either agonistic or antagonistic properties are observed.

Materials and Methods

Materials

Culture media were purchased from Invitrogen. α2M* was prepared as described previously (11, 12, 22). Antibodies were procured against the COOH-terminal domain of GRP78 (catalog number SPA-826 from Stressgen called antibody A and catalog number sc-1051 called antibody B, and catalog number sc-13968 called antibody C from Santa Cruz Biotechnology, Inc.) and against the NH2-terminal domain of GRP78 (Santa Cruz Biotechnology). These antibodies, at an IgG concentration of 200 μg/mL, were used at a 1:200 dilution in these studies. [γ32P]ATP (specific activity, 3,000 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. p53, p-p53Ser392, p-MDM2Ser166, acetyl-p53Lys382, and p21 were from Cell Signaling Technology, Inc. Antibodies against HSP70, HSP90, and PDI were purchased from Noventa. Formaldehyde (1% neutral buffered) and antiactin antibodies were procured from Sigma-Aldrich. Goat serum was from Biomedia. Hoechst 33342 was from Invitrogen, and crystal violet solution and staurosporine were from Sigma-Aldrich. Other reagents of the highest available purity were procured locally.

Cancer Cell Lines

We used two prostate cancer lines (i.e., 1-LN and DU145) and one melanoma cancer cell line (A375), which express GRP78 on the cell surface, and the PC-3 prostate cancer line, which does not express GRP78 on the cell surface. The highly metastatic prostate carcinoma cell line 1-LN is derived from the less metastatic PC-3 cells and was a kind gift of Dr. Philip Walther (Duke University Medical Center, Durham, NC). The PC-3, DU145, and A375 cell lines were obtained from the American Type Culture Collection. These cells were grown in 6-well plates (500 × 103 per well) to confluency in RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L glutamine, 12.5 units/mL penicillin, 6.5 μg/mL streptomycin, and 10 nmol/L insulin in a humidified CO2 (5%) incubator at 37°C. At ∼90% confluence, the medium was aspirated, the monolayers were washed with ice-cold HHBSS, and a fresh volume of medium was added to the monolayers.

Autophosphorylation of GRP78 in 1-LN Cells Treated with α2M* and Anti-GRP78 Antibody

Autophosphorylation of GRP78 was measured essentially as described (6). 1-LN cells were grown in RPMI 1640 containing 10 nmol/L insulin, 2 mmol/L glutamine, 10% fetal bovine serum, 12.5 units penicillin, and 6.5 μg/mL streptomycin in 6-well plates (4 × 106 per well) at 37°C in a humidified CO2 incubator until 80% to 90% confluent. The medium was aspirated, the monolayers were washed with chilled HHBSS buffer, and a volume of HHBSS was added to the monolayer. The cells were lysed in a volume of buffer containing 40 mmol/L HEPES (pH 7.4), 1% NP40, 100 mmol/L NaCl, 1 mmol/L EDTA, 25 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin over ice for 15 min. The lysates were pipetted into Eppendorf tubes through a 27-gauge needle several times, and lysates were centrifuged at 1,000 × g for 5 min at 4°C to remove cell debris. The supernatants were transferred to new tubes, and their protein contents were determined by the Bradford method (23). To equal amounts of lysate proteins in the respective tubes, antibodies against cell surface-associated GRP78 (1:50) were added followed by the addition of protein A-agarose and the tubes were incubated overnight at 4°C in a rotary shaker. The immunoprecipitates were

Published Online First May 5, 2009; DOI: 10.1158/1535-7163.MCT-08-0990

Mol Cancer Ther 2009;8(5). May 2009
recovered by centrifugation (2,500 × g/5 min) at 4°C and washed twice with lysis buffer and thrice with kinase buffer containing 50 mmol/L HEPES (pH 7.5), 10 mmol/L MgCl₂, 2 mmol/L MnCl₂, and 0.2 mmol/L DTT. Autophosphorylation was measured in 50 μL of kinase buffer containing 10 μCi of [γ³²P]ATP for 20 min at 30°C. The reaction was stopped by adding a volume of 4× sample buffer and heating the tubes for 5 min at 90°C. The tubes were centrifuged and proteins were separated on a 10% gel. The protein bands on the gels were transferred to membranes, the membranes were dried, and ³²P-labeled GRP78 was detected and quantified by autoradiography in a Storm 860 Phosphorimagery.

Measurement of [³H]Thymidine Uptake by 1-LN Prostate Cancer Cells Treated with Antibodies against the COOH-Terminal Domain of GRP78

One of the cellular responses to the binding of α₂M* to cell surface–associated GRP78 is a 2- to 3-fold increase in [³H]thymidine uptake by 1-LN and DU145 but not PC-3 prostate cancer cells. The former two lines express GRP78 on the cell surface, whereas PC-3 cells do not (12). Silencing GRP78 gene expression by RNAi in 1-LN cells abrogates calcium signaling and [³H]thymidine uptake (10). We studied the effect of anti–COOH-terminal domain antibodies on [³H]thymidine uptake and cellular proliferation in 1-LN prostate cancer cells as previously described (10). Briefly, 1-LN cells were grown in RPMI 1640 to 80% to 90% confluency. The medium was aspirated and cells were scraped in RPMI 1640, the pellet was suspended in a volume of the medium, and the cells were pipetted into 48-well plates (80–100 × 10⁵ per well) and allowed to adhere and medium was replaced with fresh RPMI 1640 containing 0.5% fetal bovine serum. After incubation for 10 to 12 h, [³H]thymidine (2 μCi/mL) was added to each well followed by the addition of buffer, α₂M* (50 pmol/L), or anti-GRP78 COOH-terminal domain antibodies A, B, or C at 1:200 dilution and the cells were incubated overnight as described. The reactions were terminated by aspirating the medium, and monolayers were washed twice with ice-cold 5% trichloroacetic acid followed by three washings with ice-cold PBS and lysed in 1N NaOH (40°C/2 h). Aliquots of the lysate were used for protein quantification (23) and for determining radioactivity in a liquid scintillation counter.

Determination of 1-LN Prostate Cancer Cell Number after Treatment with Anti–COOH-Terminal Domain Antibody

Because increased DNA synthesis is normally associated with an increase in total cellularity, the effect of treatment with antagonist anti–COOH-terminal domain antibody A was determined. 1-LN cells were grown in RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L glutamine, 10 mmol/L insulin, 12.5 units/mL penicillin, and 6.5 μg/mL streptomycin to 80% to 90% confluency at 37°C in a humidified CO₂ (5%) incubator. The monolayers were washed with HHBSS twice, a volume of the medium was added, and cells were scraped into fresh tubes and centrifuged at 1,000 × g for 5 min at 4°C. The pellet was suspended in the above medium, and cells were counted in a hemocytometer. Equal number of cells in 0.5 mL medium was pipetted into 1.5 mL siliconized polypropylene tubes, and to respective tubes was added buffer, α₂M* (50 pmol/L), or anti–COOH-terminal domain antibody A (1:200 dilution) and cells were incubated up to 5 d (22). At the desired period of incubation, an aliquot of the cell suspension was removed from each group for cell counting (22).

Hoechst 33342 Staining of 1-LN Prostate Cancer Cells Treated with Anti-GRP78 Antibody

to examine the effect of antibody directed against the COOH-terminal domain of GRP78 on induction of apoptosis in 1-LN prostate cancer cells, ~50,000 cells in RPMI 1640 were plated onto 2.5 cm² glass coverslips in 35-mm dishes and incubated as above. The monolayers were treated with medium, α₂M* (50 pmol/L), anti-GRP78 antibody A (1:200), or staurosporine (5 μmol/L), and monolayers were incubated for 16 h as above. Medium was aspirated and monolayers were washed thrice with chilled PBS. The cells were fixed with 1% formaldehyde for 25 min at 37°C and washed thrice with PBS. Cells were permeabilized with a 0.5% Triton X-100 in PBS for 5 min at room temperature and washed thrice with PBS. Cells were stained with Hoechst 33342 (10 μg/mL) for 20 min at room temperature. Cells on the coverslips were washed several times with PBS and examined by a fluorescence microscope. The percentage of apoptotic cells under the experimental conditions was determined by microscopic cell counting of multiple fields in the various experiments.

Clonogenic Assay of 1-LN Cells Treated with Anti-GRP78 Antibody

1-LN cells incubated in RPMI 1640 (3 × 10⁶ per well) overnight in 6-well plates were treated with buffer, α₂M* (50 pmol/L/10 min), antibody A directed against the COOH-terminal domain of GRP78 (1:200/30 min), or staurosporine (5 μmol/L/2 h) and incubated at 37°C in a humidified CO₂ (5%) incubator. Incubations were terminated by aspiration and monolayers were washed twice with the medium. The cells were detached with trypsin/EDTA and centrifuged, the pellet was suspended in RPMI 1640 as above, and cells were counted. Three hundred cells from each group were pipetted into wells on a 6-well plate, and cells were incubated as above in 2 mL of RPMI 1640 in a humidified CO₂ (5%) incubator until colonies were visible (8–10 d). The medium was removed and cells were washed with the medium. Cells were fixed in methanol for 10 min; after aspiration, 2 mL of crystal violet solution were added to each well and cells were stained for 10 min. The stain was removed and colonies consisting of 50 to 60 cells were counted and photographed.

Assay of DNA Fragmentation in 1-LN Cells Treated with Antibody Directed against the COOH-Terminal Domain of GRP78

The measurement of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) after induction of cell death was done with a Cell Death Detection ELISA kit (Roche). 1-LN cells incubated overnight (10⁶ per well) in 96-well plates in the above RPMI 1640 were treated with buffer, α₂M* (50 pmol/L/15 min), antibody A directed...
against the COOH-terminal domain of GRP78 (1:200/2 h), or staurosporine (5 μmol/L/2 h) and incubated. The DNA fragmentation is expressed as the specific enrichment of cytosol with mononucleosomes and oligonucleosomes.

**Determining the Selectivity of Antibodies against the NH2- or COOH-Terminal Domain of GRP78 on p53 Up-Regulation in Cancer Cell Lines**

To determine the selectivity/specificity of GRP78 antibodies on p53 in 1-LN, DU145, or PC-3 prostate cancer lines, or the melanoma line A375, we chose three antibodies directed against the COOH-terminal domain and one against the NH2-terminal domain of GRP78. Cells were grown under identical conditions and treated with the respective anti-GRP78 antibodies (1:200/30 min/37°C). The reactions were terminated by aspirating the medium, cells were lysed in lysis buffer as stated in the preceding sections, and protein contents were determined (23). Equal amounts of lysate protein were then electrophoresed, protein bands on the gels were transferred to membranes, membranes were immunoblotted with antibodies against p53, and p53 bands on the immunoblots were visualized by enhanced chemiluminescence.

**Silencing p53 Gene Expression by RNAi and the Effect of Anti-COOH-Terminal Domain Antibodies on p53 Protein Levels in 1-LN, DU145, and A375 Cancer Lines**

To determine the specificity of the target of anti-COOH-terminal domain antibody A, we silenced p53 gene expression by RNAi (24). Annealed and validated small interfering RNA for the gene was purchased from Ambion (small interfering RNA ID 106141) of the sense sequence (5′→3′) GGGUUAGUUUACAAUCAGCtt and antisense sequence (5′→3′) GCUUAUGUUUAACCUACGtt and then stimulated with anti-GRP78 antibody A (1:200/30 min/37°C). The cells were transfected with 75 nmol/L of annealed p53 double-stranded RNA (dsRNA), and control cells were transfected with Lipofectamine as described previously (24). Forty-eight hours after transfection, the control cells were stimulated with either buffer or anti-GRP78 COOH-terminal antibody A (1:200/30 min/37°C). Cells for the negative controls were transfected with scrambled dsRNA (75 nmol/L/48 h; Ambion) and then stimulated with anti-GRP78 antibody A. Reactions were terminated by aspirating the medium and cells were lysed in a volume of lysis buffer as described above. Protein contents of respective lysates were determined and equal amounts of lysate proteins were used for determining p53 protein levels by Western blotting as described above. Statistical analysis where shown was done by Student’s t test.

**Overexpression of GRP78 in PC-3 Prostate Cancer Cells with Thapsigargin**

Thapsigargin was first used to induce up-regulation of GRP78 protein levels and its cell surface localization in human rhabdomyosarcoma cells (25). We have overexpressed GRP78 in PC-3 cancer cells by treating the cells with thapsigargin (1 μmol/L/3 h). The cell growth and incubation conditions were the same as outlined in the preceding sections. The transcriptional up-regulation of GRP78 was measured by qualitative determination of GRP78 mRNA by PCR and by GRP78 protein levels by Western blotting as described previously (11). Briefly, PC-3 cells in RPMI 1640 containing the additions listed above were grown (1 × 10⁶ per well) in 6-well plates in duplicates and treated with thapsigargin (1 μmol/L/3 h) and incubated as above. The reaction was terminated by aspirating the medium, and one plate was processed for qualitative determination of GRP78 mRNA by PCR (11) and the other plate was processed for quantification of GRP78 protein by Western blotting (11).

**Determination of Cell Surface Expression of GRP78 in Cells Treated with Thapsigargin by Cell Western Blotting**

Treatment of cells with thapsigargin induces the translocation of GRP78 to the cell surface in some cells (25). In this study, we determined cell surface expression of GRP78 in PC-3 cancer cells treated with thapsigargin (1 μmol/L/3 h). Cells were grown in RPMI 1640 with the additions listed above in 24-well plates and incubated in a humidified CO2 (5%) incubator until the cells formed a uniform monolayer. The medium was aspirated, a fresh volume of above medium was added to each well followed by the addition of thapsigargin (1 μmol/L/3 h), and cells were incubated. The reaction was terminated by aspirating the medium, and the monolayers were washed twice with cold PBS. Cells were fixed by adding a volume of 2% formaldehyde in PBS, and cells were incubated for 20 min at room temperature. Then, the medium was aspirated and cells were washed twice with PBS containing 0.05% Tween 20.
A volume of blocking buffer containing 3% bovine serum albumin and 5% nonimmune goat serum in PBST was added to each well, and cells were incubated at room temperature with gentle shaking for 2 h. The blocking buffer was removed, and cells were incubated with anti-GRP78 antibody against its COOH-terminal domain (1:50; antibody A) in PBST and incubated at 4°C overnight with gentle rotation. The reaction was terminated by aspirating the medium and washing the cells thrice with PBS followed by the addition of IRDye 800 DX-conjugated affinity-purified anti-rabbit IgG (goat) in PBST, and cells were incubated for 60 min with shaking at room temperature. The medium was aspirated; cells were washed twice with PBST and once with PBS, dried, and imaged; and cell surface expression of GRP78 was quantified in a LI-COR Odyssey according to the manufacturer’s instructions.

Annexin V Assay as a Measure of Apoptosis of Cells Treated with Anti-GRP78 Antibody against the COOH-Terminal Domain

Anti-GRP78 antibody–induced apoptosis of cells was measured by the Annexin V method on a Guava EasyCyte Plus flow cytometer according to the instructions provided by the manufacturer (Guava Technologies).

Results

Inhibition of Autophosphorylation of GRP78 in 1-LN Cells Treated with Anti-GRP78 Antibody

GRP78 contains an ATP binding site in the NH2-terminal domain, and in vitro autophosphorylation of tyrosine residues in GRP78 is observed (26, 27). We have shown that autophosphorylation of tyrosine residues also occurs when cell surface–associated GRP78 is ligated by α2M* (6). Treatment of 1-LN prostate cancer cells with α2M* caused a 1.6-fold increase in autophosphorylation of GRP78 compared with buffer-treated cells (Fig. 1A). Pretreatment of 1-LN cells with anti-GRP78 antibody A directed against the COOH-terminal domain of GRP78 significantly inhibited α2M*-induced autophosphorylation of GRP78 (Fig. 1A). Thus, an antibody directed against the COOH-terminal domain of GRP78 functions as antagonist to α2M*, inhibiting ligand-induced receptor activation. In addition to these effects on autophosphorylation of GRP78, this antibody also blocked up-regulation of GRP78 induced by α2M* (Fig. 1B). Interestingly, treatment of 1-LN cells with this antibody in the absence of α2M* directly decreases the levels of GRP78 observed under our experimental conditions (Fig. 1B).

Antibodies against the COOH-Terminal Domain of GRP78 Inhibit [3H]Thymidine Uptake by 1-LN Prostate Cancer Cells

Expression of GRP78 on the cell surface is essential for α2M*-dependent signal transduction, which promotes proliferation and mitogenesis (10–13). We therefore next focused on 1-LN prostate cancer cells to study the effect of antibodies directed against the COOH-terminal domain of GRP78 (antibodies A, B, and C) on DNA synthesis as assessed by the uptake of [3H]thymidine compared with that...
of cells incubated with α2M* (Fig. 2A). This cell line shows very high expression of cell surface–associated GRP78 and the greatest response to α2M* treatment among human prostate cancers that we studied (12). All three anti-COOH-terminal domain antibodies nearly abolished α2M*-induced [3H]thymidine uptake by 1-LN cells compared with cells incubated with α2M* alone (Fig. 2A). In addition, this antibody A profoundly inhibited proliferation of these cells compared with that observed with α2M* treatment alone (Fig. 2B). By contrast, antibody directed against the NH2-terminal domain of GRP78 isolated from prostate cancer patients causes an increase in [3H]thymidine uptake and promotes cellular proliferation in 1-LN or melanoma cells (8).

**Induction of Apoptosis in 1-LN Prostate Cancer Cells Treated with Anti-GRP78 Antibody**

We next evaluated the effects of anti-GRP78 antibody A on apoptosis in 1-LN prostate cancer cells by examining cell staining with Hoechst 33342 and clonogenic assays (Fig. 3A and C). 1-LN cells treated with antibody A directed against the COOH-terminal domain of GRP78 caused shrinkage of cells and nuclear condensation (Fig. 3A). Consistent with our previous observations, α2M* treatment of 1-LN prostate cancer cells promoted cell growth (Fig. 2A and B). Clonogenic assays show that 1-LN prostate cancer cells treated with antibody A or staurosporine have significantly reduced colony-forming potential in contrast to cell treated with α2M*, where seeded cells formed 60% to 70% colonies (Fig. 3C). Likewise, measurement of DNA fragmentation in 1-LN cells shows a proapoptotic effect of anti–COOH-terminal domain antibody A (Fig. 3D). Such as the DNA fragmentation method, the Annexin V flow assay showed apoptosis in 1-LN cancer cells treated with antibody directed against the COOH-terminal domain of GRP78 (Fig. 3E).

**Regulation of p53 Function in Cancer Cells Treated with Antibodies Directed against GRP78**

In view of the proapoptotic function of p53, we next chose to study the effect of anti-GRP78 antibodies on p53 levels in cancer cells. Stimulation of 1-LN prostate cancer cells with α2M* had negligible effects on p53 levels (Fig. 4A). By contrast, incubation of these cells with antibody (1:200 dilution) directed against the COOH-terminal domain of GRP78 either alone or with α2M* (50 pmol/L) caused a severalfold increase in p53 levels (Fig. 4A). p53 contains many conserved residues whose phosphorylation or acetylation affects p53 stabilization and activation. We observed a significant increase in the levels of phosphorylated and acetylated p53 in 1-LN cells treated with antibody antagonistic to GRP78 activation (Fig. 4A). p21 is a cyclin kinase inhibitor located in the tumor suppressor pathway downstream of p53, which arrests growth after DNA damage (28). Because antibodies directed against the COOH-terminal domain of GRP78 caused accumulation of p53 in 1-LN prostate cancer cells, one would expect elevated levels of p21 in these cells. In fact, antagonistic anti-GRP78 antibody treatment elevated p21 by ~2-fold compared with α2M*-treated cells (Fig. 4B). Next, we examined whether up-regulation of p53 in 1-LN cells is specific only to antibody directed against the COOH-terminal domain of GRP78, which contains the KDEL membrane–targeting sequence, or whether other KDEL proteins when ligated with antibodies might cause a similar effect. We therefore treated 1-LN cells with antibodies directed against the COOH-terminal domain of GRP78, HSP70, HSP90, PDI, or calreticulin and processed cell lysates for electrophoresis and immunoblotting. The results clearly show that only antibody directed against the COOH-terminal domain of GRP78 specifically up-regulates p53 compared with these other antibodies (Fig. 4C).

To further ascertain the requirement of cell surface GRP78 expression for up-regulation of p53, we used two more antibodies directed against the COOH-terminal domain and one antibody directed against the NH2-terminal domain of GRP78. Three cancer cell lines that express cell surface GRP78 (i.e., 1-LN, DU145, and A375) were used in this study. Among the three anti–COOH-terminal domain antibodies used, the highest increase in p53 protein levels was observed with antibody A in these cells (Fig. 5A). The other two antibodies also increased p53 protein levels in these
cells, but the increases were of lower magnitude (Fig. 5A). Antibody directed against the NH₂-terminal domain of GRP78 showed a more modest effect on increase in p53 protein levels in these cells (Fig. 5A). Antibody directed against the COOH-terminal domain of GRP78 had no effect on p53 levels in PC-3 cancer cells, which lack expression of GRP78 on their cell surface (Fig. 5A).

**Silencing GRP78 or p53 Expression by RNAi Abrogates the Increase in p53 Protein Levels Caused by Antibodies Directed against the COOH-Terminal Domain of GRP78**

If antibodies directed against the COOH-terminal domain of GRP78 increase p53 levels by down-regulating cell surface–associated GRP78 activity and function, then silencing GRP78 expression by RNAi should abrogate antibody-induced increase in p53 and this was observed (Fig. 5B). Furthermore, if cellular p53 is the direct target of GRP78 down-regulation, then silencing p53 expression by RNAi should also abrogate the induction of p53 by these antibodies and this was observed (Fig. 5C). Antibody A significantly increased p53 in 1-LN, DU145, or A375 cells, and this effect was drastically reduced by silencing either GRP78 or p53 expression by RNAi (Fig. 5B and C).

**Down-Regulation of p-MDM2 Expression in 1-LN Cells Treated with Anti-GRP78 Antibody**

The regulation of p53 activity occurs by its posttranslational modification and through its interactions with various other proteins. Many proteins respond to DNA damage by phosphorylating, acetylating, and methylating the p53 protein, resulting in changes in its activity. MDM2 is a primary regulator of p53 stability. MDM2 binds tightly (Fig. 5B). Furthermore, if cellular p53 is the direct target of GRP78 down-regulation, then silencing p53 expression by RNAi should also abrogate the induction of p53 by these antibodies and this was observed (Fig. 5C). Antibody A significantly increased p53 in 1-LN, DU145, or A375 cells, and this effect was drastically reduced by silencing either GRP78 or p53 expression by RNAi (Fig. 5B and C).
to the NH2-terminal transactivation domain of p53 and blocks expression of p53-regulated genes. During DNA damage, the interaction between p53 and MDM2 is reduced, which allows p53 to accumulate (21, 29). Posttranslational modifications of MDM2 and p53 regulate their interaction. Phosphorylation of MDM2 by Akt at Ser166 and Ser186 activates MDM2 and down-regulates p53 (18). Antibody directed against the COOH-terminal domain of GRP78 significantly decreased phosphorylation of MDM2 and increased p53 and MDM2 protein levels in 1-LN cells treated with α2M* (Fig. 4A and B).

Overexpression of GRP78 in PC-3 Cancer Cells

Transcriptional up-regulation and accumulation of GRP78 in several ER situations is induced by either pathophysiologic and/or pharmacologic treatments (refs. 1, 4 and references therein). However, increase in cellular GRP78 protein levels does not dictate its translocation onto the cell surface. For example, in breast cancer cells, although there is increased cellular GRP78 levels compared with nontransformed cells, GRP78 is not found on cell surface (8). Likewise, PC-3 cancer cells have elevated cellular GRP78 levels compared with nontransformed cells, but it

Figure 4. Antibodies directed against the COOH-terminal domain of GRP78 up-regulate protein levels of components of p53-mediated signaling in 1-LN prostate cancer cells. A, representative immunoblots of three to four experiments showing changes in protein levels of p53[□], phosphorylated p53[☆], acetyl-p53[☆], and p-MDM2[☆] in 1-LN cells treated with (1) buffer, (2) α2M* (50 pmol/L/10 min), (3) anti-GRP78 antibody A (1:200/30 min), and (4) anti-GRP78 antibody A (1:200/30 min) and then α2M* (50 pmol/L/10 min). The changes in levels of these components in groups treated as above are shown in the bar diagram as ratios of the respective protein/actin. Each immunoblot was reprobed for actin, and representative actin immunoblot of each respective protein is shown. B, up-regulation of MDM2 and p21 in 1-LN cells treated with antibody A directed against the COOH-terminal domain of GRP78. Representative immunoblots of two experiments showing changes in protein levels of MDM2 and p21 in 1-LN cells treated with (1) buffer, (2) α2M* (50 pmol/L/10 min), (3) anti-GRP78 antibody A (1:200/30 min), and (4) anti-GRP78 antibody A + α2M*. The changes in the protein levels of MDM2 (□) and p21 (●) are shown in the bar diagram as the ratios of test protein/actin. Each immunoblot was reprobed for actin, and representative actin immunoblot of each respective protein is shown. C, a bar diagram and an immunoblot showing changes in the protein levels of p53 protein in 1-LN prostate cancer cells treated with anti-GRP78 antibody A (1:200/30 min) and antibodies against cell surface-associated chaperones having KDEL COOH-terminal domain. The bars are as follows: (1) buffer, (2) α2M* (50 pmol/L/10 min), (3) anti-GRP78 antibody A (1:200/30 min), (4) anti-HSP70 antibody, (5) anti-HSP90 antibody, (6) anti-calreticulin antibody, and (7) anti-PDI antibody. Representative immunoblot of three to four experiments along with the protein loading control actin is shown.
is not expressed on the cell surface. Here, we have used thapsigargin (1 μmol/L/3 hours) to induce transcriptional up-regulation and cell surface translocation of GRP78 in PC-3 cancer cells. We have determined GRP78 up-regulation by measuring mRNA levels of GRP78 by PCR, GRP78 protein levels by Western blotting, and cell surface expression of GRP78 by cell Western blotting (8). Incubation of PC-3 cells with thapsigargin elevated GRP78 mRNA

![Figure 5](Imagelink)

**Figure 5.** The effects of antibodies generated against the COOH-terminal domain of GRP78 or against the NH$_2$-terminal domain on cancer cells expressing GRP78 on their cell surface. See Materials and Methods for details. A, p53 protein levels in 1-LN, DU145, PC-3, and A375 cancer cells incubated with (1) buffer (□), (2) α₂M* (50 pmol/L; ▪), (3) anti-COOH-terminal domain antibody A (1:200 dilution/30 min; □), (4) antibody directed against the NH$_2$-terminal domain of GRP78 (□), (5) anti-COOH-terminal domain antibody B (1:200 dilution/30 min; □), and (6) anti-COOH-terminal domain antibody C (1:200 dilution/30 min; □). The immunoblots shown are representative of three individual experiments. Each membrane was reprobed for the protein loading control actin and a representative blot of the actin control for each respective protein is shown. Antibody-induced changes in protein levels of p53 are shown as the ratio of p53/actin in a bar diagram. A representative corresponding immunoblot is shown to the left. The lanes in immunoblot are as follows: (1) buffer, (2) α₂M*, (3) anti-GRP78 antibody A, (4) antibody directed against the NH$_2$-terminal domain of GRP78, (5) antibody B, and (6) antibody C. B, down-regulation of the effect of anti-COOH-terminal domain antibody on p53 in 1-LN, DU145, and A375 cancer cells on silencing GRP78 gene expression by RNAi. The bars are as follows: (1) buffer (□), (2) anti-GRP78 antibody A (1:200/30 min; □), and (3) GRP78 dsRNA + antibody A (■). Immunoblots shown are representative of two individual experiments. Each membrane was reprobed for the protein loading control actin, and a representative immunoblot of actin for each of the respective proteins is shown. Antibody-induced changes in p53 protein levels in cancer cells are inhibited by silencing the expression of GRP78 and are shown as the ratio of p53/actin in a bar diagram. Also shown is a representative immunoblot of GRP78 protein in 1-LN cells treated with (1) Lipofectamine + buffer, (2) Lipofectamine + α₂M* (50 pmol/L/15 min), and (3) GRP78 dsRNA (75 nmol/L/48 h) and then α₂M*. C, suppression of the effects of anti-COOH-terminal domain antibody A–induced increase in p53 levels in 1-LN, DU145, and A375 cancer cells on silencing p53 expression by RNAi. The bars are as follows: (1) buffer, (2) anti-GRP78 antibody A (1:200/30 min), and (3) p35 dsRNA + antibody A. Immunoblots shown are representative of two individual experiments. Each membrane was reprobed for the protein loading control actin, and a representative immunoblot of actin for each of the respective proteins is shown. Antibody-induced changes in p53 protein levels in cancer cells are inhibited by silencing p53 expression and are shown as the ratio of p53/actin in a bar diagram. A representative corresponding immunoblot is also shown. The lanes in the immunoblots in B and C are as follows: (1) buffer, (2) anti-GRP78 antibody A, and (3) GRP78 dsRNA or double stranded p53 + antibody A. The bars in B and C are as follows: (1) buffer (□), (2) anti-GRP78 antibody A (□), and (3) double stranded GRP78 or double stranded p53 + anti-GRP78 antibody A (■).
levels by ~2-fold (data not shown), and GRP78 protein levels by 2- to 3-fold (Fig. 6A). Incubation of PC-3 and 1-LN cells, which already express GRP78 on the cell surface, with thapsigargin showed a significant localization of GRP78 on their cell surface (Fig. 6B). In the foregoing sections, we have shown that apoptosis in 1-LN cells is induced by the binding of anti-GRP78 antibodies to cell surface–localized GRP78, resulting in the activation of proapoptotic p53 signaling. To study the role of GRP78 on the cell surface of PC-3 cells treated with thapsigargin, we measured p53 protein levels (Fig. 6C) and apoptosis by the Annexin V method (Fig. 6D). Incubation of PC-3 cells with thapsigargin elevated GRP78 protein levels (Fig. 6A) but had negligible effect on p53 protein level (Fig. 6C). However, incubation of these cells with antibodies against the COOH-terminal domain of GRP78 elevated p53 protein levels by about 2- to 3-fold (Fig. 6C). Elevations in the stability of p53 protein levels should lead to activation of proapoptotic signaling and eventually apoptosis, and indeed, this was observed.

Discussion
In this study, we explored the effect on p53 of down-regulating the function of cell surface–associated GRP78 by antibodies directed against the COOH-terminal domain of GRP78. A necessary role for GRP78 in tumor promotion was first shown by inhibiting GRP78 induction in fibrosarcoma cells with antisense, which rendered them completely

Figure 6. Thapsigargin induces up-regulation of GRP78 and its translocation to the cell surface in PC-3 cells. A, total cellular GRP78 protein in PC-3 cells treated with (1) buffer, (2) α2M* (50 pmol/L/15 min), and (3) thapsigargin (1 µmol/L/3 h). The protein values are shown in arbitrary units (×10⁴). Columns, mean of two individual experiments; bars, SE. A representative immunoblot of GRP78 along with the protein loading control actin is shown below the bar diagram. B, on cell Western blot showing the cell surface expression of GRP78 in 1-LN and PC-3 cells treated with thapsigargin. The wells in each group are as follows: (1) cells treated with buffer, (2) cells treated with thapsigargin (1 µmol/L/3 h), and (3) cells treated with nonimmune IgG. The changes in cell surface expression of GRP78 in 1-LN and PC-3 cells are shown in a bar diagram above the cell Western immunoblot. Columns, mean of six independent experiments; bars, SE. C, total cellular p53 protein levels in PC-3 cells treated with (1) buffer, (2) α2M* (50 pmol/L/3 h), (3) thapsigargin (1 µmol/L/3 h), and (4) thapsigargin (1 µmol/L/3 h) and then antibody directed against the COOH-terminal domain of GRP78 (1:200/2 h). The protein values are shown in arbitrary units (×10⁴). Columns, mean of two individual experiments; bars, SE. A representative immunoblot of p53 along with the protein loading control actin is shown below the bar diagram. D, GRP78 antibody-induced apoptosis in PC-3 cells as measured by the Annexin V method. The changes in apoptotic PC-3 cells treated with (1) α2M* (50 pmol/L/3 h), (2) anti-GRP78 antibody (1:200/3 h), or (3) staurosporine (2 µmol/L/3 h) are expressed as percentage change over basal, which is taken as 100% from two to three individual experiments done in triplicates. Columns, mean of three independent experiments; bars, SE.
incapable of forming tumors in vivo (1, 4, 16). Delivery of a suicide transgene driven by the GRP78 promoter into fibrosarcoma or breast tumor cells also completely blocks formation of sizable tumors in mice (1, 4). A role for GRP78 in the progression of cancer is also suggested by the effects of targeting plasma membrane GRP78 with suicide peptides (4). Reductions in GRP78 levels in heterozygous mice impede tumor progression through multiple mechanisms (3). Thus, down-regulating the levels of cellular GRP78, including that of cell surface–associated GRP78, could be of major importance in cancer therapy. In the current study, we show that cell surface expression of GRP78 in cancer cells is required for observing an effect of antibodies directed against the COOH-terminal domain of GRP78. The major findings of this study are the following. (a) These antibodies inhibit α2M*-induced autophosphorylation and activation of the GRP78 receptor. (b) They inhibit [3H]thymidine uptake and cellular proliferation and induce apoptosis. (c) They up-regulate the tumor suppressor protein p53 and its phosphorylated and acetylated species. (d) Silencing GRP78 or p53 expression by RNAi blocks the ability of anti–COOH-terminal domain antibody to increase levels of p53. (e) The COOH-terminal domain contains the membrane-targeting KDEL sequence (1, 4). Antibodies against other KDEL-containing proteins did not show the effects seen with antibodies directed against the COOH terminus of GRP78. (f) Thapsigargin-induced overexpression of GRP78 in PC-3 cells caused its translocation to the cell surface. This increases p53 protein levels and apoptosis on ligation with anti-GRP78 antibody directed against the COOH-terminal domain.

Traditional approaches to cancer therapy involve surgery, radiation therapy, and chemotherapy. More recent efforts target specific biochemical mechanisms that are essential for the survival of cancer cells, such as apoptotic pathways (1, 30, 31). Using antibodies to affect tumor cell growth and survival has been one of the applications for monoclonal antibodies. A previous study with the fully human monoclonal IgM antibody SAM-6 has specifically shown targeting of a variant cell surface–expressed form of GRP78 as a possible therapeutic approach (17). Here, we show that exposure of high metastatic 1-LN prostate cancer cells to antibody binding to the COOH-terminal domain of GRP78 significantly increases p53-mediated apoptosis and decreases cell proliferation. In many pathophysiologic conditions, the transcription of GRP78 is up-regulated as part of a general cellular defense mechanism—the unfolded protein response (1). The microenvironment of tumors is a pathophysiologic milieu where ER stress is significant and unfolded protein response is crucial for survival of tumor cells subjected to persistent hypoxia (1). Overexpression of GRP78 occurs in many forms of cancer, where it correlates with histologic grade, metastasis, and drug resistance (4, 19, 29). Although GRP78 is an ER resident protein, it is also found on the cell surface where it shows several functions, including serving as a signaling receptor for α2M* (5, 7, 10, 11). In a recent report, we showed that ligation by α2M* of cell surface–associated GRP78 on 1-LN prostate cancer cells promotes survival and proliferation while suppressing apoptosis (11). These events require induction of Ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt, nuclear factor–κB, and unfolded protein response–dependent signaling. Autoantibodies directed against GRP78 are present in the serum of patients with prostate cancer, and these antibodies are a marker for progression and metastasis of these tumors (14, 15). These autoantibodies bind to the same amino acid sequence in the NH2-terminal domain where α2M* binds, and are agonists mimicking the proliferative and antiapoptotic effects seen when α2M* binds to cell surface–associated GRP78 (8).

In the current study, we show that α2M*-induced autophosphorylation of GRP78 was significantly inhibited by an antibody directed against the COOH-terminal domain of GRP78. By blocking receptor activation, the proproliferative and antiapoptotic signaling pathways are aborted in 1-LN prostate cancer cells. Moreover, our data suggest that anti-GRP78 antibody in the absence of α2M* induces down-regulation of GRP78. Our data imply that ligating the COOH-terminal domain of GRP78 must activate different signaling pathways than ligation of the NH2-terminal domain either by α2M* or autoantibodies from the sera of cancer patients because the latter are progrowth and antiapoptotic (8).

Antireceptor antibody-induced down-regulation and degradation of growth factor receptors also occurs for the type I insulin-like growth factor receptor and the epidermal growth factor receptor (32–37). In several cancer cell lines, such antibodies inhibited the binding of growth factors to the receptors and blocked receptor autophosphorylation and downstream cell proliferative signaling (32–37). These results are comparable with what we observed by exposing 1-LN prostate cancer cells to antibody directed against the COOH-terminal domain of GRP78.

Similar behavior also has been reported for antibodies to other cell surface resident proteins (38–43). We do not precisely understand the mechanism by which antibodies against the two terminal domains of GRP78 elicit two opposite cellular responses. GRP78 consists of a 44-kDa NH2-terminal domain, which is responsible for GRP78 ATPase activity, a 20-kDa COOH-terminal binding domain, and a variable 10-kDa COOH-terminal “tail” of unknown function (44). In the absence of a transmembrane domain in GRP78, it is translocated to the plasma membrane in association with the co-chaperone MTJ-1 (6). GRP78 itself associates into multiple oligomeric species (45, 46). The COOH-terminal domain of GRP78 is responsible for the oligomeric properties of the protein (46). Binding of unfolded peptide substrates to the COOH-terminal domain or binding of ATP to the NH2-terminal domain promotes depolymerization and stabilization of GRP78 monomer (46). Recently, it has been shown that binding of Dengue virus serotype 2 with the cell surface–associated GRP78 requires interaction with both the NH2- and COOH-terminal domains of GRP78 antibodies A directed against either domain altered the binding of the virus to the cell surface (47), showing thereby that both the NH2 and COOH termini of GRP78 are accessible.
at the cell surface. Differences in the responses elicited after binding of antibodies directed against the COOH-terminal as opposed to NH2-terminal domain of GRP78 may lie in the nature of the conformational changes induced by binding of these antibodies to their target.

In conclusion, we show that the down-regulation of cell surface-associated GRP78 by antibodies directed against the COOH-terminal domain of GRP78 blocks α2M* binding to cell surface-associated GRP78, inhibits cell surface-associated GRP78 receptor activation, and down-regulates cell survival and proliferative signaling observed in cells treated with α2M*. Furthermore, this antibody directly promotes the induction of apoptotic signaling, primarily mediated by p53, resulting in growth inhibition and cell death.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Molecular Cancer Therapeutics

Ligation of cancer cell surface GRP78 with antibodies directed against its COOH-terminal domain up-regulates p53 activity and promotes apoptosis

Uma Kant Misra, Yvonne Mowery, Steven Kaczowka, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-08-0990

Cited articles
This article cites 47 articles, 21 of which you can access for free at:
http://mct.aacrjournals.org/content/8/5/1350.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/8/5/1350.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.