Critical Role of the Stress Chaperone GRP78/BiP in Tumor Proliferation, Survival, and Tumor Angiogenesis in Transgene-Induced Mammary Tumor Development

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Abstract

The unfolded protein response (UPR) is an evolutionarily conserved mechanism that activates both prosurvival and survival pathways to allow eukaryotic cells to adapt to endoplasmic reticulum (ER) stress. Although the UPR has been implicated in tumorigenesis, its precise role in endogenous cancer remains unclear. A major UPR protective response is the induction of the ER chaperone GRP78/BiP, which is expressed at high levels in a variety of tumors and confers drug resistance in both proliferating and dormant cancer cells. To determine the physiologic role of GRP78 in in situ-generated tumor and the consequence of its suppression on normal organs, we used a genetic model of breast cancer in the Grp78 heterozygous mouse where GRP78 expression level was reduced by about half, mimicking anti-GRP78 agents that achieve partial suppression of GRP78 expression. Here, we report that Grp78 heterozygosity has no effect on organ development or antibody production but prolongs the latency period and significantly impedes tumor growth. Our results reveal three major mechanisms mediated by GRP78 for cancer progression: enhancement of tumor cell proliferation, protection against apoptosis, and promotion of tumor angiogenesis. Importantly, although partial reduction of GRP78 in the Grp78 heterozygous mouse substantially reduces the tumor microvessel density, it has no effect on vasculature of normal organs. Our findings establish that a key UPR target GRP78 is preferably required for pathophysiologic conditions, such as tumor proliferation, survival, and angiogenesis, under-scoring its potential value as a novel therapeutic target for dual antitumor and antiangiogenesis activity. [Cancer Res 2008; 68(2):498–505]

Introduction

The endoplasmic reticulum (ER) is an essential cellular organelle where secretory and membrane proteins are synthesized and modified and is also a major intracellular Ca²⁺ storage compartment. Recent discoveries further point to the ER as a novel focal point for the regulation of apoptosis (1, 2). The unfolded protein response (UPR) triggers multiple pathways to allow cells to respond to ER stress (3, 4). The UPR can be protective through activation of adaptive, antiapoptotic pathways as well as commit cells to apoptosis under severe stress. Cancer cells exhibit elevated glucose metabolism and are often exposed to tumor hypoxia, resulting in ER stress (5–7). Despite studies in xenograft models linking the UPR to tumor growth, the precise role of the UPR in the development of endogenous cancer remains unclear (5, 8–11).

The ER chaperone GRP78/BiP with antiapoptotic properties is a central regulator of ER homeostasis, and its up-regulation is widely used as a sentinel marker for ER stress under pathologic conditions (7, 12–14). GRP78, also referred to as BiP [immunoglobulin (Ig) heavy chain binding protein], is a member of the HSP70 protein family that resides primarily in the ER (13–16). As a major ER chaperone, it facilitates protein folding, preventing intermediates from aggregating, and targeting misfolded protein for proteasome degradation. GRP78 also binds Ca²⁺ and serves as an ER stress signaling regulator (3, 12, 13).

GRP78 induction occurs during embryonic development and has been widely reported in human cancer (17, 18). In cancer cell lines, GRP78 promotes survival and chemoresistance in both proliferating and dormant tumor cells (16, 18, 19) and has also been implicated in proliferation and invasion through activation of the Akt and PAK2 pathways (20–22). Autoantibodies against GRP78 in patient sera correlate with aggressive tumor behavior, and retrospective studies revealed that high-level GRP78 expression predicts poor survival for cancer patients (7, 23). Given the importance of GRP78 in cancer cell survival, it is a prime target for discovery of anticancer agents. However, because GRP78 controls UPR signaling that has both prosurvival and proapoptotic pathways, down-regulation of GRP78 may result in premature activation of the UPR. This raises the critical questions: Is this beneficial or harmful to tumor progression, and how would this affect normal organs and tissues?

To address this, we used the Grp78 heterozygous mouse model where the basal level and ER stress induction of GRP78 has been determined to be about half of wild-type (WT) level (24), thus mimicking anti-GRP78 agents that achieve partial suppression of GRP78 expression. In contrast to Grp78 homozygous knockout, which results in lethality due to proliferative defect and massive apoptosis of the inner cell mass of 3.5-day-old mouse embryos (24), the Grp78/+ mice are viable and fertile. Thus, on breeding with a transgenic mouse model of cancer, the Grp78 heterozygous mice provide a unique opportunity to examine the physiologic role of GRP78 in in situ-generated tumor progression compared with normal organ development. Here, we report that reduction in GRP78 level by about half in the Grp78 heterozygous mice has no effect on organ development or antibody production but...
significantly impedes tumor progression through multiple mechanisms. Our findings provide direct evidence that a major protecti

tive arm of the UPR, GRP78/Bip, is critical for tumor proliferation, survival, and angiogenesis, underscoring its value as a novel thera
tic target for dual antitumor and antiangiogenesis activity.

Materials and Methods

Generation of mammary tumors in genetically altered Grp78 mice and monitoring of tumor growth. The generation of the Grp78+/− mice has been described (24). Female Grp78+/− mice were mated with male MMTVPyVT heterozygous transgenic mice (25). The progenies were genotyped by PCR of tail DNA and monitored for tumor growth and incidence. The experiment on tumor growth was done in two phases. Seven Grp78+/−×PyT mice and six Grp78−/−×PyT mice were used in the initial phase. Starting 8 weeks of age, mammary tumors were detected. The primary tumor diameters were measured with a caliper weekly. The second phase was a replication of the first one, with two modifications: (a) the sample size in phase 2 was 15 mice in each group, and (b) both the width and the length of the tumor were measured in phase 2. Tumor volume was calculated using the following equation: volume = width² × length × 0.5. Following sacrifice at the end of the experiment, organs and tumors were harvested for analysis. All animal protocols were conducted with the approval of the University of Southern California Animal Care and Use Committee. The numbers of mice with tumors at each week from both phases are shown in Fig. 2A and the tumor growth curves in the second phase are shown in Fig. 2B.

Immunization and determination of Ig titers. Six- to 8-week-old Grp78+/− and littermate Grp78+/+ mice were immunized by i.p. injections of 100 μg trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH; Biosearch Technologies) in Imject alum (Pierce). TNP-KLH-immunized mice were given a booster 21 days later. Serum samples were collected before immunization and on day 7 after TNP-KLH reimmunization. Basal Ig levels in sera were quantified using Beadlyte mouse Ig isotyping kit (Millipore) following the manufacturer's protocol. To measure relative TNP-specific antibody levels, the same isotyping kit combined with Beadlyte biotin-conjugated TNP ovalbumin (Millipore) and Beadlyte streptavidin-phycoerythrin (Millipore) was used following the manufacturer's protocol.

Derivation of cell culture from tumor tissues. Primary culture of tumor cells was established as described (26). Briefly, tumor tissues were cut into fine pieces and seeded onto 6-cm-diameter culture dishes and cultured at 37°C with 5% CO2 in 1 ml of 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. After overnight culturing, an additional 2 ml medium was added. After 5 days, the tumor tissues were removed from the culture dish, and the attached cells were allowed to grow. After expansion, the cells were seeded for immunohistochemical staining and growth rate measurements. For growth measurements, the cells were stained with trypan blue and counted every 2 days until day 9.

Immunohistochemical and immunofluorescent staining. Immunohistochemical staining was performed on paraffin-embedded tumor sections (4 μm) or cell culture in a chamber slide (Nalge Nunc International). Vectastain Elite avidin-biotin complex kit (Vector Laboratories) was used for immunohistochemistry, and fluorescein or Texas red conjugated anti-rabbit or mouse IgG (H+L; Vector Laboratories) for immunofluorescent staining. Primary antibodies against Pan-cytokeratin (rabbit, 1:50), Grp78 (rabbit, 1:100), CHOP (mouse, 1:200) and proliferating cell nuclear antigen (PCNA; mouse, 1:50) were from Santa Cruz Biotechnology. Antibody against human vimentin (mouse, 1:100) was from Chemicon International. ProLong Gold antifade mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) and aqueous mounting medium were from Vector Laboratories.

Immunohistochemical staining was carried out as described previously (27). Immunofluorescent staining of cultured cells was performed on cells plated in a chamber slide. The cells were fixed with methanol for 30 min at −20°C. Three washes with PBS were followed by permeabilization with 0.1% Triton X-100 in PBS (v/v) for 10 min at room temperature and blocking for 60 min at room temperature with 1% bovine serum albumin in PBS (w/v). The treated cells were incubated with the first primary antibody at 4°C overnight in a humidified chamber. After washing with PBS thrice, first secondary antibody conjugated with fluorescein or Texas red was added at a final concentration of 10 μg/ml and incubated for 60 min at room temperature. For immunofluorescent double staining, the cells were washed with PBS thrice and the above staining procedure was repeated for the second primary and second secondary antibodies, respectively. The stained cells were mounted with aqueous mounting medium or ProLong Gold antifade mounting medium with DAPI. The processed cells were visualized using a Leica DM LB2 fluorescence microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. In Situ Cell Death Detection kit, TMR red (Roche Applied Science) was used and the sections of paraffin-embedded tumor were stained following the protocol provided by the manufacturer. The tumor sections were mounted with ProLong gold antifade mounting medium with DAPI. The apoptotic cells were visualized using a fluorescence microscope.

Microvessel density measurement. Frozen mouse tissues (tumor, kidney, heart, and brain) were sectioned at 5 μm and fixed with acetone and tissue sections were stained overnight with rat anti-mouse CD31 antibody (BD PharMingen) followed by biotinylated anti-rat antibody (Vector Laboratories) for 45 min as previously described (28). Tissues were then treated with avidin-biotin peroxidase complex (Vector Laboratories) for 30 min, and the aminosilane carbodiimide solution (Vector Laboratories) for 10 min. Counterstaining was performed with hematoxylin. The red precipitate denotes positive staining. Isotype-matched serum was used in place of the primary antibody for staining controls. Positive staining was quantified using the imaging processing program ImageJ (NIH). Tumor microvessel density (MVD) staining was analyzed in six mice, three from each genotype, and the evaluation was performed on five random fields per tumor. Organ MVD staining was analyzed in a pair of Grp78 WT and heterozygous mice from the same group, with five random fields examined per tissue.

Western blots. Pieces of tumors dissected from mice were homogenized with a Dounce homogenizer in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1% NP0, 0.5% deoxycholate, 0.1% SDS, 10% glycerol, protease inhibitors] to lyse cells followed by centrifugation at 14,000 × g at 4°C for 10 min. The supernatant obtained from each sample was subjected to immunoblot. Protocols for Western blot analysis have been described (16). The primary antibodies used were the following: rabbit anti-GRP78 (1:1,000), rat anti-poliovirus early antigens (1:1,000), mouse anti-CHOP (1:1,000), rabbit anti-ATF6 (1:1,000), and rabbit anti-ATF4 (1:1,000) from Santa Cruz Biotechnology; mouse anti-β-actin (1:5,000) from Sigma-Aldrich; mouse anti-caspase-7 (1:4,000) from BD PharMingen; and rabbit anti-cleaved caspase-3 (1:1,000) and anti-phosphorylated eIF2α and rabbit anti-eIF2α (1:1,000) from Cell Signaling. The experiments were repeated two to three times.

Xbp-1 splicing measurement. Total RNA from tumor samples was isolated using Trizol reagent (Invitrogen), and first-strand cDNA was synthesized with SuperScript II (Invitrogen). To detect both spliced and unspliced forms of Xbp-1, nested PCR was performed. The primers used for first-round PCR were as follows: 5′-TAGAAAGAAACCCGCGATGA (forward) and 5′-AAGGGAGGCTGGTAAGGAA (reverse). PCR primers for the second round were as follows: 5′-GAAGCCAGGTATAAAGACC (forward) and 5′-AGGGCAACAGTGTGAGTCCC (reverse).

Statistical analysis. The tumor volumes of the Grp78 WT and heterozygous mice were compared using the random coefficient model where the intercept and the slope for each mouse were treated as random. The tumor volumes were monitored in two experiments. Before analysis, the cell numbers were normalized so that all cell lines started at the same level with the number of
cells being $2 \times 10^5$ and log transformed. ANOVA was performed to compare the cell numbers on day 9 between the cell lines from Grp78 WT and heterozygous tumors after adjusting for the experiment. The MVD in tumors and organs of heterozygous mice was also compared with that of WT mice using ANOVA after log transforming the data. The means and 95% confidence interval (95% CI) were calculated on the log-transformed data and then transformed back to the original scale.

**Results**

**Grp78 heterozygous mice exhibit normal growth, organ development, and antibody production.** Both male and female Grp78+/− mice grew at the same rate as their WT siblings (Fig. 1A) and the size, morphology, and histology of their major organs were comparable with those of WT (Fig. 1B; data not shown). As the Ig binding protein, GRP78 is highly abundant in plasma cells where it stabilizes Ig chains and facilitates their assembly (13). Analysis of preimmune serum IgM and various subclasses of IgG antibody levels yielded no significant difference between the WT and heterozygous mice (Fig. 1C). After immunization with the antigen TNP-KLH, the relative serum levels of IgG1 and IgG2b specific to TNP remained similar (Fig. 1D). Thus, about half of WT GRP78+/− mice were crossed with the transgenic mice (MMTVPyVT) expressing the mammary tumor viral promoter (25). Cohorts of Grp78+/, PyT, Grp78+/-, PyT, Grp78+/-, and Grp78+/- mice were monitored for 15 weeks. Statistical analysis indicated that the tumor growth was significantly reduced in the Grp78+/-,PyT mice compared with the Grp78+/,PyT mice (P < 0.001; Fig. 2B), with the mean tumor volume at week 15 reduced from 201 mm$^3$ for Grp78+/,PyT mice to 92 mm$^3$ for Grp78+/- mice, a reduction of 109 mm$^3$ (95% CI, 60–159 mm$^3$). Therefore, partial reduction of a single UPR target, GRP78, is sufficient to impede tumor growth and reduce tumor size by 60%. In this mammary tumor model, which is noted for its high penetrance, the mice also developed smaller tumors in subsequent weeks. The size and number of these tumors were also reduced in the Grp78 heterozygous mice (data not shown).

**Grp78 heterozygosity prolongs the latency period and impedes mammary tumor growth.** To determine the role of GRP78 on endogenous tumor growth, the Grp78+/- mice were crossed with the transgenic mice (MMTVPyVT) expressing the polyoma middle T oncogene (PyT) driven by the murine mammary tumor viral promoter (25). Cohorts of Grp78+/, PyT, Grp78+/-, PyT, Grp78+/-, and Grp78+/- mice were monitored for the time of appearance and size of the primary mammary tumors. In the Grp78+/-,PyT mice, most tumors were first detectable between week 8 and 10, whereas most tumors in the Grp78+/-,PyT mice became detectable between week 10 and 12 (Fig. 2C). Thus, the latency period was delayed for ~2 weeks in the heterozygous Grp78 mice. As expected, neither the Grp78 WT nor heterozygous mice without the PyT allele developed any tumor (Fig. 2C). The growth of the primary tumors in mice bearing the PyT oncogene was monitored for 15 weeks. Statistical analysis indicated that the tumor growth was significantly reduced in the Grp78+/-,PyT mice compared with the Grp78+/,PyT mice (P < 0.001; Fig. 2B), with the mean tumor volume at week 15 reduced from 201 mm$^3$ for Grp78+/,PyT mice to 92 mm$^3$ for Grp78+/- mice, a reduction of 109 mm$^3$ (95% CI, 60–159 mm$^3$). Therefore, partial reduction of a single UPR target, GRP78, is sufficient to impede tumor growth and reduce tumor size by 60%. In this mammary tumor model, which is noted for its high penetrance, the mice also developed smaller tumors in subsequent weeks. The size and number of these tumors were also reduced in the Grp78 heterozygous mice (data not shown).

**Grp78 heterozygosity inhibits tumor proliferation and promotes apoptosis.** Toward understanding the mechanisms that contribute to the slower growth rate of tumors of the Grp78+/-,PyT mice, tumor sections were analyzed. H&E staining of paraffin-embedded tumor sections revealed that whereas oncogene was monitored for 15 weeks. Statistical analysis indicated that the tumor growth was significantly reduced in the Grp78+/-,PyT mice compared with the Grp78+/,PyT mice (P < 0.001; Fig. 2B), with the mean tumor volume at week 15 reduced from 201 mm$^3$ for Grp78+/,PyT mice to 92 mm$^3$ for Grp78+/- mice, a reduction of 109 mm$^3$ (95% CI, 60–159 mm$^3$). Therefore, partial reduction of a single UPR target, GRP78, is sufficient to impede tumor growth and reduce tumor size by 60%. In this mammary tumor model, which is noted for its high penetrance, the mice also developed smaller tumors in subsequent weeks. The size and number of these tumors were also reduced in the Grp78 heterozygous mice (data not shown).

**Grp78 heterozygosity preferably affects tumor growth.** In contrast, the size and morphology of major organs in all four groups of mice (Grp78+/,PyT, Grp78+/-, PyT, Grp78+/-, and Grp78+/-) were comparable (Fig. 2C). The organ weights of the Grp78+/+ and Grp78+/- mice were also similar (Fig. 2D). Thus, Grp78 heterozygosity preferably affects tumor growth.

**Grp78 heterozygosity inhibits tumor proliferation and promotes apoptosis.** Toward understanding the mechanisms that contribute to the slower growth rate of tumors of the Grp78+/-,PyT mice, tumor sections were analyzed. H&E staining of paraffin-embedded tumor sections revealed that whereas ...
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next, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed. In Grp78 WT tumors, there were very few apoptotic tumor cells; in contrast, tumor cells from the Grp78 heterozygous mice showed enhanced apoptosis in general (Fig. 3D, top). Further, in some regions of the Grp78+/−/PyT tumors, strong immunohistochemical staining of CHOP was observed, indicative of ER stress-induced apoptosis (Fig. 3D, bottom).

Tumor cells derived from the Grp78+/−/PyT mice grow slower in vitro. Primary tumor cells from the Grp78 WT and heterozygous mice were propagated in vitro. This allows us to examine whether decreased proliferation of the Grp78+/−/PyT tumor cells is an intrinsic property of the epithelial tumor cells with reduced GRP78 expression or due to alterations in the tumor microenvironment in the Grp78 heterozygous mice. Direct comparison of the proliferative rates of the cells in culture will eliminate the extrinsic factors such as tumor hypoxia and angiogenesis. The decrease in proliferation of tumor cells from the Grp78+/−/PyT group was evident from substantially lower PCNA labeling compared with the Grp78+/+,PyT group (Fig. 4A, top). The identity of the epithelial tumor cells in each group was further confirmed by costaining with fluorescent anti-keratin antibody (Fig. 4B, bottom). To confirm the staining results, the growth rates of multiple primary cell lines derived from Grp78+/+,PyT and Grp78+/−/PyT tumors were directly monitored (Fig. 4B). The growth rates began to diverge after 5 days of culture. By 9 days of culture, the mean number of tumor cells was significantly decreased from $8.9 \times 10^4$ for the Grp78+/+,PyT group to $6.3 \times 10^4$ for the Grp78+/−/PyT group, a 29% reduction ($P = 0.048$). Thus, the reduction in proliferation rate is an intrinsic property of the tumor cells of the heterozygous group rather than a consequence of the tumor microenvironment.

Grp78 heterozygosity up-regulates CHOP and caspases in tumors. The detection by immunohistochemistry that CHOP was induced in some areas of the tumor from the Grp78 heterozygous mice indicates that those tumor cells were unable to adapt to ER stress. As an ER stress-inducible transcription factor, CHOP has been reported to induce apoptotic cell death by promoting protein synthesis and oxidation in the stressed ER (29). To extend these observations, Western blot analysis was performed on cell lysates of tumors from the Grp78 WT and heterozygous mice. Tumors of different sizes were examined, and in general, tumors from the Grp78 heterozygous mice showed lower level of GRP78, confirming results obtained from immunohistochemical staining of tumor sections (Fig. 5A and B). The level of the polyoma middle T antigen (PyT) was similar or slightly higher in the Grp78+/−/PyT tumors (Fig. 5A), ruling out the possibility that decreased GRP78 may negatively affect PyT production. CHOP induction was observed in Grp78+/−/PyT tumors, which expressed the lowest level of GRP78 (Fig. 5C). GRP78 is known to bind procaspase-7 (C-7) and block its activation (16, 30, 31). Interestingly, strong activation of C-7 was also observed in large Grp78+/−/PyT tumors. Caspase-3 activation, as evidenced by cleavage products, was generally observed in Grp78+/−/PyT tumors and in large Grp78+/−/PyT tumors (Fig. 5C). The large tumors, in general, displayed necrotic regions, which were not observed in medium- or small-sized tumors (data not shown).

Next, we addressed the issue whether reduction of GRP78 level by about half in tumors from the Grp78 heterozygous mice alters the UPR signaling pathways, as GRP78 regulates their activation (3, 4, 13). ATF6 activation is examined by detection of its cleavage component (Fig. 3A). The epithelial origin of the tumor cells was confirmed by the demonstration of positive immunofluorescence staining with anti-keratin antibody (depicted by cytosolic green staining) in these cells, whereas immunofluorescence positivity for vimentin, a mesenchymal cell marker, was confined to fibroblasts (depicted by cytosolic red staining), which were more prevalent in the Grp78+/−/PyT tumors (Fig. 3B).

Sections of tumors from the Grp78 WT and heterozygous mice were further subjected to PCNA staining to determine whether Grp78 heterozygosity affects tumor proliferation. Strikingly, cell proliferation was substantially reduced in the Grp78+/−/PyT tumors that expressed lower levels of GRP78, as revealed by immunohistochemical staining (Fig. 3C). The antiapoptotic property of GRP78 predicts that lower GRP78 expression level in the Grp78+/−/PyT tumors could result in enhanced apoptosis. To test this, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed. In Grp78 WT tumors,
products in Western blot analysis. Compared with the positive control where the cells were treated with the classic ER stress inducer thapsigargin, ATF6 activation in both sets of tumors was negligible, albeit a slightly higher level in the Grp78+/−.PyT tumors in general (Fig. 5C). Due to technical difficulty, we were unable to assay directly for PERK or IRE activation in the tumor samples; however, we were able to monitor for activation of their downstream targets. Interestingly, phosphorylated eIF2α, a downstream target of PERK, was detected in Grp78+/+.PyT but not Grp78+/−.PyT tumors. The level of ATF4, another PERK target, was either not affected or slightly lower in the Grp78+/−.PyT tumors (Fig. 5C). Activation of the IRE1 pathway leads to Xbp-1 splicing. In tumors from the Grp78 WT or heterozygous mice, the level of Xbp-1 transcript was the same and no splicing was detected (Fig. 5D). Thus, although it remains possible that these pathways were affected earlier during tumor development, our results suggest that the phenotypes observed in the Grp78+/−.PyT tumors are independent of sustained activation of these UPR pathways.

**Grp78 heterozygosity inhibits tumor angiogenesis.** Another mechanism that may contribute to the slower growth of the Grp78+/−.PyT tumors is that GRP78 is required for tumor angiogenesis. To test this, tumor sections from the Grp78 WT and heterozygous mice were analyzed for MVD through staining with the antibody to the endothelial cell marker anti-CD31. In contrast to the well-vascularized Grp78+/+.PyT tumors, Grp78+/−.PyT tumors showed a dramatic reduction in MVD (Fig. 6A). Quantitation of the stained tumor vasculature revealed a decrease in the MVD from 9.1 μm² per field for the Grp78+/+.PyT tumors to 2.7 μm² for the Grp78+/−.PyT tumors, a 70% reduction (95% CI, 46–83%; P = 0.038; Fig. 6B). In contrast, the vasculature in organs and tissues from the same mice, including brain, heart, and kidney, was not affected (Fig. 6C; data not shown). These results reveal that GRP78 is preferably required for tumor angiogenesis.

**Discussion**

The emerging importance of the stress response and molecular chaperones in oncogenesis is well recognized (15, 18, 32). Compared with normal tissue, tumor cells are subject to ER stress due to elevated glycolytic activity and, in fasting growing solid tumors, inadequate blood vessel, creating a microenvironment of glucose deprivation, acidosis, and hypoxia. These combined factors lead to ER stress and the trigger of UPR adaptive responses. Recent studies using xenograft models suggest that UPR pathways may play an important role for tumor survival and angiogenesis under hypoxic conditions, conferring growth advantage to the tumor cells (5, 8, 9, 33). However, the UPR could also exert opposing effects (11, 34). Therefore, it is important to examine the role of UPR in the context of endogenous tumor development, which more closely mimics human cancer.

A prominent UPR protective response is the transcriptional activation of the ER stress chaperone GRP78, which is known to confer resistance to a variety of cancer therapy in both proliferating and dormant cancer cells in culture (7, 16, 18, 19, 22, 35). A prerequisite toward understanding the physiologic role of GRP78 in endogenous cancer requires a model organism with altered GRP78 expression. The creation of novel mouse strains with genetic disruption of the Grp78 allele affords this opportunity (24). In this report, using a genetic model of breast cancer in the Grp78 heterozygous mice, we provide direct evidence that partial suppression of GRP78 level delays the latency period and significantly inhibits *in situ*-generated tumor growth and tumor angiogenesis. In contrast, partial GRP78 level is sufficient for organ development and cellular immunity.

These observations raise several important issues. How might GRP78 confer growth advantage to the tumor cells? Our results reveal three major mechanisms mediated by GRP78: enhancement of tumor cell proliferation, protection against apoptosis, and...
promotion of tumor angiogenesis. With regard to cell proliferation, as a major ER chaperone, GRP78 may facilitate the processing and trafficking of critical growth factors and their receptors (36). Moreover, GRP78 can be detected on the surface of tumor cells (21, 30, 37). As part of a cell surface receptor complex, it is proposed that GRP78 mediates the Akt signal transduction pathway that induces tumor cell proliferation (21). Our results with the Grp78 heterozygous mice validate a novel role of GRP78 in tumor cell proliferation. With regard to apoptosis, we show that, in tumors where GRP78 expression level is reduced, there is specific induction of CHOP and activation of executive caspase-3 and caspase-7. In tissue culture systems, GRP78 has been shown to suppress CHOP induction and ER stress–induced apoptosis and that GRP78 is an interactive partner of caspase-7 and blocks its activation under stress (16, 22, 30, 31, 38).

Tumor angiogenesis plays a critical role in tumor growth and is a key target for anticancer therapy (39, 40). Interestingly, the tumor vasculature is physiologically and functionally distinct from blood vessels in normal organs (28, 41). In contrast to normal endothelial cells, primary tumor endothelial cells proliferate more slowly and are much more resistant to cytotoxic drugs. Proteomics of human endothelial cells suggests that GRP78 inhibits endothelial cell apoptosis induced by topoisomerase inhibitors (42). Thus, it is possible that the antiapoptotic properties of GRP78 protect tumor as well as tumor-associated endothelial cells against stress in the tumor microenvironment. Further, as an ER chaperone, GRP78 may be critical for processing client proteins essential for tumor vasculature formation. Cell surface GRP78 expression in
endothelial cells could also mediate their proliferation. In support of a potential role of GRP78 in angiogenesis, expression of angiogenic factors (vascular endothelial growth factor and interleukin-8) is highly responsive to ambient glutamine deprivation, which induces GRP78 (43). Although the precise mechanisms on the reduction in tumor MVD in the tumors of the Grp78 heterozygous mice await future investigations, it could be due to combinatorial effects of deficient angiogenic factor production, reduced proliferation, and enhanced apoptosis. Our discovery that GRP78 is preferably required for the maintenance of the tumor vasculature supports the notion that GRP78 function is most critical under pathophysiologic conditions in adult animals.

On the translational front, stress chaperones represent exciting therapeutic targets for cancer based on their multiple and essential roles in tumor growth (44). Recent retrospective studies reveal that high GRP78 expression level in cancer biopsies is closely associated with poor prognosis and resistance to therapy (18, 45–47). Earlier xenograft studies showed that down-regulation of GRP78 by antisense inhibits xenograft formation and tumor growth, providing the first hint that suppressing GRP78 expression is a promising new anticancer approach (10). Our findings with the Grp78 heterozygous mice validate the earlier results and further establish GRP78 as a novel therapeutic target for dual suppression of tumor growth and angiogenesis. Down-regulation of GRP78, therefore, may become an important adjunct in future cancer treatment. Recent developments of small molecules that can block GRP78 and/or its activity may make this approach clinically feasible in the near future (7). For example, a microbial compound, versipelostatin, which inhibits induction of GRP78 under glucose starvation conditions and disrupts some components of the UPR, selectively kills glucose-deprived cancer cells and acts synergistically with cisplatin in inhibiting tumor growth in xenografts (48). Recently, it was shown that (−)-epigallocatechin gallate, an active component from green tea, directly interacts with the ATP-binding domain of GRP78, blocking its interaction with C-7, thereby inhibiting its antiapoptotic function and sensitizing cancer cells to chemotherapy (31). Another agent currently in clinical trials is recombinant human plasminogen Kringle 5 (ABT 828). Preclinical studies revealed that GRP78 is the receptor for Kringle 5, which induces apoptosis of both fibrosarcoma and growth-stimulated endothelial cell–specific rat antimouse CD31 antibody and lightly counterstained with H&E. Reddish brown, positive staining; blue, nuclei. A representative tumor section stained with CD31. Bar, 100 µm. B, quantitation of the MVD in tumors from the Grp78 WT and heterozygous mice. One unit is defined as 1 µm² for one ×200 magnification field. The difference in vessel density is significant (P = 0.038). Columns, mean MVD; bars, 95% CI. The results are representative of six animals examined. C, staining of organs with H&E (top) or CD31 (bottom). Bar, 100 µm. D, quantitation of MVD in the indicated organs (brain, heart, kidney). Columns, mean MVD; bars, 95% CI.
cells (30). It is proposed that, following internalization, Kringel 5 blocks intracellular GRP78 binding to C-7, suppressing its antiapoptotic function. With GRP78 playing critical roles in tumor proliferation, survival, angiogenesis, and drug resistance of both proliferating and dormant cancer cells, as well as tumor-associated endothelial cells (7, 18, 19, 32), combination of drugs capable of GRP78 suppression with conventional therapy may represent a novel approach toward eradication of residual tumor, which remains a major challenge in cancer therapy.

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References

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