Microenvironment and Immunology

A Critical Role for GRP78/BiP in the Tumor Microenvironment for Neovascularization during Tumor Growth and Metastasis

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Abstract

Glucose-regulated protein 78 (GRP78)/BiP is a multifunctional protein which plays a major role in endoplasmic reticulum (ER) protein processing, protein quality control, maintaining ER homeostasis, and controlling cell signaling and viability. Previously, using a transgene-induced mammary tumor model, we showed that Grp78 heterozygosity impeded cancer growth through suppression of tumor cell proliferation and promotion of apoptosis and the Grp78+/− mice exhibited dramatic reduction (70%) in the microvessel density (MVD) of the endogenous mammary tumors, while having no effect on the MVD of normal organs. This observation suggests that GRP78 may critically regulate the function of the host vasculature within the tumor microenvironment. In this article, we interrogated the role of GRP78 in the tumor microenvironment. In mouse tumor models in which wild-type (WT), syngeneic mammary tumor cells were injected into the host, we showed that Grp78+/− mice suppressed tumor growth and angiogenesis during the early phase but not during the late phase of tumor growth. Growth of metastatic lesions of WT, syngeneic melanoma cells in the Grp78+/− mice was potently suppressed. We created conditional heterozygous knockout of GRP78 in the host endothelial cells and showed severe reduction of tumor angiogenesis and metastatic growth, with minimal effect on normal tissue MVD. Furthermore, knockdown of GRP78 expression in immortalized human endothelial cells showed that GRP78 is a critical mediator of angiogenesis by regulating cell proliferation, survival, and migration. Our findings suggest that concomitant use of current chemotherapeutic agents and novel therapies against GRP78 may offer a powerful dual approach to arrest cancer initiation, progression, and metastasis. Cancer Res; 71(8); 1–10. ©2011 AACR.

Introduction

Glucose-regulated protein 78 (GRP78), also referred to as BiP or HSPA5, is a member of the HSP70 protein family with established function as an endoplasmic reticulum (ER) chaperone protein and a regulator of the ER stress signaling pathway (1). As a multifunctional protein, GRP78 plays critical roles in physiologic and pathologic stress (2). GRP78 is induced in a wide variety of tumors through intrinsic factors such as altered glucose metabolism in cancer cells, compounded by extrinsic factors such as glucose starvation and hypoxia in the microenvironment of poorly perfused solid tumors (3). The induction of GRP78 leads to an increase of Grp78 in the ER compartment and promotion of GRP78 to the cell surface, where it assumes a new function of coreceptor for cell-surface signaling (4–7). Although a large number of studies have established that GRP78 is required for tumor cell proliferation, survival, and therapeutic resistance (3, 8, 9), little is known about the role of GRP78 in the tumor microenvironment, which is critical for support of tumor growth and metastasis.

Previously, we established a heterozygous knockdown mouse model of GRP78 as homozygous deletion of Grp78 leads to early embryonic lethality (10). The heterozygous Grp78+/− mice, despite reduced expression of GRP78 to about half the wild-type (WT) level in all the tissues, are phenotypically normal with respect to organ development and antibody production (11). However, through breeding of
the WT and Grp78\(^{+/−}\) mice with the transgenic mice expressing the middle T oncogene driven by the murine mammary tumor viral promoter, we discovered that Grp78 heterozygosity prolonged the latency period and significantly impeded cancer growth by suppressing tumor cell proliferation and promoting tumor cell apoptosis (11). Strikingly, the microvessel density (MVD) of the endogenous tumors in the Grp78\(^{+/−}\) mice was reduced by 70%, while the vasculature of normal organs remained unaffected. This observation provided the initial evidence that Grp78 may selectively regulate the function and survival of endothelial cells associated with the tumor.

The tumor microenvironment contains a plethora of cells that support tumor growth and progression. Tumor vasculature is essential for tumor growth and metastasis, as it supplies the nutrients and oxygen crucial for the growth and maintenance of the tumor (12, 13). Endothelial cells are therefore the requisite members of the tumor microenvironment. Previous studies on human glioma-derived endothelial cells have shown that these tumor-associated endothelial cells possess unique functional characteristics, including an activated phenotype (14). Tumors require an activated endothelial population to provide a constant new blood vessel formation at the growing border of the tumor, which allows for continued expansion and progression. Our recent investigations have also indicated that GRP78 expression is constitutively elevated within the tumor vasculature, emphasizing the constant state of activation of these tumor-associated endothelial cells (15).

In this report, we tested the role of GRP78 in host cells supporting tumor growth by using syngeneic cancer models. Our studies revealed that neovascularization during early tumor growth and tumor metastasis was potently suppressed by host Grp78 heterozygosity. We further created an endothelial cell–specific Grp78 heterozygous knockout mouse model (Grp78\(^{+/−}\)/Tie2-Cre) and showed severe reduction of tumor angiogenesis and growth of metastatic lesions, with minimal effect on normal tissue MVD. Furthermore, knockdown of GRP78 in immortalized human endothelial cells results in suppression of endothelial cell proliferation, promotion of apoptosis, and decreased migration. These findings reveal GRP78 is a regulator of endothelial cell–specific angiogenic functions and provides a mechanistic explanation for the requirement of GRP78 in the microenvironment during tumor growth and metastasis.

Materials and Methods

Cell cultures

The E0771 mouse breast cancer cell line was generously provided by Dr. Enrico Mihich (Roswell Park Cancer Institute) and was maintained in RPMI 1640, supplemented with 10% FBS, 10 mmol/L HEPES, and 1% penicillin antibiotics as described (16). The immortalized human microvascular endothelial cells (HMEC) were kindly provided by Dr. Betty Wu-Hsieh (Taiwan National University). HMECs were grown in Medium 200 (Invitrogen) supplemented with Low Serum Growth Supplement (Invitrogen). All cells were maintained at 37°C and 5% CO₂.

Generation of the B16-Fluc-A1 melanoma cell line

B16-F0 cells (American Type Culture Collection) were transduced with a lentiviral construct containing the firefly luciferase gene driven by an internal CMV promoter. Clonal cell lines were selected by limiting dilutions in 96-well plates and expanded and screened for high levels of luciferase expression, using a Promega luciferase detection kit, per the manufacturer’s instructions. The cells were maintained in high-glucose Dulbecco's modified Eagle's medium containing 4.5 mg/mL glucose, supplemented with 10% FBS, 2 mmol/L glutamine, and 1% penicillin antibiotics.

Generation of genetically altered Grp78 mouse model

The Grp78\(^{+/−}\) mice were generated as described (10) and were backcrossed into the C57BL/6 genetic background for 8 generations. To create endothelial cell–specific Grp78 heterozygous knockout mice, mice carrying the Grp78 floxed allele (in C57BL6 and 129/Sv background; ref. 17) were crossed with Tie2-Cre transgenic mice (Tek-Cre in C57BL6 background; the Jackson Laboratory; ref. 18). Genotyping for the WT, floxed, and KO alleles were carried out by PCR using genomic DNA extracted from mouse tail biopsies as described (17). Genotyping was also carried out using genomic DNA extracted from enriched primary brain endothelial cells as previously described (19), with modifications (20). The Tie2-Cre transgene was identified with forward primer: 5′-AGAACCTGTAGGACATGTTCAGGGA-3′ and reverse primer: 5′-ACGAACCTGGTCGAAATCAGTGCG-TTC-3′. Three-month-old mice were used for the tumor model studies. All animal protocols were conducted with the approval of the USC University Animal Care and Use Committee.

Generation of tumor models

The generation and monitoring of endogenous mammary tumors driven by the MMTV-PyVT transgene in Grp78\(^{+/−}\) or Grp78\(^{+/−}\) mice have been described (11). In the syngeneic tumor models, E0771 cells (4 × 10⁶/mice) were resuspended in 200 µL PBS and injected into the mammary fat pad of 4-week-old female mice; B16-Fluc-A1 melanoma cells (amount as indicated) were resuspended in 200 µL PBS and injected through the lateral tail vein. Lung metastasis was monitored by luminescence imaging (Xenogen) weekly. Following sacrifice, the lungs were removed and the number of surface metastases was counted.

Immunofluorescent and immunohistochemical staining

Immunostaining was done on paraffin-embedded tumor sections as previously described (11). The primary antibodies used were the following: mouse anti-PCNA (1:100), goat anti-VEGF (1:100) from Santa Cruz Biotechnology; and rabbit anti-Cd31 (1:50) from Thermo Fisher Scientific. The processed sections were visualized using a fluorescence microscope.

Microvessel density measurement

Mouse tumor tissues were processed for MVD analysis as previously described (11). The tissues were stained with rat
anti-mouse CD31 antibody (BD Pharmingen) and quantified using the imaging processing program ImageJ (NIH).

**siRNA transfection**

HMECs were seeded at a density of $6 \times 10^4$ per well in 6-well plates and transfected with siRNA using Lipofectamine 2000 Transfection Reagent (Invitrogen) per the manufacturer’s instructions. The siRNA against Grp78 (siGrp78) is 5’-ggagcgcaaucaauacagtt-3’ as described (21). The control siRNA (siCtrl) is 5’-aaggagacguauagcaacggu-3’, composed of a 21-base pair scrambled sequence without significant homology to any known gene sequences from mouse, rat, or human. The experiments were repeated 3 times.

**Detection of cell-surface GRP78 protein**

The biotinylation and detection of cell-surface GRP78 by Western blotting in HMECs 48 hours posttransfection were done as previously described (4). The experiments were repeated 3 times.

**MTT cell viability assays**

HMECs were seeded in quadruplicate at a density of $3 \times 10^5$ per well in 96-well plates 48 hours posttransfection and grown for an additional 48 hours. The MTT assay was done according to the manufacturer’s instructions (Sigma-Aldrich) and as previously described (15).

**BrdU cell proliferation assays**

HMECs were seeded in quadruplicate at a density of $3 \times 10^5$ per well in 96-well plates 48 hours posttransfection and grown for an additional 24, 48, or 72 hours. The bromodeoxyuridine (BrdU) cell proliferation assay (Roche Diagnostics Corp.) was conducted according to the manufacturer’s instructions.

**Migration assays**

HMECs were seeded in duplicate at a density of $4 \times 10^4$ per modified Transwell Boyden chamber (BD BioCoat) 48 hours posttransfection. Each chamber housed a 6.5-mm-diameter polyethylene terephthalate filter with 8-μm pores. Migration was stimulated by adding complete growth media to the lower compartment of the experimental apparatus. After 6 hours, the filters were fixed and stained with Harleco Hemacolor staining solution (EMD Chemicals). Cells that migrated to the underside of the filter were quantified under high-power magnification ($400 \times$). A 6-hour migration period was used to eliminate the effect of disparate rates of cell proliferation.

**TUNEL assay**

HMECs were seeded at a density of $1.5 \times 10^5$ per well on glass coverslips 48 hours posttransfection. These cells were mounted with ProLong Gold Anifade mounting medium with 4’,6-diamidino-2-phenylindole (DAPI). The apoptotic cells detected by the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science) were visualized using a fluorescence microscope. A total of 1,000 cells were counted per treatment condition.

**Statistical analysis**

For the syngeneic E0771 mammary tumor model, a linear model was used to compare tumor volume over time, with slope and quadratic and cubic terms for each mouse treated as random. The likelihood ratio test for the Group $\times$ Time interaction was used to indicate whether the tumor growth patterns were significantly different between the 2 genotypes. This analysis was based on the logarithm of tumor volume $+ 1$. For the B16 melanoma tumor model, the log-rank test was used to compare time to lung metastasis between the 2 genotypes stratifying by experiment. The Pike estimates of relative HR were calculated using the observed and expected numbers of events based on the log-rank test statistic. Kaplan–Meier plots were graphed for time-to-lung metastasis. Two-way ANOVA was carried out for comparison of pulmonary metastases and BrdU incorporation in HMECs, with genotype and weeks as the 2 factors. Prior to ANOVA, comparing the endothelial cells with and without GRP78 knockdown, logit was taken of the responses to render the data compatible with the assumptions of normality and homoscedasticity. Pair-wise comparisons among the groups were done using the least significance difference method if the overall $P$ value was less than 0.05. $t$ tests were used when only 2 groups were compared for a measurement.

**Results**

**Grp78 heterozygosity in host environment suppresses early tumor growth**

In the MMTV-PyT transgene-driven mammary tumor model, tumors were formed in both the $Grp78^+/−$ and $Grp78^+/+$ mice. Previously, we reported the latency period for tumor formation was delayed and the size of the primary tumor at 15 weeks was reduced by about 60% in the $Grp78^+/−$ mice (11). On following the mouse cohorts for a longer period (after 18 weeks), secondary tumors became apparent. In this endogenous tumor model, in which both the tumor and host cells were heterozygous for $Grp78$, there were fewer total number of tumors ($P < 0.05$) and the tumors were of smaller size ($P < 0.01$) in $Grp78^+/−$ PyT mice than in $Grp78^+/+$ PyT mice (Fig. IA and B).

To examine the role of GRP78 in tumor growth in the host environment, the growth of tumors derived from syngeneic WT murine E0771 breast cancer cells injected subcutaneously into either $Grp78^+/−$ ($n = 12$) or $Grp78^+/+$ ($n = 15$) mice was monitored. Thus, in these experiments, only the host environment was varied. The likelihood ratio test based on random coefficient model comparing the 2 curves showed a significant reduction of E0771 tumor growth in $Grp78^+/−$ mice as compared with the WT mice ($P < 0.001$; Fig. 1B, left panel). Tumor size was examined at different times during tumor development. The early phase referred to the period between 10 and 15 days following tumor implantation, and the late phase referred to the period between 20 and 25 days posttumor injection. Our results showed that $Grp78$ heterozygosity in the host primarily affected the early phase of tumor growth (Fig. 1B, right panel); as after 20 days, the tumor growth rate...
between the 2 host genotypes was parallel to each other (Fig. 1A, left panel). The slower growth of the early tumors in the \( \text{Grp78}^{+/+} / \text{C0} \) mice associated with a 50% reduction (\( P < 0.001 \)) in tumor cell proliferation compared with the \( \text{Grp78}^{+/+} \) mice, as assayed by proliferating cell nuclear antigen (PCNA)-positive staining cells, whereas during the late phase, tumor cells in both host genotypes showed similar number of proliferating cells (Fig. 1C). Collectively, these data suggest that \( \text{Grp78} \) heterozygosity in the host environment is sufficient to impede tumor growth and proliferation at the early phase of tumor growth; however, at the late stage, the WT tumor cells can overcome the host deficiency due to \( \text{Grp78} \) heterozygosity.

**Grp78 host heterozygosity suppresses tumor angiogenesis during early tumor growth**

Initiation of tumor growth requires neovascularization. To examine the effects of \( \text{Grp78} \) host heterozygosity on tumor MVD, E0771 breast tumors harvested from \( \text{Grp78}^{+/+} \) or \( \text{Grp78}^{+/-} \) mice were snap frozen and stained with anti-CD31 antibody, which is specific for endothelial cells. Tumors from both early- and late-phase periods were analyzed. The early tumors of the \( \text{Grp78}^{+/-} / \text{C0} \) mice showed 70% reduction in MVD compared with \( \text{Grp78}^{+/+} \) mice (\( P < 0.01 \)), whereas the differences in the MVD between the 2 host genotypes were not significant in the late-phase tumors (\( P = 0.68 \); Fig. 2A). These results were confirmed with fluorescence staining of CD31 in paraffin-embedded tumor sections (Fig. 2B). In contrast,
staining for the VEGF was similar between the 2 host genotypes in both early- and late-phase tumors (Fig. 2C). This suggests that VEGF, primarily produced and released by the WT tumor cells (22), was not obviously affected by Grp78 host heterozygosity.

Grp78 host heterozygosity suppresses growth of metastatic lesions

Formation of new tumor vasculature is critical for the establishment and growth of metastatic colonies. To test whether the inhibition of early-phase tumor angiogenesis by Grp78 host heterozygosity negatively impacts tumor metastasis, we utilized the syngeneic B16 melanoma tumor model. A newly derived melanoma clonal cell line (B16-Fluc-A1) was generated through transduction with a lentivirus expressing luciferase, followed by serial dilution and screening. Luciferase expression was approximately 5 times stronger in the B16-Fluc-A1 cell line than in the original B16-Fluc cell line that also expressed the green fluorescent protein, with comparable in vitro and in vivo growth characteristics (23). Following tail vein injection of the WT tumor cells (5 × 10^5/mice; n = 10 for each host genotype), the progress of metastasis was first determined by whole-body luminescence imaging and representative images are shown in Figure 3A. Visible large tumor colonies were detected in the lungs of syngeneic Grp78^+/− mice beginning at 3 weeks and more were observed at 4 weeks, whereas Grp78^+/− mice showed no visible tumor at 3 weeks and only 1 mouse developed a small visible tumor at 4 weeks (Fig. 3A). Following sacrifice at 2, 3, and 4 weeks, the lungs of the Grp78^+/− mice showed considerably larger tumor and greater number of tumors than did Grp78^+/+ mice (Fig. 3B). The number of tumors per lung for the 2 genotypes is summarized in Figure 3C. The result of stratified log-rank test showed that the risk of developing lung metastasis for Grp78^+/− mice was 47% (95% CI: 25–89) of that for Grp78^+/+ mice (P < 0.001; Fig. 3D). Thus, consistent with the notion that GRP78 is required for tumor neovascularization, Grp78 heterozygosity in the host environment suppressed the number of visible metastatic lesions and slowed time to progression of metastatic tumors.

Endothelial cell–specific Grp78 heterozygosity inhibits angiogenesis and proliferation in metastases

Endothelial cells, the principal cellular component of the angiogenesis process, are a critical cellular component of the tumor microenvironment. Therefore, to understand the role of GRP78 in endothelial cells, we created a new mouse model of heterozygous knockout of Grp78 specifically in endothelial cells by breeding the Grp78 floxed/floxed mice (17) with the Tie2-Cre transgenic mice (18). The genotypes of the mouse strains
were confirmed by PCR analysis (Supplementary Fig. S1A). The Cre-recombinase was driven by the receptor tyrosine kinase Tek promoter/enhancer, which is active predominantly in endothelial cells starting at embryonic E9.5 (24). The knockout of the Grp78 floxed allele in primary endothelial cells isolated from the Grp78F/++;Tie2-Cre mice was confirmed by PCR analysis (Supplementary Fig. S1B). Mice with the Grp78F/++;Tie2-Cre genotype, which is phenotypically normal served as the experimental group, with sibling Grp78F/+ mice as negative controls. A high dose of B16-Luc-A1 melanoma cells were injected via tail vein into Grp78F/++;Tie2-Cre and Grp78F/+ mice (2 × 10⁶/mice; n = 21 for each genotype) to generate more metastatic lesions for comparison. Lungs were removed at 2, 3, and 4 week intervals (n = 7 at each time point). The macroscopic inspection showed clear reduction in metastasis in the Grp78F/+;Tie2-Cre mice and representative images are shown in Figure 4A. The visible pulmonary surface metastases were fewer in the Grp78F/+;Tie2-Cre mice and the difference became greater as time increased (P < 0.001) for the interaction between group and time. The mean difference (and associated 95% CI) in number of colonies per lung between the 2 groups of mice was 60 (32–88), 167 (139–195), and 355 (327–383) at 2 weeks, 3 weeks, and 4 weeks (all with P < 0.001), respectively (Fig. 4B). Thus, Grp78 heterozygosity in endothelial cells causes significant delay and decrease in metastatic growth. In these same groups of mice, CD31 staining of cryostat sections of normal organs such as brain, liver, and heart showed minimal difference in the blood vessel density (Fig. 4C and D). In contrast, immunofluorescence staining of paraffin-embedded lung sections of these mice with the CD31 antibody revealed fewer vessels at the border between the tumor and the adjacent normal tissue in the Grp78F/+;Tie2-Cre tumor sections than in Grp78F/+ controls (Fig. 5A), correlating with reduced PCNA staining in the Grp78F/+;Tie2-Cre tumor sections (Fig. 5B). Thus, endothelial cell–specific heterozygous knockdown of GRP78 is sufficient to impede growth of metastatic lesions and vascularization at the growing edge of the tumor with minimal effect on normal organs.

**Knockdown of GRP78 suppresses proliferation, migration, and promotes apoptosis in immortalized endothelial cells**

To identify the functional consequence resulting from decreased GRP78 levels in endothelial cells mimicking those in the tumor microenvironment, *in vitro* studies were conducted with HMECs. The immortalized HMECs are SV40-transformed endothelial cells (25), which constitutively express high levels of GRP78, as observed in tumor-associated endothelial cells (14, 15). The HMECs were transiently transfected with siRNA specifically targeting Grp78 (siGrp78) or control siRNA (siCtrl). Western blot analysis of whole-cell lysates indicated that a greater than 85% knockdown of GRP78 expression was achieved (Fig. 6A). In the HMECs, biotinylation of cell-surface protein followed by avidin agarose pull down and Western blot revealed that approximately 1% of total intracellular GRP78 was present on the cell surface, and following siGrp78 treatment, cell-surface GRP78 was below detection limits (Fig. 6A). Investigation of the overall viability of cells using the MTT assay showed a 55% (95% CI: 49–61; P < 0.001) decrease in the number of viable HMECs at 48 hours following GRP78 knockdown (Fig. 6B). Analysis of cell proliferation via incorporation of BrdU showed that knockdown of GRP78 reduced cell proliferation and the reduction became greater as time increased (P < 0.001 for the interaction between cell type and time). Knockdown of GRP78 reduced cell proliferation by 24% (95% CI: 4–40; P = 0.024), 61% (95% CI: 50–69; P < 0.001), and 79% (95% CI: 73–83; P < 0.001) when compared with siCtrl-transfected HMECs at 24, 48, and 72 hours, respectively (Fig. 6B). In addition to proliferation, within 6 hours of the knockdown of GRP78, HMECs showed reduced overall cell migration by 31%
Partial loss of GRP78 led to cell death; however, it required at least 48 hours. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining revealed that apoptosis in siGrp78-transfected cells at 48 hours posttransfection was significantly increased, from 2.5% in siCtrl-transfected cells to 23% (P < 0.001; Fig. 6C). Thus, reduced GRP78 expression in immortalized endothelial cells suppresses cell proliferation, promotes apoptosis, and decreases migration.

**Discussion**

In an array of tissue culture and tumor models, GRP78 has been established to be important for tumor progression and

(95% CI: 16–43; P = 0.001) cells per hpf (Fig. 6B).
chemotherapeutic drug resistance, correlating with clinical studies that patients with high GRP78 expression levels in their tumors generally have a poorer prognosis and experience earlier relapse (3, 9, 11, 21, 26, 27). These discoveries suggest that GRP78 can be a potent therapeutic target for anticancer therapy. Previous studies on GRP78 function in cancer progression have largely focused on tumor cells and how GRP78 exerts its protective antiapoptotic effect to allow them to escape immunosurveillance and stress associated with tumor progression (3, 9). Here, we examined the role of GRP78 in the tumor microenvironment. In 2 syngeneic tumor models, WT tumors in the Grp78 heterozygous host environment were significantly impeded in tumor angiogenesis and proliferation. Through the creation of a new mouse model with endothelial cell–specific heterozygous knockout of Grp78, and coupled with in vitro studies on GRP78 knockdown in immortalized endothelial cells, we further showed that GRP78 is an important mediator of endothelial cell proliferation, survival, and migration and provided a new mechanistic explanation for the requirement of GRP78 in the microenvironment during tumor growth and metastasis.

How might GRP78 regulate function and promote survival of endothelial cells supporting tumor growth? Expression of GRP78, with well-established chaperone function (28), is induced in hypoxic endothelial cells (29). Induction of GRP78 leads to increase in both intracellular form of GRP78 and cell surface of GRP78 (4). Upregulation of GRP78 in the ER can protect endothelial cells against ER stress, maintain ER calcium homeostasis, and suppress onset of stress-induced apoptosis through suppression of caspase 7 and CHOP induction, which mediate cytotoxicity in cells undergoing ER (17, 30). On the other hand, cell-surface GRP78 is reported to colocalize with proangiogenic growth factor receptors, promoting growth signaling, proliferation,

Figure 6. GRP78 regulates function and viability of endothelial cells. A, Western blot analysis of total and cell-surface GRP78 expression level in HMECs transfected with control siRNA (siCtrl) or siRNA against Grp78 (siGrp78), with β-actin serving as a loading control for the total lysate samples and a control free of cytoplasmic contamination for the cell-surface protein samples. The amount of lysate applied onto the gel represented 20% of input. B, left, analysis of cell viability at 48 hours posttransfection by the MTT assay showed that knockdown of GRP78 significantly reduced cell survival (**, \( P < 0.001 \)). Middle, BrdU incorporation in HMECs showed knockdown of GRP78 significantly decreased cell proliferation at 24, 48, and 72 hours (**, \( P = 0.024 \) at 24-hour time point and ***, \( P < 0.001 \) for 48- and 72-hour time points). Data are expressed as the mean OD of the siGrp78 group relative to the mean OD of the siCtrl group at each time point. The 95% CIs are indicated. Data are based on 2 independent experiments. Right, HMEC migration was assessed by the modified transwell Boyden chamber assay. After a 6-hour migration period, the number of migrated HMECs was significantly lower in the siGrp78-transfected group than in the siCtrl-transfected group (***, \( P < 0.001 \)). Data represent the mean number of cells per hpf that migrated to the underside of the porous filter and are representative of 2 independent experiments. The 95% CIs are indicated. C, left, detection of apoptotic cells (in red indicated by arrows) by TUNEL assay in the transfected HMECs. Bar, 50 μm. Right, quantitation of apoptosis cells in the transfected groups with significant differences detected (***, \( P < 0.001 \)).
and cell migration (5, 6, 8). GRP78 cell-surface expression is induced by VEGF in human umbilical vein endothelial cells and is required for VEGF-induced proliferation and angiogenic signaling (31). Therefore, the observed decrease in HMEC proliferation and migration, 2 critical angiogenic functions, correlates well with the consequences of GRP78 knockdown in these cells. Interestingly, our studies indicate that the effects of Grp78 host heterozygosity are most evident in a minimal growth factor environment at the early stage of tumor growth. A possible explanation is that as the tumor grows bigger, sufficient levels of proangiogenic growth factors are produced and/or secreted by the WT tumor cells to stimulate tumor endothelial cell growth, hence can compensate for the effects of GRP78 loss in the host microenvironment.

Given the critical role of GRP78 both in tumor cell survival and in endothelial cells supporting tumor growth, it represents a dual target for anticancer therapy. GRP78 is the receptor for Kringle 5, which induces apoptosis of both cancer cells and endothelial cells (29). Sensitization of Kringle 5–induced apoptosis of brain microvessel endothelial cells by radiation requires GRP78 (32). Proteomic studies suggest that GRP78 reduces the efficacy of topoisomerase inhibitors to induce endothelial cell apoptosis (33). Furthermore, some tumor cells are capable of secreting GRP78, which confers bortezomib resistance to endothelial cells (34). We show here that reducing GRP78 affects several critical aspects of angiogenesis: endothelial cell number and endothelial cell migration. Migration is especially important because blood vessel growth involves the migration of endothelial progenitor cells to the tumors and the expansion of neighboring blood vessels (35). Furthermore, other antiangiogenic drugs [i.e., bevacizumab (Avastin)] alter the VEGF levels in the patient, resulting in a change in the delicate balance of angiogenic growth factors. Such an imbalance can cause severe blood vessel regression and resistance to drugs (36). It is recently reported that angiogenesis inhibitors targeting the VEGF and other proangiogenic growth factor pathways, while showing antitumor effects, concomitantly elicit tumor adaptation leading to accelerated metastasis (37, 38). Here, we showed that Grp78 host heterozygosity does not seem to affect VEGF staining in the tumor sections, suggesting that VEGF production in the tumor tissue is not altered. Furthermore, the decrease in tumor metastatic growth in both Grp78+/− and Grp78−/− Tie2-Cre mice persisted throughout the entire course of the experiments, which was terminated at 4 weeks when animals required euthanasia. One explanation is that altering the cytokine balance by decreasing VEGF activation is likely to induce a response that is different from decreasing GRP78 expression, as GRP78 regulates a much wider repertoire of cellular function (2, 7). Thus, concomitant use of current chemotherapeutic agents and novel therapies against GRP78 may offer a powerful new approach to arrest cancer initiation, progression, and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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