Bcl-2/Adenovirus E1B 19 kDa Interacting Protein-3 Knockdown Enables Growth of Breast Cancer Metastases in the Lung, Liver, and Bone

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Abstract
The mouse breast cancer cell lines 4T1, 4T07, and 67NR are highly tumorigenic but vary in metastatic potential: 4T1 widely disseminates, resulting in secondary tumors in the lung, liver, bone, and brain; 4T07 spreads to the lung and liver but is unable to establish metastatic nodules; 67NR is unable to metastasize. The Bcl-2/adenovirus E1B 19 kDa interacting protein-3 (Bnip-3) was recently shown to be absent after hypoxia in pancreatic cancer cell lines whereas its overexpression restored hypoxia-induced cell death. We found that Bnip-3 expression increased after 6 hours of hypoxia in all cell lines tested but was highest in the nonmetastatic 67NR cells and lowest in the highly metastatic 4T1 cells. Hypoxia-induced expression of Bnip-3 in the disseminating but nonmetastatic 4T07 cells was intermediate compared with 4T1 and 67NR cells. Cleaved caspase-3, a key downstream effector of cell death, increased after 6 hours of hypoxia in the 67NR and 4T07 cells by 1.9- and 2.5-fold, respectively. Conversely, cleaved caspase-3 decreased by 45% in the highly metastatic 4T1 cells after hypoxia. Small interfering RNA targeting endogenous Bnip-3 blocked cell death and increased clonigenic survival after hypoxic challenge in vitro and increased primary tumor size and enabled metastasis to the lung, liver, and sternum of mice inoculated with 4T07 cells in vivo. These data inversely correlate the hypoxia-induced expression of the cell death protein Bnip-3 to metastatic potential and suggest that loss of Bnip-3 expression is critical for malignant and metastatic evasion of hypoxia-induced cell death. (Cancer Res 2005; 65(24): 11689-93)

Introduction
In the United States in 2003, 211,240 new cases in of breast cancer were diagnosed and 40,410 patients died. Metastases were the main cause of death, spreading to the bone, liver, lung, and brain. The 5-year survival rate of metastatic breast cancer is 20% and at 10 years is a grim 5%. When a mammary tumor reaches 1.5 cm in diameter, the likelihood that it has already metastasized is 50%. Despite considerable investment from private and public sources, currently available treatments remain highly toxic and often lethal. A better understanding of the molecular mechanisms underlying breast cancer metastases is essential to identify better targets for therapy.

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The metastatic cascade proceeds through a sequence of events as tumor cells spread throughout the body: (a) invasion of surrounding healthy tissue, (b) intravasation of and (c) systemic transport through the lymphatic and vascular supply, (d) arrest on and (e) extravasation through the vascular endothelium of distant organs, and, finally, (f) growth into a secondary tumor (1, 2). As metastatic tumor cells disseminate throughout the organism and complete metastasis, they likely experience hypoxia after lodging in distant organs. Hypoxia is also a common characteristic of solid tumors and is thought to develop very early in carcinogenesis before initiation of the angiogenic switch (3). As a solid tumor grows, the vascular supply lags behind or is dysfunctional, resulting in hypoxic regions with little (hypoxia) or no oxygen. Cellular resistance to hypoxia-induced cell death is thought to be a requirement for primary tumor growth and may be crucial for metastasis.

The Bcl-2 protein family regulates cell death in response to various stimuli. Members of the Bcl-2 family oppose each other by acting to promote (Bax and Bak) or inhibit (Bcl-2, Bcl-xL, Bcl-w, A1, and Mcl-1) cell death. A subgroup of the Bcl-2 family, the Bcl-2 homology region 3-only (BH3-only) proteins, effect cell death in response to different stresses by neutralizing prosurvival Bcl-2 family members (4, 5). The pro-death Bcl-2/adenovirus E1B 19 kDa interacting protein-3 (Bnip-3) and the closely related Bnip-3L are unique members of the BH3-only proteins in that their genes contain a functional hypoxia response element in the promoter, which is a target for the hypoxia-inducible transcription factor-1 (HIF-1) that is itself stabilized by low oxygen (6). Bnip-3-mediated cell death seems to be specific to low oxygen and not other cellular stresses, such as hypoglycemia, etoposide, or staurosporine.1

Transcriptional silencing of the Bnip-3 gene has recently been observed in multiple human cancer cell lines, including colorectal, gastric (7), pancreatic (8, 9), and hematopoietic tumors (10). Bnip-3 silencing in these models rendered the cell lines resistant to hypoxia-induced cell death, which could be reversed with restoration of Bnip-3 expression. Loss of Bnip-3 expression also correlated with a poor prognosis and increased chemoresistance in patients with pancreatic cancer (11). The role of Bnip-3 silencing in vivo or in metastatic disease is unknown.

67NR, 4T07, and 4T1 murine breast cancer cell lines form primary tumors in syngeneic BALB/c mice with similar kinetics but vary in their ability to disseminate and establish metastatic secondary tumors in distant tissues. After inoculation of the mammary fat pad of host mice, 67NR cells do not metastasize at all, whereas 4T07 cells can be isolated from lung homogenates.

Circulating 4T07 cells apparently die shortly after being trapped in pulmonary vascular beds, however, and disappear from the lung following removal of the primary mammary tumor (12). In contrast, 4T1 cells metastasize to the lung, liver (12), and bone (13), and form secondary tumors that eventually lead to death.

In this study, we investigate for the first time the in vivo effect of Bnip-3 silencing on primary tumorigenesis and extend our findings to breast cancer metastasis using an experimental model of metastatic breast cancer in mice. We show that expression of Bnip-3 protein is inversely proportional to the ability of cells to metastasize. Furthermore, knockdown of Bnip-3 enhances hypoxic survival in vitro, increases primary tumor size and metastatic burden in the lungs, and induces robust metastases in the sternum and liver.

Materials and Methods

Cell culture and RNA interference. The malignant cell lines 67NR, 4T07, and 4T1 were established from a single spontaneously arising mouse mammary tumor in a BALB/c mouse and were a gift from Dr. Fred Miller, (Prentis Cancer Research Center, Karmanos Cancer Institute, Detroit, MI) (12). Cells were cultured in DMEM with 4.5 g/dL glucose and 10% fetal bovine serum and passaged no more than four times. For hypoxia treatment, plates were placed in an incubator set to 1% O2 and 5% CO2. For RNA interference (RNAi) 5,000 67NR or 4T07 cells were plated per 60 mm plate and transfected 3 days later with Bnip-3 or scrambled control small interfering RNA (siRNA) oligonucleotides from Dharmacon (Lafayette, CO) to ensure that equal amounts of protein were loaded onto SDS-PAGE for immunoblotting.

SDS-PAGE and Western blotting. Cells were lysed by sonication in cell fractionation buffer (250 mMol/L sucrose, 2.5 mMol/L EDTA, 1 mMol/L phenylmethylsulfonyl fluoride, 20 mMol/L MOPS, and 100 mMol/L each of phosphatase inhibitor cocktails I and II from Sigma-Aldrich, St. Louis, MO). Centrifugation at 4°C for 5 minutes at 500 × g yielded the nuclear fraction and the supernatant was further centrifuged at 4°C for 20 minutes at 20,000 × g to obtain the mitochondrial pellet and cytosolic supernatant. CHAPS (1-2%) was added to dissolve the membranes and total protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA) to ensure that equal amounts of protein were loaded onto SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) and an anti-Bnip-3 antibody (1:2,000) from Sigma (St. Louis, MO) was used for immunoblotting.

Caspase assay. The amount of cleaved caspase was determined to be proportional to the amount of active caspase, the effectors of apoptosis, or programmed cell death. The amount of cleaved caspase in cell lysates was quantified by the PathScan Cleaved Caspase-3 Sandwich ELISA kit (Cell Signaling, Beverly, MA) according to the instructions of the manufacturer.

Survival analysis. Cells were trypsinized and seeded at a density of 100 cells per 100 mm culture dish and allowed to recover and adhere under normoxic conditions (21% O2) for 4 hours before 24 hours of hypoxic (1% O2 and 5% CO2) or control normoxic conditions as previously described (14). Cells were then allowed to recover for 10 days and colonies were fixed with methanol and acetic acid, stained with crystal violet, and counted.

In vivo metastatic breast cancer. BALB/c mice from The Jackson Laboratory (Bar Harbor, ME) were inoculated by s.c. injection into the abdominal mammary fat pad with 50,000 4T07 cells in 50 μL of RPMI that had been transfected 3 days earlier with either Bnip-3 or scrambled siRNA. Tumor growth was monitored and measured weekly by two-dimensional ultrasound with a 12 mHz linear transducer on a Toshiba Apilo 80. The maximum diameter was measured in three orthogonal dimensions (d1, d2, and d3) by rotating the transducer 90 degrees and tumor volume V was computed by the formula $V = \frac{1}{2} (d_1 \times d_2 \times d_3)$ (15).

Six weeks after inoculation, mice were sacrificed. Moreover, the lungs, liver, and sternum were harvested, postfixed with Shandon Glyo-Fixx (Thermo Electron Corporation, Pittsburgh, PA), dehydrated, and embedded in paraffin. Four-micrometer-thick sections were stained with H&E, coverslipped, and examined under a light microscope.

Results

Western blot analysis revealed that Bnip-3 expression was induced after 6 hours of 1% oxygen (hypoxia) in all mouse breast cancer cell lines tested (Fig. 1). Interestingly, the amount of Bnip-3 protein was highest in the malignant but nonmetastatic 67NR cell line and lowest in the highly metastatic 4T1 cell line (Fig. 1A). Bnip-3 protein levels were intermediate in the 4T07 cell line, which metastasizes to the lung in vivo, but does not form grossly visible nodules and disappears upon removal of the primary tumor. Likewise, after 6 hours of hypoxia, there was a 2-fold increase in cleaved caspase in both 67NR and 4T07 cells but a 50% reduction in 4T1 cells compared with normoxic controls (data not shown). This indicates that hypoxia-induced Bnip-3 expression correlates...
with cell death and is inversely proportional to the metastatic potential of these murine breast cancer cells. Bnip-3 appeared in the cytosol (Fig. 1B) only in those cell lines with low (4T07) or no metastatic potential (67NR), suggesting a role for Bnip-3 translocation in the execution of its apoptotic function. Hypoxia-induced Bnip-3 protein expression was reduced significantly by 50 nmol/L siRNA oligonucleotides in both 67NR (Fig. 1C) and 4T07 (Fig. 1D) cells.

Our finding that hypoxia-induced Bnip-3 expression inversely correlates with metastatic potential led us to hypothesize that evasion of Bnip-3-mediated cell death confers a survival advantage to metastatic breast cancer cells. To test this hypothesis, we measured the clonogenic survival after hypoxic challenge of 4T07 and 67NR cells transfected with a siRNA specific to Bnip-3. We found that Bnip-3 RNAi increased the ability of both 67NR and 4T07 cells to survive after 24 hours of hypoxia and form colonies. In hypoxic 4T07 cells, Bnip-3 RNAi resulted in a 38% increase in the number of colonies compared with the scrambled oligonucleotide (58 ± 10 versus 42 ± 8, P < 0.01), evidence of an increase in surviving cells following hypoxic challenge (Fig. 2, top). Interestingly, Bnip-3 siRNA increased survival in 67NR cells in both normoxia and hypoxia (Fig. 2, bottom), although the effect was more dramatic in hypoxia with nearly a 3-fold increase in the number of colonies with Bnip-3 compared with scrambled siRNA (27 ± 6 versus 10 ± 3, P < 0.01). To test whether Bnip-3 RNAi translates to an increase in in vivo tumor growth, we injected 50,000 4T07 cells into the abdominal mammary fat pads of BALB/c mice 4 days after transfection with Bnip-3 siRNA or control scrambled siRNA. We found that knockdown of Bnip-3 protein increased tumor volume by 2- to 3-fold at every time of examination, from 3 weeks until sacrifice at 6 weeks after implantation (Fig. 3).

4T07 cells are able to complete the entire metastatic cascade except for the last step, which is the formation of large, growing tumors in distant tissues that threaten the life of the organism. When injected into the abdominal mammary fat pad, they can be found in the lung but disappear upon removal of the primary tumor. This suggests a continuous homing, complete turnover, and death of cells that disseminate to the lung. We tested the hypothesis that Bnip-3 RNAi would enable 4T07 cells to survive metastatic dissemination and establish large, durable tumors in the lungs, livers, and bones of mice inoculated with cells transfected with either Bnip-3 or scrambled siRNA oligonucleotides. We found this to be the case; Bnip-3 but not scrambled siRNA dramatically induced grossly visible tumors of the liver and sternum 6 weeks following implantation of 4T07 cells in the abdominal mammary fat pad in five of nine mice (Fig. 4). In agreement with the findings of previous investigators (12), we found that control (scrambled siRNA) 4T07 cells formed small perivascular tumors only in the lung, but not the liver or sternum (Fig. 5). The lung micrometastases were not grossly visible, no larger than 230 μm in diameter, and occurred in only two of nine mice. Importantly, we found that Bnip-3 siRNA enabled 4T07 cells to establish grossly visible hepatic and sternal metastases and form tumors in the lungs of six of nine mice that were 3-fold larger (0.7 ± 0.5 versus 0.2 ± 0.3 mm, P < 0.0025) and more numerous (32 metastases in eight lungs versus five metastases in three lungs) compared with scrambled control.

**Discussion**

This study is the first to show a role for Bnip-3 in either primary or metastatic tumor growth. Our finding that hypoxia-induced expression of Bnip-3 protein inversely correlates with metastatic potential extends the observations of other groups of endogenous Bnip-3 transcriptional silencing in multiple cancers to include a role for Bnip-3 silencing not only in primary tumor growth but also the metastatic cascade (7, 9, 10). We hypothesized and confirmed that exogenous knockdown of Bnip-3 is sufficient for Bnip-3 to function as a negative regulator of cell death in metastatic cancer cells.
to enable metastasis of primary breast cancer tumors to multiple organs, further supporting a critical function for Bnip-3 silencing in enabling malignant cells to make the leap to metastasis. The increased survival following Bnip-3 knockdown under hypoxic conditions, which are prevalent in most solid tumors, suggests an important mechanism of metastatic breast cancer that includes evasion of Bnip-3-induced cell death when oxygen becomes scarce.

Bnip-3 translocation from the mitochondria to the cytosol was observed in nonmetastatic cell lines, but was lost in metastatic cells, suggesting an additional mechanism for evasion of Bnip-3-mediated cell death beyond transcriptional silencing. This mechanism may hinge, at least in part, on the posttranslational modification of Bnip-3 with acetylglucosamine bound to serine and threonine hydroxyl groups, a highly regulated, reversible, and labile covalent modification analogous to phosphorylation known as O-linked glycosylation (16). Results from our group in a cardiomyocyte cell line indicate that chemical inhibition of O-linked deglycosylation prevents Bnip-3 translocation and delays cell death, whereas siRNA silencing of the enzyme responsible for O-linked glycosylation increases Bnip-3 translocation from the mitochondria to the cytosol. Indeed, the 4T1 cell line has an increased level of O-linked glycosylated Bnip-3, with no change in the total level of Bnip-3 in metastatic cells recovered from the lung compared with naïve 4T1 cells before implantation (data not shown). Taken together, these results suggest that increased O-linked glycosylation of Bnip-3 is an additional mechanism beyond transcriptional silencing of the Bnip-3 death program that enhances metastatic survival.

Decreasing oxygen in solid tumors stabilizes HIF-1, which correlates with poor prognosis, chemoresistance, tumor invasion, angiogenesis, and metastasis (17–19). HIF-1 transactivates hundreds of genes that paradoxically may either promote or inhibit the growth and survival of individual tumor cells (20). Thus, the selection of an individual tumor cell that, on the one hand, maintains the expression of beneficial HIF-1 transcriptional

Figure 4. Gross dissection of BALB/c mice 6 weeks after s.c. inoculation in the abdominal mammary fat pad with 50,000 4T07 cells transfected with either Bnip-3 or scrambled siRNA oligonucleotides. The liver and xiphoid process of the sternum are shown with visible metastatic nodules in the liver and a greatly enlarged xiphoid process in mice receiving Bnip-3 siRNA-transfected cells.

Figure 5. H&E-stained sections of mice 6 weeks after s.c. inoculation in the abdominal mammary fat pad with 50,000 4T07 cells transfected with either Bnip-3 or scrambled siRNA oligonucleotides. Small perivascular metastatic nodules are present only in the lung (arrow) in the scrambled siRNA mice, whereas the liver and sternum are normal. Large nodules are present in the lung, and significant perivascular metastatic dissemination is visible in the liver (arrows) and sternum (arrows) in Bnip-3 siRNA mice, with extensive necrosis (N) in the large sternal metastasis. Original magnification, ×100.
targets whereas, on the other hand, silences pro-death hypoxia-induced signals would result in a dangerous malignancy. Our study indicates that silencing hypoxia-induced Bnip-3-mediated cell death is a crucial step in the emergence of a metastatic phenotype. Our finding that Bnip-3 silencing induces or increases metastatic growth in the lung, liver, and bone, which are the three most common metastasis sites of breast cancer at autopsy (21), support the relevance of this study. Likewise, the size of metastases to the lung was dramatically increased by Bnip-3 knockdown in our model of experimental metastatic breast cancer. This finding may be important because the size, but not the number of lung metastases, had a significant negative influence on survival in patients with metastatic breast carcinoma and was the second-best prognostic indicator of survival after disease-free interval (22).

Reconstitution of Bnip-3 signaling by a small molecule mimetic may cause targeted death of not just primary tumors, but metastatic colonies in the lung, liver, and bone. In fact, transient ectopic overexpression of epitope-tagged Bnip-3 resulted in the death of MCF-7 breast carcinoma cell within 12 hours (23). Likewise, a small molecule BH3 mimetic called ABT-737 binds to and antagonizes Bcl-2-like proteins (24). This compound was found to kill most chronic lymphocytic leukemias and small cell lung cancers in vitro and increased survival in murine xenograft models of the same tumors. Metastasis was not examined, and many cell lines derived from solid tumors did not respond; nevertheless, our results indicate that specifically reconstituting Bnip-3-mediated cell death in metastatic breast cancer is a worthwhile target.

Acknowledgments

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References

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