XBP1 Is Essential for Survival under Hypoxic Conditions and Is Required for Tumor Growth

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Abstract

Hypoxia within solid tumors is a major determinant of outcome after anticancer therapy. Analysis of gene expression changes during hypoxia indicated that unfolded protein response genes were one of the most robustly induced groups of genes. In this study, we investigated the hypoxic regulation of X-box binding protein (XBP1), a major transcriptional regulator of the unfolded protein response. Hypoxia induced XBP1 at the transcriptional level and activated splicing of its mRNA, resulting in increased levels of activated XBP1 protein. After exposure to hypoxia, apoptosis increased and clonogenic survival decreased in XBP1-deficient cells. Loss of XBP1 severely inhibited tumor growth due to a reduced capacity for these transplanted tumor cells to survive in a hypoxic microenvironment. Taken together, these studies directly implicate XBP1 as an essential survival factor for hypoxic stress and tumor growth.

Introduction

Hypoxia is a physiologically important endoplasmic reticulum (ER) stress common to all solid tumors. Numerous clinical studies have demonstrated that tumor hypoxia predicts for decreased local control, increased distant metastases, and decreased overall survival in a variety of human tumors (1). Hypoxia selects for tumors with an increased malignant phenotype (2) and increases the metastatic potential of tumor cells (3). Understanding the role of hypoxia in tumorigenesis and its influence on anticancer therapy is crucial to improving current cancer treatments (4).

The ER is an extensive intracellular membrane network that extends throughout the cytoplasm and functions primarily to process newly synthesized secretory and transmembrane proteins. Accumulation of unfolded proteins in this compartment causes ER stress and prolonged ER stress ultimately results in cell death. The cellular response to ER stress consists of at least two coordinated pathways: (1) rapid translational arrest mediated by pancreatic ER kinase or PKR-like ER kinase (PERK); and (2) transcriptional activation of unfolded protein response target genes (5, 6). In addition to cancer (7), the unfolded protein response has therapeutic implications in diseases such as diabetes, atherosclerosis, viral infection, conformational diseases, and cerebrovascular disease (8).

Koumenis et al. (9) previously demonstrated that translational control of protein synthesis during hypoxia occurs through the activation of PERK. These investigators showed that PERK−/− mouse embryonic fibroblasts where unable to phosphorylate eIF2α and had decreased survival during hypoxia when compared with wild-type mouse embryonic fibroblasts. They concluded that PERK plays an important role in hypoxia-induced translation attenuation, additionally supporting a role for hypoxia as an inducer of ER stress (9). When the PERK-dependent translational attenuation pathway is activated, activating transcription factor-4 (ATF-4) mRNA is selectively translated, leading to an induction of ATF-4 downstream target genes (10). Moreover, ATF-4 protein has also been shown to be stabilized in a hypoxia-induced factor 1(HIF-1)-independent manner during anoxia (11).

In this study, we analyzed global changes in gene expression during hypoxia and found that unfolded protein response-related genes, including genes specifically regulated by XBP1, were most robustly induced during severe hypoxia/anoxia. Because of its role as a major transcriptional activator of these genes and its overexpression in some tumor types (14), we investigated the effect of hypoxia on XBP1 regulation. Most importantly, we present evidence that loss of XBP1 increases the sensitivity of transformed cells to killing by hypoxia and severely impedes tumor growth. Because loss of XBP1 had little effect on the secretion of proangiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, the mechanism of impaired tumor growth in XBP1-deficient cells is likely not dependent upon angiogenesis and is a direct consequence of impaired survival of XBP1-deficient cells under hypoxic conditions. These studies support the concept of targeting XBP1 as a therapeutic strategy to eliminate hypoxic cells and inhibit tumor growth.

Materials and Methods

Cell Culture and Hypoxia Treatments. HIF-1α wild-type and knockout mouse embryonic fibroblasts, XBP1 wild-type and knockout mouse embryonic fibroblasts, and HT1080 cells (human fibrosarcoma cell line) were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 incubator. For all hypoxia experiments, cells were treated at 70 to 80% confluence and maintained in an anaerobic chamber (Sheldon Corp., Cornelius, OR) with pO2 levels < 0.02%.

Gene Expression Studies. Mouse genome MGU74Av2 GeneChip arrays (Affymetrix, Santa Clara, CA) were used for mRNA expression profiling. The preparation of samples and hybridization were carried out as described by Affymetrix. In brief, total RNA from ras/c-myc–transformed mouse embryonic fibroblasts exposed to 8 hours of hypoxia were prepared using Trizol

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(Invitrogen, Carlsbad, CA). Biotinylated RNA was then synthesized using the BioArray RNA transcript labeling kit (Enzo, Farmingdale, NY). Arrays were hybridized with biotinylated in vitro transcription products for 16 hours at 45°C according to the manufacturer’s instructions. The Fluidic Station 400 (Affymetrix) was used for washing, and a three-step staining protocol was used to enhance detection of the hybridized biotinylated RNA. Arrays were scanned by the Affymetrix GeneChip reader (Hewlett Packard). The raw image DAT data files were initially processed using Affymetrix GeneChip software (version 5) to create CEL files. Higher level analysis of microarray CEL files was performed using dChip v1.13 (15) Thorou...
Results and Discussion

Ras and c-myc transformed mouse embryonic fibroblasts were exposed to severe hypoxia/anoxia (0.02% O₂) for a period of 8 hours before total RNA was isolated from both normoxic and hypoxic samples for microarray analysis. This experiment was repeated six times to minimize experimentally introduced variations. Among the 12,000 genes present on the MGU74Av2 GeneChip, 285 were found to be induced by at least 2-fold (2.4%). Of the top 30 most robustly induced genes under severe hypoxia/anoxia, 25% were involved in the unfolded protein response. Fig. 1A is a compilation of known unfolded protein response genes that were induced during hypoxia. We confirmed by Northern blotting that many of these unfolded protein response genes were transcriptionally induced under hypoxia (Fig. 1B).

Next, we investigated the kinetics of unfolded protein response induction under severe hypoxia/anoxia. Overall, the time course for transcriptional activation of unfolded protein response genes was slower than for HIF-1-regulated genes (Fig. 2A). The induction of VEGF by severe hypoxia/anoxia was rapid compared with the unfolded protein response genes (GRP78, GRP94, and XBP1), and the expression of unfolded protein response genes remained elevated during prolonged periods of severe hypoxia/anoxia (48 hours) while VEGF mRNA decreased (Fig. 2A). This decrease in VEGF mRNA corresponded with a similar decrease in HIF-1α protein (data not shown). Furthermore, the induction of unfolded protein response genes was not evident under less severe hypoxic (2% O₂) conditions (data not shown).

The generation of the active spliced form of XBP1 was assayed by PCR amplification with primers specific to the spliced and unspliced forms of XBP1 in HIF-1 wild-type and HIF-deficient mouse embryonic fibroblasts. The splicing occurred with equal efficiency during hypoxia in both cell lines, indicating that XBP1 activation occurs in a HIF-1-independent manner (Fig. 2B). Spliced XBP1 mRNA was efficiently translated into the proteins of XBP1 during severe hypoxia/anoxia as evidenced by immunoblotting with XBP1-specific antibody (Fig. 2C).

To determine the role for XBP1 in cell survival during these conditions, we compared the amount of apoptosis in cell cultures of XBP1 wild-type and XBP1-deficient mouse embryonic fibroblasts by TUNEL staining (Fig. 3A). After 48 hours of hypoxia, 62.7 ± 11.8% of the XBP1-knockout cells stained positive for TUNEL staining, whereas only 38.6 ± 9.3% of the wild-type cells stained positive for TUNEL. Under normoxic conditions, 4.5 ± 3.7% of the XBP1-deficient cells and 7.8 ± 4.0% of the wild-type cells stained positive for TUNEL. These data represent the mean of four independent experiments ± SD. These results indicate that XBP1-deficient cells are more sensitive to hypoxia-induced apoptosis than XBP1 wild-type cells and are consistent with published data showing increased apoptosis during ER stress in myeloma cells, expressing either a dominant negative or a siRNA vector to inhibit XBP1 expression (17).

Fig. 2. A, GRP78, GRP94, XBP1, and VEGF mRNA expression increased during hypoxia in HT1080 cells. Treatment with 300 nmol/L thapsigargin (Tg), an inhibitor of the Ca-ATPase, was included as a positive control for UPR induction. B, HIF-1 wild-type and knockout mouse embryonic fibroblasts (MEFs) demonstrated similar splicing of XBP1 mRNA during hypoxia. C, Spliced XBP1 protein was induced during hypoxia in HT1080 cells.

Fig. 3. Comparison of apoptosis after 48 hours of hypoxia between (A) XBP1 wild-type and knockout MEFs. B, comparison of clonogenic survival in XBP1 wild-type and knockout MEFs (left panel). Comparison of clonogenic survival in control MEFs (neo-XBP1) and siRNA XBP1-knockdown cells (ix-XBP1; right panel). Far right panel was included to demonstrate that XBP1 expression was reduced in cells stably transfected with XBP1 siRNA construct.
Fig. 4. XBP1 is essential for tumor growth. A, Each SCID mouse was implanted subcutaneously with two tumors consisting of 2 × 10⁶ XBP1 wild-type cells in one flank and either 2 × 10⁶ knockout cells or 2 × 10⁶ ix-XBP1 cells in the contralateral flank. In a separate experiment, 5 × 10⁶ XBP1 wild-type or -knockout cells were implanted into separate mice. The XBP1-knockout cells were not able to grow as tumors, and the XBP1-knockdown cells demonstrated a 4-fold tumor growth delay. B, summary of the tumor volume and weights at the completion of the tumor growth delay experiments. C, comparison of in vitro growth rates between XBP1 wild-type and -deficient cells. D, comparison of secretion of proangiogenic factors VEGF and basic fibroblast growth factor in the XBP1 control and -knockdown cells. Error bars represent 1 SD of the mean from two independent experiments.
ROLE OF XBP1 DURING HYPOXIA AND TUMORIGENESIS

Clonogenic survival was reduced 65-fold in the XBP1-knockout mouse embryonic fibroblasts compared with wild-type mouse embryonic fibroblasts during severe hypoxia/anoxia. (Fig. 3B). To further support the role of XBP1 in mediating survival during hypoxia, we also analyzed clonogenic survival in XBP1 wild-type mouse embryonic fibroblasts stably transfected with a siRNA vector to inhibit XBP1 expression. These XBP1 knockout cells (ix-XBP1) exhibited reduced expression of XBP1 (<10% compared with control cells) and showed a similar decrease (19-fold) in clonogenic survival during severe hypoxia/anoxia as compared with the knockout cells (Fig. 3B). Interestingly, although inhibition of XBP1 by siRNA reduced clonogenic survival under severe hypoxia/anoxic conditions, the small amount of XBP1 remaining affords significant protection to these cells. Therefore, complete loss of XBP1 has far more profound effects on survival than inhibition of XBP1.

We implanted XBP1 wild-type, XBP1-deficient, or XBP1 siRNA-transfected cells (either $2 \times 10^6$ or $5 \times 10^6$ cells) into the flanks of SCID mice to determine whether the differences that we found in clonogenic survival during hypoxic conditions in vitro translated into impaired tumor growth in XBP1-deficient cells. In each mouse, we implanted XBP1 wild-type cells in one flank and either XBP1-knockout cells or XBP1 siRNA-transfected cells (ix-XBP1) in the contralateral flank. We performed serial tumor measurements up to 63 days (XBP1-knockout cells; Fig. 4A, left panel) and 49 days (ix-XBP1, Fig. 4A, right panel) after implantation. The XBP1-knockout cells were unable to grow as tumors, whereas the ix-XBP1 cells showed a 4-fold delay in tumor growth rate. We attribute the slower tumor growth rate in the ix-XBP1 cells to incomplete suppression of XBP1 by the siRNA vector. The tumor weights at the time the mice were sacrificed are summarized in Fig. 4B. Mice bearing XBP1 wild-type tumors were also received injections of EF5, a nitroimidazole that forms intracellular adducts under hypoxic conditions (18). Immunohistochemical staining of these tumor sections with EF5- and XBP1-specific antibodies showed excellent colocalization of XBP1 expression with areas of hypoxia (data not shown).

To confirm that the differences in tumor growth rates could not simply be attributed to a difference in cell proliferative capacity, we compared the in vitro growth rates of XBP1 wild-type and XBP1-deficient cells. The growth rates between these cell types were similar. In fact, the XBP1-deficient cells grew slightly faster than the wild-type cells (Fig. 4C).

Because tumor growth requires cells to survive long enough to form a multicellular mass and secrete proangiogenic growth factors, we investigated whether the differences in tumor growth between XBP1 wild-type and -deficient cells were due to a defect in the secretion of proangiogenic factors such as VEGF or basic fibroblast growth factor. We found that this was not the case as XBP1-deficient cells actually secreted a higher basal level of fibroblast growth factor, whereas VEGF secretion was similar at baseline and in response to severe hypoxia/anoxia in XBP1 wild-type and -deficient cells (Fig. 4D).

Taken together, these studies strongly support a role for XBP1 in mediating survival during hypoxia and in regulating tumor growth. Before the establishment of an adequate blood supply, tumor cells must be able to survive in a microenvironment that is deficient in oxygen, ATP, and other essential nutrients. Under these conditions, the response to ER stress is critical for cell survival. The unfolded protein response pathway functions independently of HIF-1α and directly links XBP1 with tumorigenesis, leading us to conclude that hypoxic regulation of the unfolded protein response is fundamentally important in the development of tumors. Although the exact mechanism for this observation remains to be elucidated, other components of the unfolded protein response pathway are also likely involved. Furthermore, because XBP1-deficient cells were unable to grow as tumors, it is unclear whether XBP1 is essential for establishment of a tumor, regulation of its subsequent growth, or both processes. Future experiments are directed at defining this mechanism.

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