Hyperinducibility of Hypoxia-responsive Genes without p53/p21-dependent Checkpoint in Aggressive Prostate Cancer

Konstantin Salnikov, Max Costa, William D. Figg, and Mikhail V. Blagosklonny

Abstract

Hypoxia limits tumor growth but selects for higher metastatic potential. We tested the functional activity of hypoxia-inducible factor-1 (HIF-1) in prostate cell lines ranging from normal epithelial cells (PrEC), hormone-dependent LNCaP, hormone-independent DU145, PC-3 to highly metastatic PC-3M cancer cell lines. We found that HIF-1-stimulated transcription was the lowest in PrEC and LNCaP cells and the highest in PC-3M cells. The induction by hypoxia of the HIF-1-dependent genes Cap43 and GAPDH was the highest in the most aggressive PC-3M cancer cells. Because these advanced prostate cancer cell lines have lost p53 function, this further shifts a balance from p53 to HIF-1 transcriptional regulation, and a high ratio of HIF-1-dependent:p53-dependent transcription was a marker of the advanced malignant phenotype. Transient transfection of HIF-1α expression vector induced transcription from p21 promoter construct in prostate cancer cell lines. Furthermore, hypoxia slightly induced p21 mRNA in these cells. However, neither expression of p21 nor hypoxia caused growth arrest in PC-3M cells. Therefore, high inducibility of HIF-1-dependent genes, loss of p53 functions with high ratio of HIF-1-dependent:p53-dependent transcription, and loss of sensitivity to p21 inhibition is a part of hypoxia phenotype associated with aggressive cancer behavior.

Introduction

Tumor progression toward aggressive and metastatic potential is a fundamental process in neoplasia, but stimuli that drive this progression are poorly understood. Hypoxia limits tumor growth, and tumors with poor vascularization fail to grow and form metastases (1, 2). On the other hand, hypoxia selects for more aggressive and metastatic cancer phenotypes that are associated with poor prognosis (3). Hypoxia in tumors develops early because of inadequate vascularization of the tumor. The transcriptional response to hypoxia is mediated by HIF-1 (4, 5). Lack of HIF-1 retards solid tumor growth and vascularity because of the reduced capacity to produce VEGF during hypoxia (1, 2). Increased glycolysis may protect cells from hypoxia, and most glycolytic enzymes are HIF-1-dependent (5). Hypoxia inhibits cell growth and may cause a p53-dependent apoptosis (6, 7).

Taking into account that hypoxia, while limiting tumor growth, is inevitably associated with tumor progression, we envision the ability of cancer cells to survive hypoxia as a natural test that on successful completion allows further tumor progression. We propose that the adverse conditions associated with hypoxia provide a driving force for selection of aggressive, autonomous, and metastatic phenotypes. Interestingly, hypoxia and carcinogenic nickel exert almost identical effects on gene expression. Furthermore, nickel, a potent nonmutagenic carcinogen, induces gene expression, in part through HIF-1 transcription factor (8, 9).

Previously, we have demonstrated an increase in HIF-driven transcription versus a p53-driven transcription in nickel-transformed cells (8). If hypoxia plays a significant role in tumor progression, we predict that not only nickel-transformed cells but also natural human cancer cells would have HIF-1:p53 alterations. In fact, Zhong et al. (10) have demonstrated that elevated amounts of HIF-1α protein exist in PC-3 prostate cancer cells under normoxic conditions linking HIF-dependent transcription under normoxia with tumor progression (11). Here we evaluated HIF-1- and p53-dependent transcription in a panel of prostate cell lines ranging from normal PrECs to the most aggressive PC-3M cells, previously selected for increased metastatic potential in mice. The comparison of PC-3M cells with less aggressive cells revealed more pronounced “hypoxic” features of the aggressive cancer phenotype. Because hypoxia already exists in primary prostate carcinomas (12), our data suggest that an increased inducibility of HIF-dependent genes may be a hallmark of the hypoxia-driven selection. Furthermore, we have shown that rather high levels of HIF-1 are required for transcriptional activation of p21<sup>waf1/cip1</sup>. This activation occurs in prostate cancer cells in a p53-independent manner. The accumulation of p21 did not result in growth arrest in either PC-3M or DU-145 cells. Using flow cytometry, we have shown that prostate cancer cells lost their p21-dependent cell cycle control, whereas p53-dependent cell cycle control was still intact in these cells.

Materials and Methods

Cell Lines and Reagents. The human prostate cancer cell lines, LNCaP, DU-145, and PC-3 cells were obtained from American Type Culture Collection (Manassas, VA). PC-3M cells, a highly metastatic clone of PC-3 cells, were described previously (13). PrEC, a nontransformed primary cell line, were obtained from Clonetics (San Diego, CA) and incubated in PrECM medium with supplements according to supplier’s instructions. MEF and MEF HIF-1α−/− were obtained from Dr. R. Johnson (University of California San Diego) and were described previously (9). DFX was obtained from Sigma (St. Louis, MO) and prepared as a stock solution in water. Ad-p21, a wt p21-expressing adenovirus was obtained from Dr. W. S. El-Deiry (University of Pennsylvania, Philadelphia, PA), and viral titer was determined as described previously (14).

Plasmids and Transient Transfection. WWP-Luc, a p21 promoter-luciferase construct, was obtained from Dr. W. S. El-Deiry (University of Pennsylvania). Bax-Luc, a Bax promoter-luciferase construct was obtained from K. Vousden (ABL Basic Research Program, NCI-FCRDC). pC53-SN3, containing wt p53 in a pCMV-Neo-Bam vector, was obtained from Dr. B. Vogelstein (Johns Hopkins University Baltimore, MD). pCMV-Vb.HA-HIF-1α expression plasmid was obtained from Dr. D. Livingston (Dana-Farber Cancer Institute, Boston, MA). pCMV-β-galactosidase was purchased from Clontech (Palo Alto, CA). GFP-expressing plasmid was obtained from Promega.
A HIF-responsive, VEGF promoter-derived luciferase construct containing four amplified HIF-1 binding sites (VEGF-Luc), inserted into a pGL2-promoter vector (15) was obtained from A. J. Giaccia (Stanford University, Palo Alto, CA). A partial VEGF promoter (p7) Luc construct was described previously (4) and obtained from Dr. G. Semenza (John Hopkins University, Baltimore, MD). A HIF-responsive, erythropoietin promoter-derived luciferase construct (Epo-Luc) inserted into a pGL3-Promoter vector was obtained from F. Bunn and E. Huang (Harvard Medical School, Boston, MA). A HIF-1 responsive element promoter luciferase construct (NOS-Luc or HRE-Luc), a gift from Dr. G. Melillo (National Cancer Institute), was described previously (8).

A total of 50,000 cells were plated in 24-well plates and, on the next day, were transfected with plasmids using Lipofectamine (Life Technologies, Inc.) or TransFast Transfection Reagent (Promega) according to the manufacturers’ recommendations. After 2–6 h of incubation with the plasmid-lipid suspension, the medium was changed, and cells were grown for an additional 16 h, unless otherwise indicated; then cells were lysed and analyzed for luciferase activity. For inducing HIF-1 transcription factor, cells were incubated with 260 μM DFX as described previously (8) or at 1% oxygen (hypoxia). All of the measurements were performed in duplicate.

**Immunoblot Analysis.** Proteins were harvested in TNESVF buffer [50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1% NP40] with protease inhibitors. For HIF-1α protein, nuclear extract was prepared as described previously (8). Equal amount of proteins were resolved on 7.5% SDS-PAGE for HIF-1α (8).

Immunoblot was performed using anti-HIF-1α antibodies (Oncora Vision, Fremont, CA).

**DNA Synthesis.** DNA synthesis was monitored by [3H]thymidine incorporation as described previously (14). In brief, 2,000 cells were plated in 96-well flat-bottomed plates, or 15,000 cells were plated in 24-well plates. The next day, cells were incubated under either normoxic or hypoxic conditions (1% oxygen) for 24 h and then were incubated with 1 μCi (methyl-3H)thymidine (Amersham) for an additional 4 h after which, acid-insoluble radioactivity was determined.

**Cell Cycle Analysis.** Cells were harvested by trypsinization, washed with PBS, and resuspended in 75% ethanol in PBS and kept at 4°C for at least 30 min. Before analysis, cells were washed again and analyzed with PBS, resuspended, and incubated for 30 min in propidium iodide staining solution containing 0.05 mg/ml propidium iodide (Sigma), 1 mM EDTA, 0.1% Triton X-100 and 1 mg/ml RNase A in PBS. The suspension was then passed through a nylon mesh filter and analyzed on a Becton Dickinson FACScan.

For cell cycle analysis of GFP-transfected cells, PC-3M were transfected with a vector-expressing GFP and cotransfected with vectors expressing either wt p53 or HIF-1α or with an empty vector. Cells expressing GFP were analyzed on a Becton Dickinson FACScan. Cells were excited at 488 nm. GFP and propidium iodide were measured at 520 nm and 585 nm, respectively. Cell cycle analysis was performed on 520-nm-positive cells.

**Northern Blotting.** Total RNA was extracted from cells immediately after treatment using RNezol B (Cinna/Biotek) and following manufacturer’s instructions, electrophoresed (15–20 μg of total RNA/lane) in 1.2% agarose/formaldehyde gels, and transferred to a nylon membrane in 7.5 mM NaOH buffer overnight. Probes representing a coding part of GAPDH or p21, or Cap43 gene were labeled with [α-32P]dCTP using a Random Primed DNA Labeling kit (Boehringer Mannheim). The membrane was prehybridized for 2 h, hybridized with the probe for 2 h, washed, and exposed to Kodak X-ray film overnight (9).

**Results**

**Basal and Inducible Levels of HIF-dependent Transcription.** To evaluate the functional activity of HIF-1 transcription factor in several prostate cell lines, we compared the expression of three HIF-1-dependent promoter-luciferase constructs (Epo-Luc, VEGF-Luc, HRE-Luc) in these cells. Because the expression of each promoter, especially Epo and VEGF, depends on the activity of multiple factors, we used all of the three constructs to determine a common trend. To normalize for transcription efficiency and other unrecognized factors, we also measured expression of a CMV-driven promoter-Luc construct. CMV-Luc expression was not significantly affected by hypoxia or hypoxia-mimicking conditions (data not shown). In contrast, all three of the HIF-dependent promoters were activated after exposure to DFX (hypoxic conditions; Fig. 1A). We observed several trends in the expression of these HIF-1-responsive constructs. First, basal expression of HIF-1-dependent constructs was very high in PC-3M, even compared with PC-3, cells and were the lowest in PrEC and LNCaP cells. Thus, the comparison of PC-3 cells with their highly metastatic subclone (PC-3M) demonstrates several-fold elevation in the basal levels of HIF-dependent transcription in PC-3M. Second, the inducibility of HIF-responsive constructs was minimal in PrEC but was high in all prostate cancer cells, including LNCaP, PC-3, PC-3M (Fig. 1A), and DU-145 (data not shown). Like the basal expression,
the induced expression of the HIF-1-responsive reporter constructs was especially high in PC-3M cells.

**Expression of Hypoxia-dependent Genes.** We next evaluated basal and hypoxia-inducible expression of the hypoxia-inducible genes such as GAPDH and Cap43 (9). In addition to HIF-1, numerous factors regulate expression of hypoxia-inducible genes, on transcriptional and posttranscriptional levels (e.g., mRNA stabilization). Thus, Cap43 mRNA was shown to be up-regulated by testosterone (16). Not surprisingly, basal levels of Cap43 mRNA was high in normal PrEC cells, which grow in the media containing testosterone. Only marginal induction of Cap43 by hypoxia was observed in these cells (Fig. 1B). Therefore, in contrast to cancer cells, normal prostate epithelial cells display testosterone-dependent rather than hypoxia-dependent regulation of Cap43. Basal levels of the GAPDH and Cap43 mRNA were higher in PC-3M and PC-3 cells than in LNCaP cells (Fig. 1B). Hypoxia induced expression of Cap43 in all cancer cell lines, particularly in PC-3 and PC-3M (Fig. 1B). Inducibility was much weaker for GAPDH than for Cap43. This indicates that Cap43 is very sensitive to hypoxia, whereas GAPDH, which encodes a glycolytic enzyme, is more constitutively expressed. Although the expression of the hypoxia-responsive genes under normoxic conditions did not differ dramatically in all of the cell lines, the inducible levels correlated with tumor progression. The induced levels of mRNA of these genes including GAPDH was the highest in PC-3M cells (Fig. 1B). In fact, exposure to hypoxia especially strongly stimulated acidification of the culture medium by PC-3M cells, reflecting high induction of glycolytic enzymes.

**Comparison of PC-3 and Highly Metastatic PC-3M Cells.** Previously we found that a ratio of HIF-dependent:p53-dependent transcription is increased in the nickel-transformed cells (8). Here we calculated the ratio of HRE-Luc (HIF-dependent):WWP-Luc (p53-dependent) transcription in prostate cell lines. We found that an increased ratio of HRE-Luc expression:WWP-Luc expression, especially under hypoxia, was a marker of the advanced cancer cell lines (Fig. 1C).

The high ratio in PC-3 and PC-3M cells in part determined by mutations in p53 in PC-3 and PC-3M. However, even in these two cell lines (with a similar background and p53 status) a higher ratio correlated with a higher metastatic potential of PC-3M. We further compared these two cell lines (Fig. 2A). Under hypoxic conditions, PC-3M cells had higher levels of expression of VEGF-Luc, Epo-Luc, HRE-Luc (Fig. 2A) as well as of HIF-1α protein (Fig. 2C) than PC-3 cells. Importantly, expression not only of HIF-dependent constructs but also of p21 promoter Luc construct (WWP-Luc) was higher in metastatic PC-3M than in PC-3 cells (Fig. 2B). This indicates that an increased ratio of HIF-dependent transcription:p53-dependent transcription (shown in Fig. 1C) is not a result of down-regulation of p21 expression. In contrast to p21, expression of another p53-dependent promoter, namely Bax, was not increased in PC-3M cells (Fig. 2B).

**HIF-1 Transactivates p21 Promoter.** High HIF-dependent transcription, which was associated with higher expression of p21 (but not of Bax) in p53-mutant PC-3M cells, may be a result of transactivation of p21 by HIF-1. In line with this suggestion, it has been proposed previously that HIF-1 may be involved in p21 induction because hypoxia induced p21 mRNA in parental EC cells but not in HIF-1-null EC cells (17); however, wt p53 status of these cells precluded firm conclusions. It has been shown that growth arrest caused by hypoxia does not depend on wt p53 (7). We observed that MEF cells lacking the HIF-1α gene (9) grow faster than wt MEF cells, with doubling time of 1.2 days versus 2.8 days, respectively. Hypoxia slightly induced p21 mRNA in these cells (data not shown); however, the wt p53 status of MEF cells precludes firm conclusions.

Here we demonstrate that transient transfection of HIF-1α-expressing vector induced p21-promoter-Luc construct in PC-3M cells (Fig. 2D). This is the first direct evidence that HIF-1 can transactivate p21 promoter. A dose-dependent response demonstrates that HIF-1α-expressing plasmid should be transfected in excess of p21-promoter construct to achieve its activation (data not shown), which indicates that a very high level of HIF-1 is required for p21-Luc activation.

Hypoxia slightly induced the p21 mRNA in these cell lines (Fig. 3A) supporting the notion that HIF-1 might be involved in p21-dependent inhibition of growth of normal cells (17). Nevertheless, hypoxia did not inhibit the proliferation of prostate cancer cells, as evidenced by unchanged [3H]thymidine incorporation immediately after hypoxia (Fig. 3A, bar graphs).

To further analyze the effect of hypoxia on the proliferation of prostate cancer cells, we used flow cytometry and transient transfections with HIF-1α or p53 expression vectors, or infection with p21 adenovirus. For flow cytometry analyses, cells were cotransfected with GFP-expressing plasmid to mark and analyze only transfected cells. Sub-G1 peaks observed in these experiments reflected toxicity.
that resulted from transient transfections with lipofectamine (Fig. 3B).
The cells transfected with HIF-1α did not differ from the cells transfected with empty vector only (control). In contrast, transfection with wt p53 induced G1 phase arrest in PC-3M cells (Fig. 3B).

Interestingly, the infection with Ad-p21-expressing p21 did not induce growth arrest in PC-3M cells (Fig. 3B), which suggests that, downstream, components of the p21-inhibitory pathway are impaired in these cells.

Importantly, hypoxia was accompanied by acidosis. It is not surprisingly that, in a high cell density, hypoxia caused acidification of the culture medium because of lactic acid production. Such acidosis induced G2 phase cell cycle arrest in PC-3M cells (Fig. 3C), with similar G2 arrest caused by lactic acid without hypoxia (data not shown).

Discussion

It has been shown that hypoxia arrests the growth of normal rodent fibroblasts but causes cell death in oncogene-transformed fibroblasts (18). These effects of hypoxia parallel the effects of growth factor withdrawal, i.e., growth arrest in normal cells and apoptosis in oncogene-transformed fibroblasts (19). In contrast to oncogene-transformed rodent fibroblasts, human cancer cells are selected in vivo for the most malignant phenotype. Thus, human cancer cell lines, with a few exceptions, neither arrest growth nor die after growth factor withdrawal (14). Here we show that human prostate cancer cells neither arrest growth nor die under hypoxic conditions. Such tolerance of hypoxia in the advanced prostate cancers is characterized by high hypoxia-induced levels of HIF-1-dependent transcription, loss of p53 function, and the inability of HIF-1 and p21WAF1/CIP1 to induce growth arrest.

We previously observed an increased ratio of HIF-driven transcription to p53-driven transcription in nickel-transformed cells (8). Here we described a hypoxic phenotype of prostate cancer cells with high inducibility of a HIF-dependent transcription, accompanied by the loss of wt p53 function and a low p53-dependent transcription. In brief, HIF-1 substitutes for p53, as a stress regulator, in highly metastatic prostate cancer cells.

Zhong et al. have described detectable expression of HIF-1α pro-

Fig. 4. Characterization of “hypoxic” phenotype in PC-3M cells. Normally, hypoxia induces HIF-1, which promotes both cell survival and growth arrest because of the activation of hypoxia-dependent genes including p21. At more severe hypoxia, stabilization of p53 contributes to apoptosis and may suppress HIF-1. After selection of “hypoxic” phenotype, p53 is lost or mutated, whereas HIF-1 is overexpressed. A balance is strongly shifted from p53 to HIF. Further evolution of the tumor is aimed toward the loss of p21-inhibitory effects, possibly downstream of p21, which allows cell survival without growth arrest.
tein in normoxic conditions (10), which leads to the notion that the increased HIF-dependent transcription is accompanying tumor progression. Here we found that in normal prostate epithelial cells, hypoxia only slightly affected two hypoxia-regulated genes, Cap43 and GAPDH, but dramatically increased their expression in cancer cells, further providing evidence that the increased HIF-dependent transcription is a part of tumor progression. Additionally, aggressive behavior corresponded to higher expression of HIF-responsive constructs in PC-3M and to very high ratio of HIF-1-dependent:p53-dependent transcription. Although p53 mutations in primary prostate cancer are relatively infrequent, they often occur at later, metastatic stages of the disease (20); therefore, prostate cancer progression indeed involves p53 inactivation (21).

Growth control is impaired in prostate cancer. Recently, p21 was shown to be significantly expressed in highly proliferating prostate tumors but not in normal or hyperplastic prostate epithelium (22). The expression of p21 did not correlate with wt p53, which suggests that other factors were involved in p21 up-regulation. Here we tested the direct ability of HIF-1 to activate p21 promoter construct and effects of hypoxia on p21 mRNA expression in p53-mutated prostate cancer cells. Indeed, we found that p21 is transactivated by HIF-1. The p21 promoter contains ACGTG sequence, which has been implicated in the regulation of lactate dehydrogenase A by hypoxia (23). Interestingly, HIF-1-null MEF cells grow faster than wt cells, which indicated that HIF-1 may inhibit proliferation. It has been shown that hypoxia failed to induce p21 in cells lacking HIF-1 but induced p21 in parental cells (17). However, both cell lines have wt p53, and, therefore, p21 induction can be attributed to wt p53 function. In this study, we found that hypoxia up-regulated p21 mRNA in DU-145 and PC-3M, both of which are cell lines with mutated p53. However, neither a high level of HIF-1α protein nor hypoxia arrested cell growth, which suggests that induction of p21 is dissociated from growth arrest in the advanced prostate cancer cells (Fig. 4). Similarly, despite the induction of p21, phorbol ester did not cause growth arrest in PC-3M, PC-3, or DU-145 cells (13) but caused p21-mediated growth arrest in LNCaP and PrEC. Our data are in agreement that 88% of prostate cancers have a high level of p21 that is dissociated from growth arrest (22). Furthermore, whereas HIF-1 negatively regulates the growth of normal fibroblasts (17), it is required for solid tumor growth independently of VEGF production (24). We conclude that the loss of growth-inhibitory components downstream of p21, along with increased HIF-1-dependent transcription, is a characteristic of aggressive metastatic phenotype in prostate cancer (Fig. 4).

Acknowledgments

We thank Drs. El-Deiry, Giaccia, Livingston, Semenza, Vogelstein, and Vousden for the plasmids and reagents used in the study. We also thank Robert Robey for assistance with the flow cytometry.

References


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