Increased Expression of Hypoxia Inducible Factor-1α in Rat and Human Prostate Cancer

Hua Zhong, Fatou Agani, Angelo A. Bacalla, Erik Laughner, Natalia Rioseco-Camacho, William B. Isaacs, Jonathan W. Simons, and Gregg L. Semenza

James Buchanan Brady Urological Institute Research Laboratories [H. Z., A. A. B., N. R.-C., W. B. L., J. W. S.], the Center for Medical Genetics [G. L. S.], Department of Pediatrics and Medicine [F. A., E. L., G. L. S.], and Oncology Center [H. Z., J. W. S.], Johns Hopkins Hospital, Baltimore, Maryland 21287-2411

Abstract

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that regulates genes involved in adaptation to hypoxia. Expression of HIF-1α was evaluated in rat and human prostate cancer cell lines. Increased expression of HIF-1α mRNA in rat prostate cancer cell lines and hypoxia-induced expression of HIF-1α protein in human prostate cancer cell lines are associated with increased cell growth rates and metastatic potential. HIF-1α mRNA was undetectable in the normal rat ventral prostate by Northern blot hybridization. HIF-1α protein expression and HIF-1 DNA binding activity were detected in normoxic PC-3 cells. Human prostate cancer cells plated at low density manifested higher functional HIF-1α expression than cells plated at high density independent of O2 tension. HIF-1α may become dysregulated in prostate cancer and thus drive the transcription of hypoxia-adaptive genes involved in tumor progression. This is also the first evidence that human cancer cells can express functional HIF-1α protein under normoxic conditions.

Introduction

Tissue hypoxia is critical in tumor formation, where it has been associated with malignant progression and resistance to radiotherapy and chemotherapy. For example, patients with cervical carcinomas measured in vivo to have pO2 <10 mm Hg have poorer disease-free survival (1). The intratumoral pO2 has been determined in different cancer xenograft models to vary between 14 mm Hg (2% O2) and 0 mm Hg (2, 3). These pO2 levels activate expression of HIF-1 both in vitro and in vivo (4–6). HIF-1α protein levels, which determine HIF-1 DNA binding activity and transcription of HIF-1-regulated genes, increase exponentially as intracellular pO2 is reduced (4, 7).

HIF-1 is a heterodimeric basic helix-loop-helix transcription factor that regulates many genes adaptive for hypoxic survival via binding to hypoxia response elements often located within the promoters of those genes (4, 8, 9). HIF-1-regulated genes include glucose transporters 1 and 3 and glycolytic enzymes such as LDH-A, pyruvate kinase, phosphofructokinase L, phosphoglycerate kinase 1, aldolase A, and GAPDH. These gene products are essential for the high glycolytic rates of cancer cells (Warburg effect; Ref. 10). Expression of these HIF-1-regulated glycolytic enzyme genes is thus essential in the bioenergetics of malignant transformation. Although the Warburg effect was described in solid tumor seven decades ago, little molecular information has been reported on altered gene expression in hypoxic cells of common solid tumors such as PCA. Recent studies suggest that HIF-1 is also involved in tumor angiogenesis and progression. HIF-1 activity-deficient hepatoma (Hepa-1) cells are suppressed in angiogenesis and growth characteristics (11, 12). Tumor vascularization may be stimulated by HIF-1α in part as a result of up-regulation VEGF (9, 11, 13, 14).

The pivotal role of HIF-1 in oxygen homeostasis suggests that its expression may be critical in the lethal phenotype of PCA. Several published results implicate HIF-1 in metastatic PCA, which causes the death of about 40,000 United States men yearly. First, in the only available spontaneously arising animal model of PCA, intratumoral pO2 levels are low enough to induce HIF-1 (15). In addition, pO2 values of the anaplastic and highly metastatic Dunning rat prostate tumors are even lower than those of well-differentiated and minimally metastatic tumors (15). Second, the endothelin-1 gene, which has previously been demonstrated to be involved in the pathophysiology of osteoblastic human PCA bone metastases (16), has been shown to be transcriptionally regulated by HIF-1 (17). Third, elevated GAPDH expression, which is regulated by HIF-1 (9), is associated with increased cell motility, invasion, and metastatic potential of rat prostatic adenocarcinoma (18). Fourth, another HIF-1-regulated glycolytic enzyme gene, LDH-A, has been used extensively as a serum marker of bone metastatic PCA disease activity (19). Yet, to our knowledge, analysis of HIF-1α expression in PCA has not been reported. Therefore, we tested the hypothesis that increased HIF-1α expression was associated with PCA progression and metastasis.

Materials and Methods

Cells, Culture Condition, and Animals. Dunning rat PCA cell lines (AT2.1, AT6.1, AT6.3, G, Mat-Lu, and Mat-LyLu) were generously provided by Dr. J. T. Isaacs (Johns Hopkins Oncology Center, Baltimore, MD) and were cultured as described previously (20). The human PCA cell lines (PC-3, DU-145, TSU, LNCaP, and PPC-1) were maintained with RPMI 1640 supplemented with 10% heat-inactivated FCS. The cells were subjected to hypoxia (1% O2 for 24 h), and Hep3B cells were described previously (4). The low-density (50% confluence with sparse cell-cell contact) and the high-density (90% confluence with plate-wide cell-cell contact) were monitored by phase contrast microscopy by two independent observers. Rat ventral prostates were isolated from Copenhagen (Harlan) rats at 8 weeks of age under methoxyflurane anesthesia. The prostate samples were immediately stored in liquid nitrogen. The animal study protocols were conducted according to approved institutional guidelines for animal use.

Total RNA Isolation and Northern Blot Analysis. Total RNA was isolated with RNeasy mini kit (Qiagen). Northern blot was performed as described previously (5). Human HIF-1α cDNA probe (593-bp HindIII/MstI fragment) and β-actin cDNA probe (1.8-kb; Clontech) were used. Autoradiographic signals were quantitated by Eagle eye computerized densitometry (Stratagen). The densitometric values of HIF-1α were normalized to the values of β-actin to control for variation in sample loading and transfer.

Immunoblot Analysis and EMSA. Crude nuclear extract and immunoblot analysis were performed as described previously (4). Proteins were detected
**Table 1** Biological characteristics of Dunning rat and human prostate cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Doubling time (days)</th>
<th>Androgen sensitivity</th>
<th>Metastatic ability</th>
<th>PSA*</th>
<th>Host survival (days)</th>
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<tr>
<td>AT2.1</td>
<td>2.5 ± 0.2</td>
<td>No</td>
<td>Low</td>
<td>NA</td>
<td>63 ± 3</td>
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<tr>
<td>AT6.1</td>
<td>4.0 ± 0.3</td>
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<td>High</td>
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<tr>
<td>AT6.3</td>
<td>4.0 ± 0.3</td>
<td>Yes</td>
<td>Low</td>
<td>NA</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>G</td>
<td>4.0 ± 0.2</td>
<td>Yes</td>
<td>High</td>
<td>NA</td>
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<tr>
<td>MatLu</td>
<td>2.7 ± 0.3</td>
<td>No</td>
<td>High</td>
<td>35 ± 1</td>
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<tr>
<td>Mat-LyLu</td>
<td>1.5 ± 0.1</td>
<td>No</td>
<td>High</td>
<td>26 ± 1</td>
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<tr>
<td>PC-3</td>
<td>1.1</td>
<td>No</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>DU-145</td>
<td>1.2</td>
<td>No</td>
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</table>

*References 20-25.

**Results**

**HIF-1α mRNA Expression Is Elevated in Rat and Human Prostate Cancer Cells.** HIF-1 consists of HIF-1α and HIF-1β subunits, and HIF-1α is the O2-regulated subunit (4, 5, 7). We first analyzed whether HIF-1α mRNA is expressed in cultured rat and human PCA cell lines as compared with normal prostate tissue. Dunning rat PCA cell lines are derived from a spontaneously arising parental R-3327 tumor (20). These sublines exhibit a wide range of tumor phenotypes with regard to androgen sensitivity, growth rate, histological and biochemical differentiation, and metastatic ability (Table 1). Expression of HIF-1α mRNA was found in every tested rat and human PCA cell line under standard culture condition (20% O2 and 5% CO2; 37°C), whereas no detectable HIF-1α mRNA was detected in the normal adult rat ventral prostate in two independent experiments (Fig. 1).

The basal levels of HIF-1α mRNA expression varied between the different cell lines. The Mat-Lu and Mat-LyLu cells have higher metastatic ability, faster growth rates, and higher basal HIF-1α mRNA levels than those sublines with low metastatic potential (AT2.1 and G) or those which have high metastatic potential but have slower growth rates (AT6.1 and AT6.3). The relative HIF-1α mRNA expression ratio of Mat-LyLu cells, which have the highest metastatic ability (>90%) and the highest growth rate (1.5 ± 0.1 days) among these cells, was 50% greater than that of G cells. G cells have the lowest metastatic potential (<5%) and slow growth rate (4.0 ± 0.2 days; Ref. 26).

**Expression of HIF-1α Protein in Human Prostate Cancer Cells.** HIF-1α protein expression and HIF-1 DNA binding activity were analyzed in cultured human PCA cell lines. Every cell line tested was

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**References 20-25.**

**PSA, prostate-specific antigen; NA, not applicable.**

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**Fig. 1.** HIF-1α mRNA expression in rat prostate cancer cell lines. All cells were incubated under standard culture conditions (20% O2 and 5% CO2; 37°C). Total RNA (15 μg) was isolated from normal rat prostate (Normal RP), Dunning rat prostate cancer cell lines (AT2.1, AT6.1, AT6.3, G, Mat-Lu, and Mat-LyLu), and human prostate cancer cell line (DU-145). RNA samples were blotted onto a nylon membrane and hybridized a 32P-labeled HIF-1α cDNA probe as described in "Materials and Methods." The blots were rehybridized under the same conditions with a 32P-labeled β-actin probe. Radioactive signals were detected by autoradiography and quantified by densitometry. The relative expression ratio represents the mean of two independent experiments.

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**Fig. 2.** Analysis of HIF-1α protein expression and HIF-1 DNA binding activity in human prostate cancer cell lines. Cells in high density were incubated under normoxic (+) or hypoxic (-) conditions (20% and 1% O2, respectively) at 37°C for 24 h before cell harvesting. In A, HIF-1α was detected in nuclear extracts by immunoblot assay. Relative density represents the mean of two independent experiments. In B, HIF-1 was detected in nuclear extracts by EMSA using an oligonucleotide probe. C, constitutive DNA binding activity, NS, nonspecific DNA binding activity. Thick arrow, HIF-1 DNA binding activity was detected in normoxic PC-3 cells.

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shown to induce HIF-1α protein expression and HIF-1 DNA binding activity in response to 24 h of continuous hypoxia (1% O₂), with Hep3B serving as a positive control (Fig. 2). The levels of hypoxia-induced HIF-1α protein at 24 h varied between the different human PCA cell lines. Hypoxia-induced HIF-1α protein levels were highest in PC-3 cells and lowest in LNCaP cells. Thus far, detectable HIF-1α protein in normoxic cells is only reported present in oncogene-transformed fibroblasts (12). Surprisingly, HIF-1α protein and HIF-1 DNA binding activity were detected in PC-3 cells under normoxic conditions (20% O₂; Figs. 2 and 4). Furthermore, HIF-1α protein was also detected in both hypoxic and normoxic PC-3 cells by immunocytochemistry, with staining being prominent in the nucleus. The nucleoli appear to have the most intensive staining (Fig. 3).

In conducting these experiments, we noted that another factor affected the degree of induced HIF-1α expression in PCA cells: cell plating density in vitro. HIF-1α protein levels and HIF-1 DNA binding activity were assayed in cultured cells at different plating densities. In both PC-3 and LNCaP cells, which express respectively the most and least induced protein, increased HIF-1α protein and HIF-1 DNA binding activity were present in cells plated at low density compared with cells plated at high density under both hypoxic and normoxic conditions (Fig. 4).

Discussion

In this study, we characterized the expression of HIF-1α and HIF-1 DNA binding activity in a panel of rat and human PCA cell lines that vary greatly in their phenotypes. Constitutive HIF-1α mRNA was expressed in every studied Dunning rat PCA subline at variable levels, which is consistent with their heterogeneity with respect to many biological properties including cell growth rates and metastatic potential. The lethal phenotype of PCA is associated with androgen independence. The only two available androgen-dependent cell lines (Dunning rat PCA cell line G and human PCA cell line LNCaP) had the lowest HIF-1α gene expression in mRNA (G cells) or protein (LNCaP cells) levels, suggesting that HIF-1α expression may increase in progression to androgen-refractory PCA. Our present results have shown that HIF-1α mRNA was expressed at higher levels in those rat PCA cells characterized by fast growth and high metastatic potential. In contrast, HIF-1α mRNA expression was not detectable in total RNA by Northern blot in normal adult rat ventral prostate. Furthermore, in human prostatectomy specimens, we have found lower HIF-1α mRNA levels in normal prostate tissue compared with adjacent cancer tissue. Taken together, these data strongly suggest that elevated HIF-1α mRNA expression in PCA cells may be important in their neoplastic phenotype and may be up-regulated in the process of cell transformation and tumorigenesis.

More than seven decades ago, Warburg demonstrated that there was an increased rate of glycolysis in tumor cells, resulting in the excess-

![Fig. 3. HIF-1α immunostaining in PC-3 cells. Cells were exposed to 20% (A and B) or 1% (C) O₂ for 24 h. A, negative controls with no primary antibody. Nuclear localization of HIF-1α staining shows intense staining in nucleoli. Arrow, nucleoli staining.](cancerres.aacrjournals.org)
sive production of lactic acid from glucose under normoxic conditions (10). The molecular genetics of the Warburg effect has remained poorly elucidated. Previous studies have demonstrated the ability of HIF-1 to up-regulate genes encoding virtually all of the glycolytic enzymes (7–9). GAPDH, ENO-1, and LDH-A mRNA expression are also up-regulated by hypoxia in all human PCA cell lines. These results, taken together, suggest that HIF-1α may play a critical role in mediating the Warburg effect in PCA.

Normally, HIF-1α protein expression is very tightly regulated by cellular O2 tension and is undetectable by Western blots in normoxic cells (4–6). Indeed, hypoxia induced stable and functional HIF-1α protein across the panel of biologically diverse human PCA cell lines. However, we discovered constitutive expression of HIF-1α protein in human PCA PC-3 cells at normoxic conditions (20% O2). To our knowledge, this is the first evidence that stable HIF-1α protein expression and function can be decoupled from O2 tension in human cancer cells, and yet can be further induced by hypoxia. Apparently, an O2-independent mechanism is affecting HIF-1α protein in PC-3 cells. Alterations in the HIF-1α gene sequence and potential signal transduction pathways in PC-3 cells are under investigation. Of note, PC-3 cells are cloned from a PCA bone metastases, and compared with the other human PCA cell lines studied, are characterized by the highest rates of Matrigel-independent xenograft formation, vascularization, and metastasis.6

Other genetic factors can influence HIF-1α gene expression. For example, the v-Src oncogene product has been demonstrated to increase the expression of HIF-1α mRNA and protein, HIF-1 DNA binding activity, and the expression of HIF-1-regulated genes (VEGF and ENO1) under both hypoxic and normoxic conditions (12). These observations suggest that genetic alterations in tumor cells may lead to increased HIF-1 activity, which may in turn allow tumors to adapt to tissue hypoxia, such that they can maintain cellular proliferation, prevent apoptosis, and undergo angiogenesis and metastasis. Heterogeneity of genetic alterations between our PCA cell lines may account for the observed differences between tumors in expression of HIF-1α mRNA and protein.

In addition to genes encoding glucose transporter, glycolytic enzymes, and VEGF (7–9, 13), HIF-1 target genes include those encoding inducible nitric oxide synthetase, heme oxygenase-1, and endothelin-1 (17, 27, 28). These genes encode very important factors for angiogenesis, vasodilation, tumor progression, and osteoblastic activation in PCA bone metastasis (16, 29, 30). Prostatic acid phosphatase has been used as a biomarker for osseous PCA metastasis, late stage, angiogenesis, vasodilation, tumor progression, and osteoblastic activity (17, 27, 28). These genes encode very important factors for angiogenesis, vasodilation, tumor progression, and osteoblastic activity

HIF-1α protein induced by hypoxia. In the present study, the cells with higher growth rates consistently manifested higher HIF-1α expression than the cells with lower growth rates. Interestingly, in embryonic stem cells, complete HIF-1α deficiency was associated with significantly decreased rates of cell proliferation (9).

These preliminary results have characterized HIF-1α gene expression in rat and human prostate cancer cells. Unexpectedly, HIF-1α protein expression is detected in normoxic PC-3 cells, suggesting that HIF-1α may be a direct or indirect target of genomic alterations occurring during tumor progression. The functional consequences of increased HIF-1α expression in PCA now require elucidation based on these studies. Characterization of the HIF-1α-regulated genes involved in cell cycle, apoptotic pathways, angiogenesis, motility, bioenergetics, signal transduction, cytokine expression, and metastases all may provide insights into the molecular mechanisms that allow PCA cells to adapt to hypoxia. Finally, the development of therapeutic approaches directed against HIF-1α in metastatic PCA may provide novel approaches to the management of this presently intractable malignancy.

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References

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