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HIF-1 α : A key survival factor for serum-deprived prostate cancer cells

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Abstract

Background—Hypoxia-inducible factor-1 α (HIF-1 α) is commonly overexpressed in prostate cancer (PCa) cells. As PCa cells are known to survive serum deprivation, we investigated the effect of prolonged serum deprivation on HIF-1 α expression, and the function of HIF-1 α in regulating the survival of normoxic serum-deprived PCa cells.

Methods—HIF-1 α protein was assessed by immunoblots. Cell viability and proliferation were assessed by trypan blue assay and flow cytometric analysis. Transcriptional activity was assessed by luciferase reporter assay and RT-PCR. HIF-1 α expression was suppressed with siRNA. Activities of HIF-1 α -target genes were inhibited with neutralizing antibody.

Results—Prolonged serum deprivation is a potent inducer of HIF-1 α in PC-3 and LNCaP PCa cells, despite normal oxygen conditions. In contrast, cells grown in the presence of serum did not show HIF-1 α protein accumulation. Moreover, HIF-1 α protein increase during serum deprivation correlated with increased cell survival, while suppression of HIF-1 α expression significantly decreased PCa cell viability. Our results further demonstrate that HIF-1 α protein increase is due to increased HIF-1 α protein synthesis. First, there was no significant increase in HIF-1 α mRNA. Secondly, cycloheximide, a protein synthesis inhibitor, prevented HIF-1 α protein increase in serum-deprived PCa cells. Moreover, the expression of HIF-1 α -target genes, VEGF and IGF-2, was concomitantly increased in serum-deprived PCa cells, while suppression of HIF-1 α expression significantly inhibited their induction. Furthermore, inhibition of IGF-2 activity resulted in a significant decline in PCa cell survival.

Conclusion—PCa cells counteract the stress of prolonged serum deprivation by upregulating HIF-1 α protein which increases IGF-2 expression to promote cell survival.

Keywords

HIF-1 α ; IGF-2; survival; serum deprivation; prostate cancer

INTRODUCTION

The hypoxia inducible factor (HIF)-1 is a key transcription factor that has been implicated in promoting tumor cell survival, proliferation and invasion following the onset of tumor hypoxia (1). HIF-1 is a heterodimer, consisting of a hypoxia-inducible HIF-1 α subunit, and a constitutively expressed HIF-1 β subunit (2–5). The degradation of HIF-1 α is regulated predominantly by O₂-dependent mechanisms (6,7). Under normoxic conditions, HIF-1 α protein is hydroxylated at two key proline residues by O₂-dependent HIF-1 α -prolyl

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hydroxylases (8,9). This hydroxylation serves to target HIF-1 α for proteasomal degradation (10). However, under hypoxic conditions, HIF-1 α -prolyl hydroxylase is inactivated thereby resulting in the stabilization of HIF-1 α (8,11). The stabilized HIF-1 α subunit translocates to the nucleus where it dimerizes with HIF-1 β subunit, and the dimer upregulates the expression of its target genes by binding to hypoxia response elements located in the promoter/enhancer regions of these genes (12).

The HIF-target genes have been shown to regulate various processes involved in tumor adaptation to hypoxia, such as glucose metabolism, tumor cell survival, proliferation and invasion (1). Increased HIF-1 α expression in PCa cells has been correlated with faster tumor growth and higher metastatic potential (13). HIF-1 α expression has also been observed to increase as prostate tumors progressed from androgen-dependent to androgen-independent states (14).

Tumors frequently outgrow their blood supply during the course of their progression to advanced states. This deficiency in blood supply can deprive tumor cells of oxygen and essential growth factors present in serum. Moreover, cancer cells can also be deprived of serum growth factors following treatments such as radiotherapy or anti-angiogenic therapy, as these treatment strategies frequently disrupt tumor vasculature (15,16). Limitations in growth factor availability and/or signaling can lead to cell death (17–19). However, studies have shown that PCa cells can survive prolonged serum growth factor deprivation (20).

An exogenous growth factor-deficient microenvironment is a relatively common occurrence in rapidly growing solid tumors, and HIF-1 α is commonly overexpressed in PCa cells when compared to the expression in the surrounding normal prostate epithelium. Therefore, this study investigated the effect of prolonged serum deprivation on HIF-1 α expression, as well as the function of HIF-1 α in regulating the survival of normoxic serum-deprived PCa cells.

MATERIALS AND METHODS

Reagents

HIF-1 α primary antibody was from Santa Cruz Biotechnology and anti- β -actin antibody was from Sigma. Secondary antibodies, horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG, M-PER mammalian protein extraction reagent and Supersignal West Femto Chemiluminescence substrate were from Pierce. Dual Luciferase reporter assay system, RNase A, oligo dT primers, random primers, dNTPs and reverse transcriptase were from Promega. Lipofectamine 2000 transfection reagent was from Invitrogen. HIF-1 α siRNA and control siRNA were purchased from Dharmacon. Propidium iodide was obtained from Roche. IGF-2 and VEGF neutralizing antibodies were obtained from R&D Systems.

Tumor cell lines and culture

The PC-3 and LNCaP PCa cell lines were obtained from ATCC. PC-3 and LNCaP cells were maintained in F-12K Nutrient Mixture (Kaighns Modification) (Invitrogen/Gibco) and RPMI (ATCC), respectively, supplemented with 10% fetal bovine serum (FBS), 100 μ g/ml streptomycin sulfate and 100 units/ml penicillin G sodium. All cultures were maintained in a humidified 5% CO₂ incubator at 37°C, and routinely passaged when 80–90% confluent.

Establishment of serum-deprived conditions

PCa cells were grown to 70–80% confluency in medium containing 10% FBS (complete medium). On day 0, the cells were first washed with serum-free (SF) medium and fresh SF medium was added. The cells were then grown under normoxic conditions for up to 4 days.

Cell cycle analysis

The PCa cells were harvested on day 0, 1, 2, 3 and 4, washed with PBS and fixed in 70% ethanol overnight at -20°C . The fixed cells were rehydrated by washing with PBS and resuspended in 1 ml of propidium iodide (PI) staining solution (20 $\mu\text{g}/\text{ml}$ PI and 20 $\mu\text{g}/\text{ml}$ RNase A in PBS) for 1 h at RT. Samples were then analysed by flow cytometry using the Beckman Coulter Cytomics FC 500 Flow Cytometer.

Assessment of cellular morphological changes

Cellular morphology was evaluated using phase-contrast microscopy, and photographs were captured with a computer-imaging system (Olympus Q-Color 3RTV camera and Adobe Photoshop for image analysis).

Trypan blue dye exclusion assay

Following culture in serum-free media, cells were washed with PBS, detached with trypsin-EDTA, neutralized with complete medium, centrifuged and re-suspended in PBS. An aliquot of cell suspension was diluted with 0.4% trypan blue (Sigma Aldrich), pipetted onto a hemocytometer and counted under a microscope at 40X magnification. Live cells excluded the dye, whereas dead cells admitted the dye and consequently stained intensely with trypan blue. The number of viable cells for each experimental condition was counted and represented on a bar graph.

Plasmid constructs and Luciferase reporter gene assay

pGL3-6xHRE-Luc, a generous gift from Dr. Peter Ratcliffe (University of Oxford), contained six copies of hypoxia response element (HRE) from the erythropoietin gene promoter linked to the thymidine kinase basal promoter and firefly luciferase gene (21). pRL-TK-Luc (Promega) was used as a transfection efficiency control and contained the thymidine kinase promoter linked to the renilla luciferase gene.

PC-3 and LNCaP cells were grown in 48-well plates in complete medium without antibiotics until 90% confluent. The cells were then transiently transfected with pGL3-6xHRE-Luc test plasmid along with pRL-TK-Luc as internal control using Lipofectamine 2000 (Invitrogen). At 4 h post-transfection, medium was replaced with fresh complete medium and incubation continued. At 24 h post-transfection, i.e. on day 0, day 0 serum-free cells were harvested, and for the day 1 serum-free analysis, cells were washed with SF medium, fresh SF medium added, and cells incubated under normoxic conditions for a further period of 24 h. Cells were harvested for the dual luciferase assay (Promega) to determine HRE-mediated transcriptional activity (procedure is as described previously) (22). Briefly, the firefly luciferase expression from pGL3-6xHRE-Luc, and renilla luciferase expression from pRL-TK were measured sequentially from a single sample, in a TD-20/20 Luminometer (Turner Designs) according to the Dual-Luciferase Reporter System protocol (Promega). The activity of pGL3-6xHRE-Luc was normalized to the activity of the pRL-TK internal control, and represented as relative luciferase activity on a bar graph.

Whole cell lysate preparation and immunoblot analysis

PC-3 and LNCaP cells were harvested by scraping and washed in ice-cold PBS. Cells were then lysed in M-PER reagent (Pierce Chemical, Rockford, IL) supplemented with protease inhibitors leupeptin (5 $\mu\text{g}/\text{ml}$), pepstatin (1 $\mu\text{g}/\text{ml}$) and aprotinin (1.7 $\mu\text{g}/\text{ml}$), and phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (2 mM). After a 15-min incubation on ice, lysates were cleared by centrifugation at 16,000 rpm, at 4°C . The resulting lysates were stored at -80°C until they were used for western blot analysis. Protein concentration was measured by bicinchoninic acid (BCA) assay (Sigma). HIF-1 α

immunoblotting was performed as described previously (22). After stripping, the blot was reprobed with β -actin antibody (Sigma) to ensure equal loading of proteins.

RNA isolation and RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA (2 μ g) isolated from cells was reverse transcribed to cDNA using oligo-dT and random primers. The cDNA was amplified by PCR using the following specific primers:

HIF-1 α : forward 5'-CTCAAAGTCGGACAGCCTCA -3'
reverse 5'-CCCTGCAGTAGGTTTCTGCT -3'

IGF-2: forward 5'-AGTCGATGCTGGTGCTTCTCA -3'
reverse 5'-GTGGGCGGGGTCTGGGTGGGTAG-3'

Primer sequences and PCR cycling parameters for VEGF and β -actin are as previously described (23). PCR cycling parameters for IGF-2 and HIF-1 α were as follows: IGF-2: 95 °C for 15 min, 94 °C for 45 s, 54 °C for 45 s, 72 °C for 1 min; 34 cycles. HIF-1 α : 95 °C for 15 min, 94 °C for 45 s, 53 °C for 45 s, 72 °C for 1 min; 30 cycles. The amplified products were visualized on 1% agarose gels.

siRNA transfection

PC-3 cells that were 40–50% confluent were transfected with 100 nM HIF-1 α siRNA or a negative control siRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen) transfection reagent. 24 h following transfection, cells were washed once with SF medium, and fresh SF medium was added. Cells were then incubated under normoxic conditions for the indicated time periods prior to analysis.

The siRNA transfection procedure for LNCaP cells was similar to that of the PC-3 cells, except that the LNCaP cells were transfected with 100 nM siRNAs for 2 consecutive days prior to serum deprivation.

RESULTS

Prostate cancer cells upregulate HIF-1 α protein levels under normoxic, serum-deprived conditions

To study the effect of prolonged serum deprivation on HIF-1 α protein expression in normoxic PCa cells, PCa cells were grown in serum-free (SF) medium for 1–4 days, followed by HIF-1 α protein analysis. We detected a significant, time-dependent increase in HIF-1 α protein levels in the serum-deprived PCa cells, despite normal O₂ conditions (Fig. 1A). We observed a 4.2-fold increase in HIF-1 α protein levels in PC-3 cells (Fig. 1B), and a 6.3-fold increase in LNCaP cells by day 4 of serum deprivation (Fig. 1C), when compared to HIF-1 α protein levels in PCa cells grown in the presence of serum, i.e. day 0 cells.

To determine if the increase in HIF-1 α protein levels in the normoxic, serum deprived PCa cells was mainly in response to serum deprivation, we assessed HIF-1 α protein levels in PCa cells of a similar confluency that were grown in the presence or absence of serum for 3 days under normoxic conditions. In contrast to the increase in HIF-1 α protein levels in serum-deprived PCa cells, we did not observe a significant increase in HIF-1 α protein levels in PCa cells that were grown in the presence of serum for the same time duration (Fig. 1D). This result indicated that the normoxic PCa cells upregulated HIF-1 α protein levels mainly in response to serum deprivation.

Serum-deprived PCa cells upregulate HIF-1 α protein levels by increasing HIF-1 α protein synthesis

To gain insight into the mode of upregulation of HIF-1 α protein during prolonged serum deprivation, we first assessed HIF-1 α mRNA levels by RT-PCR analyses to determine if the increase in HIF-1 α protein was due to increased transcription of HIF-1 α mRNA. We did not observe a significant increase in HIF-1 α mRNA levels during the entire duration of serum deprivation (Fig. 2A), suggesting that the increase in HIF-1 α protein during serum deprivation was not due to increased transcription of HIF-1 α mRNA.

We next assessed the effect of prolonged serum deprivation on HIF-1 α protein synthesis. PCa cells were serum deprived for 2 days, and then treated with 10 μ M cycloheximide (CHX), a protein synthesis inhibitor, for 0, 4, 8 and 24 h followed by cell lysate preparation. Vehicle-treated PCa cells served as control. As shown in Figs. 2B and 2C, CHX treatment abolished the serum-deprivation-induced increase in HIF-1 α protein when compared to that in the vehicle-treated cells (Fig. 2B and 2C). These results suggested that the increase in HIF-1 α protein levels during serum deprivation was most likely due to an increase in HIF-1 α protein synthesis.

Increase in HIF-1 α protein promotes PCa cell survival during serum deprivation

PCa cells have been known to survive prolonged serum deprivation (20). As HIF-1 α has been attributed with pro-survival roles during hypoxia (1), we investigated if the increase in HIF-1 α protein levels correlated with PCa cell survival during serum deprivation.

First, trypan blue dye exclusion assay revealed a modest increase in cell number during this stress (Fig. 3A, left and right panels). We determined a 1.9 and 1.4-fold increase in viable PC-3 and LNCaP cell numbers respectively by day 4 of serum deprivation when compared to the number of viable cells on day 0. The number of viable cells on day 0 was set at 1 (Fig. 3A, left and right panels).

Secondly, we employed flow cytometric analysis to determine cell-cycle distribution of the serum-deprived PCa cells. Interestingly, although a large percentage of the cells were in the G₀ phase of the cell cycle by day 4 of serum deprivation (78.9% for PC-3 and 75.5% for LNCaP), an appreciable percentage of G2-M phase PCa cells were still undergoing mitosis during serum deprivation (13.3% for PC-3 and 9.5% for LNCaP) (Fig. 3B, left and right panels).

Thirdly, we assessed morphology of the serum-deprived PCa cells. The PCa cells did not exhibit any obvious morphological changes characteristic of apoptosis such as reduction in cell volume or membrane blebbing. (Fig. 3C, top and bottom panels).

Lastly, we suppressed HIF-1 α expression by HIF-1 α -siRNA transient transfection, and determined viability of the serum-deprived PCa cells by the trypan blue dye exclusion assay. As shown in Fig. 4A, HIF-1 α suppression resulted in a significant decline in viable cell number in both the PC-3 (Fig. 4A, left panel) and LNCaP cells (Fig. 4A, right panel) when compared to the control siRNA-transfected cells. HIF-1 α suppression was shown by RT-PCR analyses (Fig. 4B, left and right panels).

Effect of HIF-1 α protein upregulation on HRE-mediated transcription, and expression of HIF-1 α -target genes, VEGF and IGF-2

HIF-1 α 's pro-survival role under hypoxic conditions has been attributed to increased transcription of pro-survival HIF-1 α -target genes such as vascular endothelial growth factor (VEGF) and insulin-like growth factor-2 (IGF-2) (1,24). We thus investigated

transcriptional activity of HIF-1 α , and expression of its target genes, VEGF and IGF-2, in normoxic, serum-deprived PCa cells. We performed a luciferase reporter gene assay to determine the transcriptional activity of the serum-deprivation-induced HIF-1 α protein. Interestingly, we observed a significant increase in HRE-mediated luciferase reporter gene activity in serum-deprived PC-3 as well as LNCaP cells when compared to that in the control cells (Fig. 5A, left and right panels). As HIF-1 α upregulates the expression of its target genes by binding to HREs found in the promoter regions of these genes, our luciferase assay results suggested that the HIF-1 α induced during serum deprivation was transcriptionally active.

We next investigated the expression of HIF-1 α -target genes, VEGF and IGF-2. RT-PCR analyses of the serum-deprived PCa cells revealed a marked time-dependent increase in the mRNA expression of VEGF as well as IGF-2 (Fig. 5B). We observed a marked increase in the mRNA levels of the VEGF₁₈₉ and VEGF₁₆₅ isoforms by day 4 of serum deprivation in the PC-3 cells (Fig. 5B, top left panel), whereas the LNCaP cells exhibited a modest increase in VEGF mRNA levels by day 4 (Fig. 5B, top right panel). We also observed an increase in IGF-2 mRNA levels in the serum-deprived PCa cells. Although the PC-3 cells exhibited an initial decrease in IGF-2 mRNA levels on day 1 of serum deprivation when compared to that on day 0, the IGF-2 mRNA levels continued to increase during the entire period of serum deprivation (Fig. 5B, bottom-left panel). The LNCaP cells exhibited a marked increase in IGF-2 mRNA levels during the entire period of serum deprivation (Fig. 5B, bottom-right panel).

To assess if the increase in VEGF and IGF-2 mRNA levels was due to HIF-1 α protein upregulation, we suppressed HIF-1 α expression by transient transfection with siRNA, followed by RT-PCR analyses of VEGF and IGF-2 mRNA levels in serum-deprived PCa cells. As shown in Fig. 5C, HIF-1 α suppression markedly decreased IGF-2 mRNA levels in serum-deprived PC-3 and LNCaP PCa cells when compared to that in the control siRNA-transfected cells. HIF-1 α suppression also appreciably decreased VEGF mRNA levels in PC-3 cells (Fig. 5C, left panel). On the other hand, HIF-1 α suppression did not markedly decrease VEGF mRNA levels in the LNCaP cells (Fig. 5C, right panel).

IGF-2 neutralizing antibody significantly inhibits survival of serum-deprived PCa cells

As VEGF and IGF-2 are known to promote survival of cancer cells under various stresses (20,24,25), we investigated if blocking the activity of these growth factors would inhibit PCa cell survival during serum deprivation. Inhibition of VEGF activity via use of VEGF neutralizing antibody during serum deprivation did not have any inhibitory effect on PCa cell survival, regardless of the dose of the VEGF neutralizing antibody used in the experiment (Fig. 6A). On the other hand, there was a significant decline in viability of the serum-deprived cells in the presence of IGF-2 neutralizing antibody when compared to the cell viability following control-antibody treatment (Fig. 6B).

DISCUSSION

As the name implies, the hypoxia inducible factor-1 α (HIF-1 α), is upregulated during hypoxia, and promotes cell survival under hypoxic stress (1,26). Our study demonstrates for the first time that prolonged serum growth factor deprivation is a potent inducer of HIF-1 α protein in normoxic PCa cells, and that HIF-1 α plays a key role in promoting PCa cell survival under this apoptotic stress. Our study further shows that the increase in HIF-1 α protein during serum deprivation occurs at the translational level, and that the HIF-1 α protein is transcriptionally active and upregulates the expression of its target genes, VEGF and IGF-2.

Our study presents several lines of evidence to prove that upregulation of HIF-1 α protein in serum-deprived PCa cells is mainly due to increased HIF-1 α protein synthesis and not due to reduced degradation. First, a steady time-dependent increase in HIF-1 α protein levels was detected during serum deprivation (Fig. 1B and Fig. 1C). If the increase in HIF-1 α protein were due to reduced degradation, then HIF-1 α protein levels would remain constant during the entire course of serum deprivation, without further increase. Second, CHX, a protein synthesis inhibitor, abolished an increase in HIF-1 α protein levels in the serum-deprived PCa cells, indicating that continuous protein synthesis is required for HIF-1 α protein increase. On the other hand, proteasome-mediated degradation of proteins requires energy (ATP) consumption. It is possible that serum-deprived PCa cells may have reduced energy (ATP) and, therefore, decreased proteasomal activity. Thus, we cannot completely rule out the possibility that there is a reduction in HIF-1 α protein degradation during serum deprivation. Nevertheless, our data strongly argues that the increase in HIF-1 α protein levels in serum-deprived PCa cells is most likely due to increased HIF-1 α protein synthesis.

Serum deprivation-induced HIF-1 α upregulation occurs in androgen-independent PC-3 cells as well as in the androgen-dependent LNCaP cells thereby suggesting that the upregulation of HIF-1 α during prolonged serum growth factor deprivation is independent of the androgen-dependent status of the PCa cells. However, both the PC-3 and LNCaP cells lack a functional PTEN, a tumor suppressor and a negative regulator of the phosphatidylinositol-3-kinase (PI3K) signaling pathway (27). Studies show that the activation of the PI3K pathway increases HIF-1 α protein synthesis by increasing the rate of HIF-1 α mRNA translation in an O₂-independent manner (1,28). In light of these observations, our results suggest that PTEN deficiency may be a strong stimulus for PCa cells to upregulate HIF-1 α protein expression during stress of serum deprivation.

Furthermore, siRNA-mediated suppression of HIF-1 α expression in PC-3 and LNCaP cells during serum deprivation led to a significant decline in cell survival (Fig. 4A). Studies have demonstrated the pro-survival role of HIF-1 α in serum-deprived renal clear cell carcinoma (RCC) cells that are deficient in VHL, a key protein involved in HIF-1 α degradation (33,34). Thus, these observations as well as our current results point to an essential role of HIF-1 α in promoting cancer cell survival during stress of prolonged serum growth factor deprivation.

Surprisingly, although the serum-deprived PC-3 cells exhibited a marked increase in VEGF mRNA levels, correlating with HIF-1 α protein levels, there was only a modest increase in VEGF mRNA levels in the serum-deprived LNCaP cells. This differential regulation of VEGF expression in serum-deprived PC-3 and LNCaP cells is further reflected in the effect of siRNA-mediated suppression of HIF-1 α expression on VEGF mRNA levels. HIF-1 α suppression resulted in a marked decline in the mRNA levels of VEGF₁₈₉ and VEGF₁₆₅ isoforms in serum-deprived PC-3 cells when compared to that in the control (Fig. 5C). In contrast, HIF-1 α suppression brought about a modest decrease in the mRNA levels of VEGF₁₈₉ isoform, but no significant effect on the VEGF₁₆₅ isoform when compared to that in the control (Fig. 5C). It is not known as to why PC-3 and LNCaP cells differ in their expression of VEGF despite the observation that both cell lines have elevated levels of HIF-1 α protein during serum deprivation. One possible explanation is that HIF-1 α may not have a major role in the regulation of VEGF expression in LNCaP cells. A recent study by Mizukami *et al* reported that the expression of VEGF in hypoxic colon cancer cells is regulated through HIF-dependent and HIF-independent pathways (35). Thus, it is likely that the differential regulation of VEGF expression in different cell lines may be due to cell-type specific characteristics of the cell lines. Nonetheless, as HIF-1 α suppression markedly reduced mRNA expression of HIF-1 α -target genes, VEGF and IGF-2, in the serum-deprived PC-3 cells (Fig. 5C), and IGF-2 in serum-deprived LNCaP cells, our results indicate that the

increase in VEGF and IGF-2 mRNA expression during serum deprivation is largely mediated by HIF-1 α .

Interestingly, although there was a significant, time-dependent increase in VEGF and IGF-2 protein secretion during serum deprivation (unpublished data, Thomas, R and Kim, MH), a significant decline in PCa cell survival was achieved by the inhibition of IGF-2 activity, and not by the inhibition of VEGF activity (Fig. 6A and 6B). The concentration of neutralizing antibody used in the experiments were optimized based on the amount of protein secreted by the cell lines and also the neutralization dose₅₀ (ND₅₀) of the antibody as recommended by the manufacturer. This observation suggests that IGF-2 may act in an autocrine manner to promote PCa cell survival during serum deprivation. Although VEGF did not have any direct pro-survival effects on serum-deprived PCa cells, we cannot exclude the possibility that the VEGF secreted by the PCa cells may indirectly promote the survival of PCa cells within tumors due to their well-established roles in promoting prostate tumor angiogenesis (36–38).

We are currently investigating the mechanism by which HIF-1 α is upregulated during serum growth factor deprivation. The observation of increased expression of HIF-1 α -target gene products, VEGF and IGF-2, has pointed to the possible involvement of a HIF-1 α -dependent autocrine growth factor loop in HIF-1 α induction during serum deprivation. Future studies will help elucidate the precise signaling pathway involved in HIF-1 α upregulation.

Taken together, the data presented in this study demonstrates that HIF-1 α is a key survival factor for serum growth factor-deprived PCa cells, and thus our study provides a strong rationale for the therapeutic targeting of HIF-1 α under both normoxic and hypoxic conditions for more effective inhibition of PCa cell survival and proliferation.

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Figure 1.

Prostate cancer cells upregulate HIF-1α protein levels under normoxic, serum-deprived conditions. (A) PCa cells were cultured in serum-free medium for 0–4 days. Cells were harvested at the indicated time points, and whole cell lysates were analyzed for HIF-1α protein by western blot analysis. The blots were stripped and reprobed for β-actin, which served as a loading control. The western blot is representative of 1 of 3 experiments. (B and C) In PC-3 cells (B) and LNCaP cells (C), the intensity of the HIF-1α protein band at each time point was normalized to that of the β-actin band at the same time point and expressed as a fold-change, with HIF-1α expression on day 0 set at 1. Data are the means ± S.D. (n=3). * indicates p<0.05 and ** indicates p<0.01 versus day 0. (D) PC-3 cells were grown to 50% confluency in complete medium (medium +10%FBS), the medium was then replaced with fresh serum-free medium or fresh complete medium and cells were cultured for up to 3 days. Whole cell lysates were prepared at the indicated time points and assayed for HIF-1α protein

by western blot analysis as described in (A). β -actin served as a loading control. HIF-1 α expression in each sample was normalized to that of β -actin in the same sample, and HIF-1 α fold change in the absence or presence of serum was expressed relative to the HIF-1 α protein levels on day 1 serum free or day 1 complete medium respectively, which was set at 1.

Figure 2.

Serum-starved PCa cells upregulate HIF-1α protein levels by increasing HIF-1α protein synthesis. (A) PCa cells were cultured in serum-free medium for 0–4 days. Total RNA was isolated from the cells at the indicated time points, and HIF-1α mRNA levels in PC-3 cells (left panel) and LNCaP cells (right panel) were assessed by RT-PCR analyses. β-actin served as a control. (B) PC-3 PCa cells were cultured for 2 days in serum-free medium, and then treated with 10 μM cycloheximide or vehicle (ethanol) for 0, 4, 8 and 24 h followed by lysate preparation. Whole cell lysates were analyzed for HIF-1α protein expression by western blot analysis as described in materials and methods. β-actin served as a loading control. The blot is representative of 1 of 3 experiments. (C) The western blot results of HIF-1α fold change in the vehicle- and cycloheximide-treated PC-3 PCa cells were

summarized in a line graph. Data are the means $\pm$ S.D. (n=3). ** indicates $p<0.01$ versus 24 h vehicle-treated control cells.

(A)

(B)

(C)

Figure 3.

Increase in HIF-1 α protein promotes PCa cell survival during serum deprivation. (A) PCa cells were cultured in serum-free medium for 0–4 days. Cells were trypsinized at the indicated time points, and live cell numbers determined by trypan blue dye exclusion assay. Viable PC-3 (left panel) and LNCaP (right panel) cell numbers were then expressed as fold increase, with viable cell number on day 0 set at 1. Data are the means $\pm$ S.D. of triplicate wells/experiment, and each experiment was repeated at least twice. * indicates $p < 0.05$ versus day 0. (B) PCa cells were harvested after culture in serum-free medium for 0–4 days, and cell cycle distribution of the PC-3 cells (left panel) and LNCaP cells (right panel) was assessed by flow cytometry according to the procedure described in Materials and Methods. (C) PCa cells were cultured in serum-free medium for 0–4 days. Pictures of cellular

morphology were taken at the indicated time points for the PC-3 (top panel) and LNCaP cells (bottom panel).

Figure 4.

Increase in HIF-1 α protein promotes PCa cell survival during serum deprivation. (A) PC-3 (left panel) and LNCaP (right panel) cells were transfected with HIF-1 α siRNA or control siRNA, and then cultured for 2 days in serum-free medium under normoxic conditions. Subsequently, cells were trypsinized and live cell numbers determined by trypan blue dye exclusion assay. Cell numbers were represented on bar graphs. Data are the means $\pm$ S.D. of triplicate wells/experiment, and each experiment was repeated at least twice. * indicates $p < 0.05$, and *** indicates $p < 0.001$ versus control siRNA-transfected cells. (B) HIF-1 α expression in the siRNA-transfected PC-3 (left panel) and LNCaP cells (right panel) was determined by RT-PCR analyses. β -actin served as the control.

Figure 5.

Effect of HIF-1 α protein upregulation on HRE-mediated transcription, and expression of HIF-1 α -target genes, VEGF and IGF-2. (A) Firefly luciferase activity from pGL3-6xHRE-Luc was measured in control (day 0) or serum-deprived cells (day 1), and normalized to renilla luciferase activity from pRL-TK and represented as relative luciferase activity on a bar graph. Data are the means $\pm$ S.D. of triplicate wells/experiment. ** indicates $p < 0.01$ and *** indicates $p < 0.001$ versus day 0 relative luciferase activity. (B) PCa cells were cultured in serum-free medium for 0–4 days. Total RNA was isolated from the cells at each time point, and VEGF and IGF-2 mRNA levels in PC-3 cells (left panel) and LNCaP cells (right panel) were assessed by RT-PCR analyses. (C) PC-3 and LNCaP cells were transfected with HIF-1 α siRNA or control siRNA, and then cultured for 2 days in serum-free medium under

normoxic conditions. IGF-2, VEGF and HIF-1 α mRNA levels in the control siRNA and HIF-1 α siRNA-transfected PC-3 (left panel) and LNCaP cells (right panel) were assessed by RT-PCR analyses. β -actin served as the control.

(A) **(B)** **Figure 6.**

IGF-2 neutralizing antibody significantly inhibits survival of serum-deprived PCa cells. (A) PC-3 cells were cultured for 3 days in serum-free medium containing 0, 10, 30 or 60 ng/ml VEGF neutralizing antibody. Subsequently, live cell numbers were determined by trypan blue dye exclusion assay. Cell numbers were represented on bar graphs. Data are the means $\pm$ S.D. (n=3). (B) PC-3 cells were cultured in serum-free medium for 2 days in the presence of 3.3 μ g/ml IGF-2 neutralizing antibody or isotype-matched control antibody. Thereafter, cells were trypsinized, and live cell numbers determined by trypan blue dye exclusion assay. Cell numbers were represented on bar graphs. Data are the means $\pm$ S.D. of triplicate wells/

experiment, and each experiment was repeated at least twice. ** indicates $p < 0.01$ versus isotype-matched-control-antibody treatment.