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Award Number: DAMD17-98-1-8475

TITLE: Oncolytic Gene Therapy for Prostate Cancer

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Medicine

Baltimore, Maryland 21205-2196

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 98 - 31 Aug 99)	
4. TITLE AND SUBTITLE Oncolytic Gene Therapy for Prostate Cancer		5. FUNDING NUMBERS DAMD17-98-1-8475	
6. AUTHOR(S) Jonathan W. Simons, M.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University School of Medicine 600 N. Wolfe Street, Marburg 409 Baltimore, Maryland 21205-2196 E-MAIL: jsimons40@hotmail.com (cc: kcordwell@jhmi.edu)		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES <p style="text-align: center;">This report contains colored photos</p>			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The overall purpose of this award has been to study the molecular pharmacology of replication restricted adenoviral vectors for prostate cancer gene therapy. The scope of the research is to identify relevant transcription units, which are prostate and cancer selective for the creation of potent oncolytic adenoviral vectors for ultimate translation to human clinical trials. Major findings of this award have been several. First, we have developed a first generation vector, CN706, which is prostate selective for PSA based on regulation of the E1A gene by the prostate specific enhancer promotor PSE from the PSA gene. Second, our DOD award research studies show antineoplastic action by killing by apoptosis. These vectors kill hormone refractory prostate cancer clones, which can be synergized by ionizing radiation at doses, which are clinically administered. We also have found these vectors have preclinical efficacy when given intravenously. Second, we have identified a new transcription unit, and the HIF gene as potent constructs and targets for improved gene therapy killing of hypoxic prostate cancer cells <i>in vitro</i> and <i>in vivo</i> . Our studies show that HIF is expressed as a gene therapy target in prostate cancer, but not normal prostate cells in humans and transgenic models. Basic research and creation of HIF PSA chimeric Ad 5 vectors has disclosed HIF is an important transcription target for oncolytic vectors. These new discoveries are currently being exploited in the creation of second generation vectors. The molecular pharmacology of oncolytic vectors suggests this is a new antineoplastic modality for hormone refractory prostate cancer.			
14. SUBJECT TERMS Prostate Cancer, gene therapy, adenoviral vector, PSA, HIF-1			15. NUMBER OF PAGES 41
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

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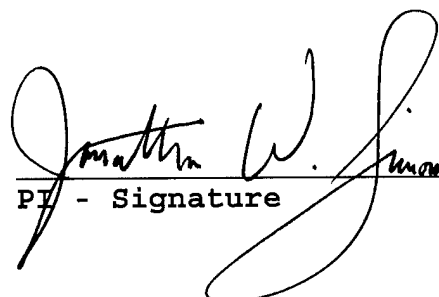

PI - Signature 10-6-99
Date

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INTRODUCTION

The overall purpose of this award has been to study the molecular pharmacology of replication restricted Adenoviral vectors for prostate cancer (PCA) gene therapy. Oncolytic gene therapy vectors kill prostate cells by replicating in tumor cells selectively. The scope of the research is to identify relevant transcription units, which are prostate and cancer selective for the creation of potent oncolytic adenoviral vectors for ultimate translation to human clinical trials. PSA is expressed by >95% of clinical prostate cancers, and is organ unique at the level of transcription. The 5' regulatory region of the PSA allows promotor specific cyto-reductive gene therapy research. Major findings of this award have been several. First, we have developed a first generation vector CN706, which is prostate selective for PSA based on regulation of the E1a gene by the prostate specific enhancer promotor PSE from the PSA gene. Our DOD award research studies show antineoplastic action by killing by apoptosis. This class of vector kills hormone refractory PCA clones. AD5 replication is essential as UV inactivated vectors have no activity on their own. This confirms the potential usefulness of combining oncolytic vectors with their own cytotoxic genomes with additional conventional therapeutic approaches to PCA in the clinic. We also have found these vectors have preclinical efficacy when given intravenously. Second, we have identified a new transcription factor, and its DNA binding sites, HIF-1. The HIF-1 gene overexpressed in PCA compared to normal tissue. HIF-1 binding elements can act as potent constructs and targets for improved gene therapy killing of hypoxic prostate cancer cells *in vitro* and *in vivo*. Our studies show that HIF-1 is expressed as a gene therapy target in PCA, but not normal prostate cells in humans and transgenic models. Basic research and creation of HIF-PSA chimeric Ad 5 vectors, has disclosed HIF is an important transcription target for oncolytic vectors. These new discoveries are currently being exploited in the creation of second generation vectors. We are currently studying HIF-1 as an important new target to block prostate cancer angiogenesis.

BODY

Described below are both negative, and important new positive finding of the research supported by this award. For ease of reference, the Task and its research milestones are listed, with the overall aim in italics prior to discussion of findings.

Task Number 1. *Evaluate the Preclinical Pharmacodynamics and Pharmacokinetics of CN706 as intravenous antineoplastics for CAP. (Months 1-24)*

1. **Prepare CN706 in macro cultures and titre for entire experiments. (1-3 months)**
2. **Define ED50 iv bolus months. (1-12 months)**
3. **Evaluate 293 microtitre vs E1A elisa in serum. (8-12 months)**
4. **Define t1/2 iv bolus. (12-16 months)**
5. **Evaluate effects of castration. vs intact. (14-18 months)**
6. **Prepare reports for investigator initiated FDA pre IND meeting. (14-18 months)**
7. **NIH RAC FDA IND filing for phase I trial trial, Simons P.I. (18-24 months)**
8. **Initial Manuscripts Submitted. (20-24 months)**
Estimated 10x2x12 athymic mice for statistically powered clinical trial simulations = 240+ animals.
9. **Evaluate optimum schedule and dose on LA-PC model and/or CWR22 model. (20-28 months)**
Estimated 10x2x5experiments with athymic mice for statistically powered clinical trial simulations = 100+ SCID or nu/nu mice.
10. **Compare histology and immunohistochemistry with data in task 2 for LNCaP for I.V. versus intratumoral delivery. (28-30 months)**
11. **Prepare Final Report and Manuscripts. (28-30 months)**

Under task number 1 we have successfully completed a number of steps. These steps have been completed along the projected time line. We have indeed been able to prepare CN706 in macrocultures and make clinical trial simulation stocks. We have used the 293 microtitre system, and have been able to generate high titre virus at $>1 \times 10^{13}$ particles per ml. Using this stock, we have defined the t1/2 given an IV bolus in clinical trial simulations. We have employed both the LAPC-4 (PSA expressing) and LNCaP (PSA expressing) cell lines. Importantly, the LNCaP model has different genetic alterations than the LAPC-4 model. It is a new model for prostate cancer gene therapy, and the DOD award research has supported its' testing. For example, the LAPC-4 model does not have an androgen receptor mutation, while the LNCaP model does.

We have generated data with the animals that suggest that the t1/2 for IV bolus is 1 hour. The data can now be used for translation to IV clinical trials. This data will be published in a forthcoming manuscript on the clinical trial simulations when the histologic evaluations are complete in item #10. Furthermore, we have identified the effects of androgen via both castration and *in vitro* by the use of R1881 and Casodex. This completes item #6. Data on the antineoplastic action and pharmacokinetics of CN706 given intravenously has been presented at a plenary session of the AACR Special Conference in Prostate Cancer by Dr. Simons in December 1998, and at the international 90th annual AACR meeting held 12 April 1999, in Philadelphia, PA. Reference may be made

to the data presented in the Proceedings of the *American Association of Cancer Research*, Volume 40, abstract #4156 (see Appendix 1), Ramakrishna, N *et al*, 1999. These findings are being submitted for publication

Positive New Findings. In conducting this research, we became aware that the HIF-1 gene could be a potential gene therapy target for vectors like CN706. This hypothesis was generated by the discovery that HIF-1 α could drive endothelin 1. The Simons laboratory had previously reported the endothelin 1 overexpression is involved in the metastatic phenotype of hormone refractory prostate cancer. Dr. Zhong, 100% supported by this DOD grant, made major discoveries on HIF-1 α as a candidate transcription factor for oncolytic vectors for this research award -- in addition to conducting the CN706 experiments. As described in Appendix 2, Dr. Zhong discovered that HIF-1 α was overexpressed in human prostate cancer cell lines and rat cell lines.

For the creation of the oncolytic vectors, a screening system of HIF-1 α -dependent genes specific for prostate cancer is required. Dr. Zhong has been able to, with RNase protection, develop a rapid high throughput screening evaluation system for calibrating hypoxic gene induction in prostate cancer cell lines. As described in Appendix 4, the baseline levels are non-hypoxia-inducible genes and can be set for arrayed genomics using either affymetrix based chips, or spotted arrays. In this DOD award, this data allows the direct search of HIF-dependent genes for the creation of oncolytic vectors for prostate cancer. Using this new technique, we can validate hypoxia-inducible prostate-specific genes. This data will be used to submit a separate NIH R01 grant on the identification of HIF-1 α -dependent genes in PCA by Dr. Simons' laboratory. This research area will not deflect from the completion of tasks 2 and 3, as these are well underway as described below. Nevertheless, the DOD award, and focusing the laboratory on the creation of transcriptionally unique vectors, has made a major difference in the identification of new transcription vector targets for antineoplastic gene therapy approaches to human prostate cancer.

Task Number 2. Evaluate *in vivo* the histology and mechanism of action of CN706 as a lead compound for Prostate Specific, oncolytic Ad5 gene therapy vectors.

1. Prepare CN706 in macro cultures and titre for entire experiments. (1-3 months)
2. Establish and treat xenografts for time course. (3-6 months)
3. Evaluate H+E at each time point. (7-10 months)
4. Perform immunohistochemistry on mechanisms of action. (10-18 months)
5. Quantitate with Apotag assay time course/mm3 apoptosis. (10-18 months)
6. Generate hypotheses for further mechanisms of action studies. (10-18 months)
7. Define post treatment histopathology for clinical trial database. (18-24 months)
8. Define optimum assays for human clinical trial biopsy material. (24-30 months)
9. Manuscript and Final Report. (24-30 months)

As described in Appendix 5 by Zhong and colleagues, we have established and developed expertise in immunohistochemistry, and interrogation at the molecular level of xenografts for all the relevant prostate cancer cell lines for

oncolytic vector molecular pharmacology. This now includes the new LAPC-4 model from UCLA, as well as PC-3, DU145, TSU, and the PPC1 xenograft. Steps 1 – 3 in task #2 have been completed. Currently, immunohistochemistry is being performed by Colleen Hanrahan, Sr. Research Technician, on mechanism of action. As described in Appendix 1, the Apotag TDT assay in fact does detect large apoptotic index changes at in 7 days post treatment. Co-localization studies using immunohistochemistry for the Ad5 hexon protein has suggested that high accumulation of hexon protein, and E1A protein is associated directly with prostate cancer and apoptosis. With respect to item #7, pilot data suggests that there is also an antivasular aspect. Currently, we are testing this new hypothesis that in the apoptotic death of CN706 infected PSA expressing prostate cancer cell lines, that there are microinfarctions. These microinfarctions may devascularize areas of uninfected tumor in a favorable fashion. We are currently looking at 2 new candidate markers of antivasular action, including the drop out of PE-CAM staining and the deposition of mouse compliment SC3 by immunohistochemistry. With this data in hand, item #9 will be possible with respect to the appropriate assays on immunohistochemistry for clinical trial monitoring.

Task Number 3. Evaluate selectivity and antineoplastic activity of a second generation PSE-E1A/PSE-E1B regulated Ad5 oncolytic vectors.

1. **Construct PSE1B plasmid.**
2. **Restriction digests and sequence of cloning joints.**
3. **Co-transfect and isolate single plaques.**
4. **Expand individual recombinant viral isolate.**
5. **PCR and Southern blot confirmation of PSE1A/PSEcloning**
6. **Sequence confirm cloning joints and open reading frames.**

7. **Macroculture expansion of lead compound clone.
Western blot confirmation PSE regulated Comparative Titre experiment
PSA+/PSA- cell lines.**
8. **R1881 androgen dependence of packaging.
(Steps 1-7, 1-16 months)**
9. ***In vitro* comparative cytotoxicity assays vs. CN702 and CN706**
10. ***In vivo* comparative antitumor action head to head CN706
(Months 16-20) N= 50 athymic mice.**
11. **Interim analysis of specificity and potency. (Months 16-20)**
12. **If candidate second generation antineoplastic PSE driven oncolytic virus, see steps
1-11 in Task Number 1. (Months 20-30) If less specific or less potent, construct
PSE-p53 wildtype E1A- Ad 5.**
13. **See steps 1-7, 9-11 starting with PSE-p53 plasmid creating sites for Eag1 using PCR.
(Months 20-30)**
14. **Prepare initial manuscripts, if study positive for superiority to CN706, prepare
investigator sponsored IND for FDA and final reports (Months 28-30).**

Given the surprising and important results with the HIF-1 α genome expression as a new transcription target for oncolytic adenoviral vectors attacking hypoxic prostate cancer cells, task 3 has only been completed through items 1 – 6. We have successfully generated the PCE E1B regulated virus in collaboration with Dr. D.C. Yu at Calydon, Inc. under a material transfer agreement signed with

Johns Hopkins. We successfully completed the creation of a HRE-PSE-E1a Ad5 virus. It appears to have lost specificity for PSA expressing cells (Drucker and Simons, unpublished observations). It replicates in breast and ovarian cells which do not express PSA but are likely HIF-1 activated. Thus the HRE of the enolase 1 gene used will overdrive the specific transcription elements of the prostate specific enhancer and promotor. We have thus concentrated on further basic research on the HIF-1 protein and pathway activation in cancers in order to inform better vector design as well as elaborate other therapeutic intervention strategies for prostate cancer therapeutics. Items 9-14 remain the same for task 3.

KEY RESEARCH ACCOMPLISHMENTS

- Proof of concept that prostate-specific PSA adenoviral vectors are antineoplastic. These vectors can kill intravenously.
- The vectors killed by apoptosis by direct viral replication can elements in the adenoviral vector can synergized with radiation for killing intratumorally.
- Vectors kill independent of androgen *in vivo* and *in vitro*.
- Oncolytic PSA selective Ad5 vectors can be monitored *in vivo* by biopsy and immunohistochemistry as intermediate endpoints for clinical trials.
- The HIF-1 α gene is a new transcription target for prostate cancer oncolytic vectors.
- HIF-1 α is overexpressed as an oncolytic vector target metastatic studies relative to primary tumors and is androgen independent making it an ideal transcription target.
- HIF-1 α is activated by an novel pathway used by oncogenes and blocked by some tumor suppressor genes: PIP3-AKT-1-mTOR and is inhibited by the angiogenesis inhibitor rapamycin.
- HIF-1 α may be a new biomarker for angiogenesis and can be detected by the monoclonal and immunohistochemistry technique we have developed in routine clinical specimens.

REPORTABLE OUTCOMES

- Manuscripts, Abstracts, Presentations

See Appendices 1 – 5

Appendix 1 – Ramakrishna, N.R., Rioseco-Camacho, N., Sawyers, C.L., Yu, D.C., Henderson, D., Simons, J.W., and DeWeese, T.L. Synergism of ionizing radiation and gene therapy with the replication competent CN706 adenovirus in the LAPC-4 prostate cancer cell line. *Proceedings of the American Association for Cancer Research*, #4156, Volume 40, March 1999.

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- patents and licenses applied for and/or issued

NONE

-degrees obtained that are supported by this award

NONE

-development of cell lines, tissue or serum repositories

NONE

-informatics such as databases and animal models, etc

NONE

-funding applied for based on work supported by this award

NIH R01 on HIF-1 α in prostate cancer to be applied for in 2000 based on this DOD award.

-employment or research opportunities applied for and/or received on experiences/training supported by this award.

NONE

CONCLUSIONS

The implications of this research are that replication competent adenoviral vectors could be an important new treatment for locally recurrent and metastatic prostate cancer following radiation or surgery. Over 40,000 men a year die from this clinical problem. Chemotherapy and radiation therapy are not entirely effective when the disease recurs. The research supported by this DOD award clearly shows that these selective adenoviral vectors kill by apoptosis like antineoplastic drugs do, but kill drug and hormone-resistant clones. Specifically, our data shows now that these do kill by apoptosis after replication, and can be effective in patients who are on hormonal therapy as the vectors can be PSA-specific, but kill independent of androgen levels. As 100% of advanced prostate cancer patients with obvious metastases have castrate levels of testosterone, this is an important new translational research discovery observation. Already, Dr. Simons has had preliminary conversations with the Food and Drug Administration and the Office for Recombinant DNA Activities for the requirements for a pharmacology toxicology section for an investigational new drug (IND) application for this approach. All of this has been supported by DOD award research.

At the molecular level, the DOD award research has shown that these vectors can be combined with radiation. Interestingly, a single dose of radiation, as described in Appendix 4, can highly synergize with CN706. This data now suggests that CN706, and second generation vectors like it, could be combined with radiation treatment by the radiation oncologist to expand the therapeutic index of radiation therapy. We are currently looking at the genomics of the virus in accelerating the death of prostate cancer cells following a single dose of sublethal radiation only. This data suggests that less radiation might be given for a greater therapeutic index if combined with vectors like CN706.

In addition to these important translational research results, which have direct potential for clinical translation, we have discovered an entirely new gene that may be useful as a target for new drugs as well used as a transcription factor to drive oncolytic vectors. The HIF-1 α gene was discovered during this DOD award research based on the identification of prostate overexpressed transcription factors, which might be used for Ad5 vectors. HIF-1 α is differentially expressed in tumors versus normal prostate in multiple models as described in Appendices 2-5. Adenoviral gene therapy requires the differential use of transcription factors. Tumors which are angiogenic are required to be killed by any gene therapy strategy. HIF-1 α has been associated with, and drives genes critical in angiogenesis like VEGF. Moreover, our data in the xenograft models in task 2 suggests that there is attenuation of efficacy in large tumor burdens which are hypoxic. We can detect HIF-1 α in clinical biopsies to potentially match treatment to the tumor. Thus, a new rationale has been created by this DOD award for the creation of HIF-1 α driven prostate-specific oncolytic Ad5 vectors. Other groups have tested hypoxia-

inducible constructs with suicide gene therapy and have found these to have potential activity *in vivo* and *in vitro*. With this DOD award, however, we find that oncolytic vectors and small molecules that inhibit directly HIF-1 action may be a rational new approach to antiangiogenesis gene therapy.

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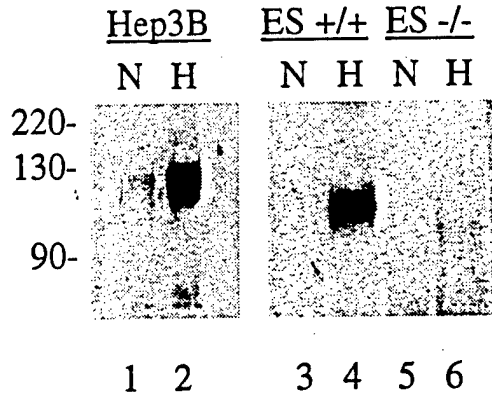
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#4156 Synergism of ionizing radiation and gene therapy with the replication competent CN706 adenovirus in the LAPC-4 prostate cancer cell line. Ramakrishna, N.R., Rioseco-Camacho, N., Sawyers, C.L., Yu, D.C., Henderson, D., Simons, J.W., and DeWeese, T.L. *The Johns Hopkins University Oncology Center, Baltimore, MD 21287; University of California Los Angeles School of Medicine, Los Angeles, CA 90095 and Calydon Corporation, Sunnyvale, CA 94025.*

A replication competent adenovirus CN706 preferentially replicates in PSA-positive prostate cancer cells. This vector is being tested in a phase I clinical trial utilizing stereotactic intraprostatic injection. We tested whether the efficacy might be augmented by combining cytoreductive gene therapy using CN706 with ionizing radiation in the PSA-positive LAPC-4 prostate cancer cell line. XRT was administered to LAPC-4 cells in doses of 1-4 Gy prior to or following viral infection with 0.1-10 moi of CN706. The combined effects of XRT and virus on cell growth were measured with a growth inhibition assay. Infection of the androgen receptor wild-type LAPC-4 cells with CN706 results in dose dependent growth inhibition and cell death. The use of virus alone or XRT alone resulted in decreased cell ATP to approximately 35% of that seen in untreated controls at 10 days post-infection. The combination of XRT and virus resulted in a decrease in cell ATP to approximately 8% of the untreated controls. The effect of the combination of radiation and viral infection on growth inhibition was approximately 35% greater than expected if the actions of both agents were purely additive. These data suggest that a combination of radiation and oncolytic virus may result in synergistic tumoricidal effects. Isobologram analyses and further experiments are underway to determine the underlying mechanisms for this interaction.

FIGURE 1

A



B

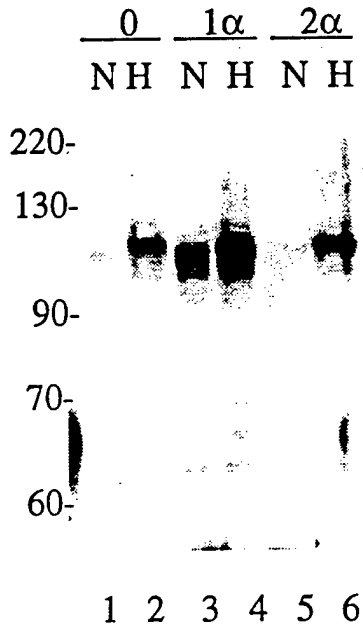


FIGURE 2

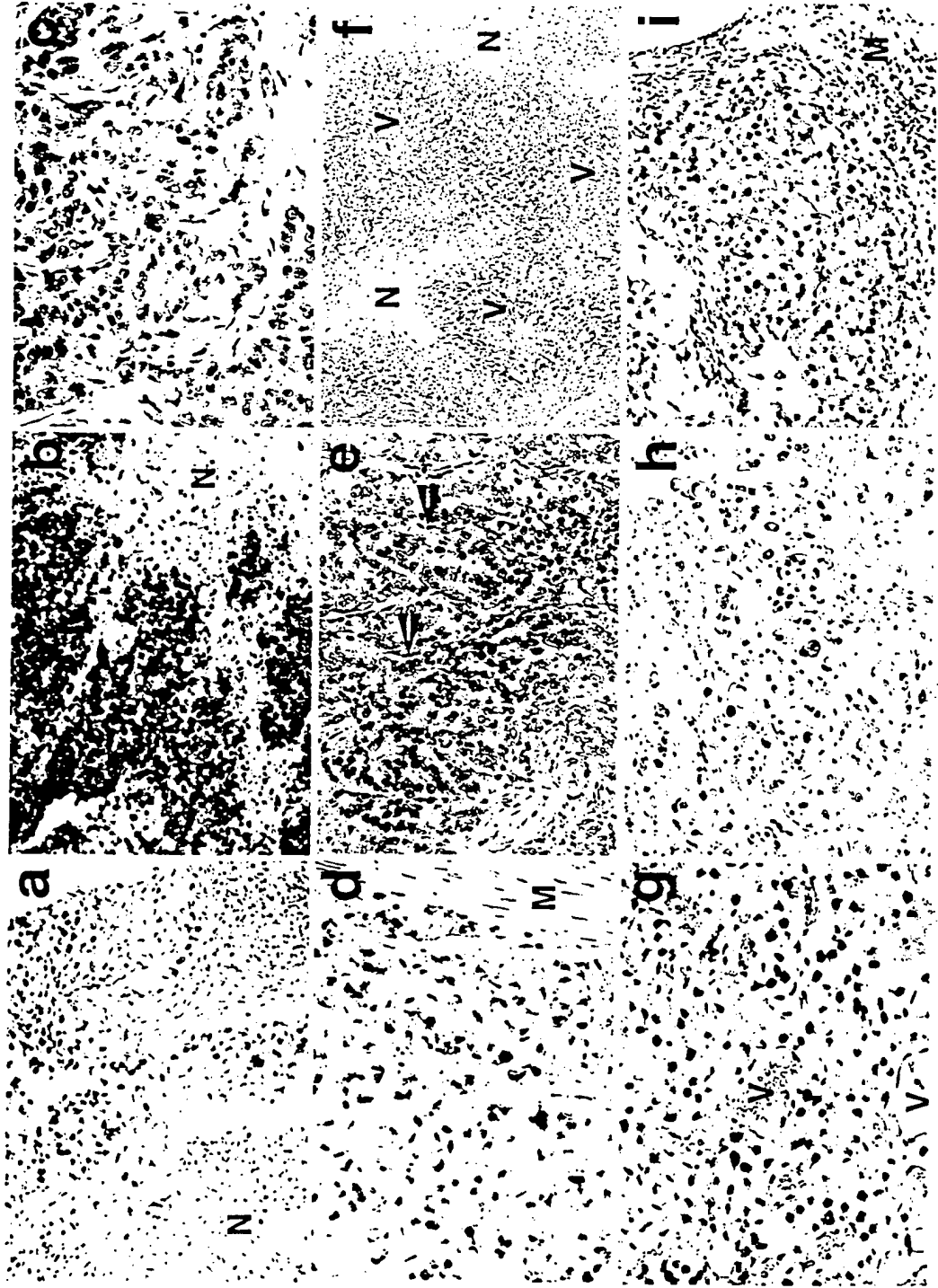
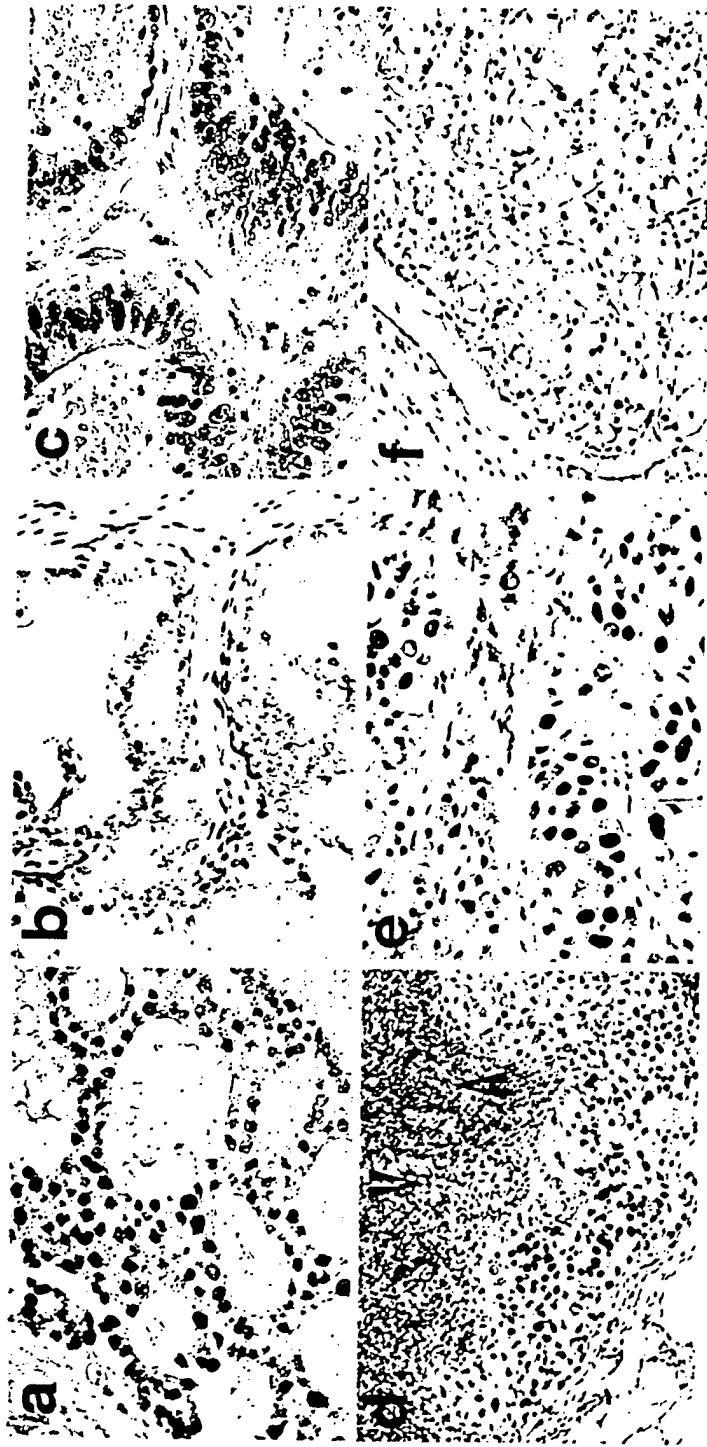


FIGURE 3



Increased Expression of Hypoxia Inducible Factor-1 α in Rat and Human Prostate Cancer¹

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that regulates genes involved in adaptation to hypoxia. Expression of HIF-1 α was evaluated in rat and human prostate cancer cell lines. Increased expression of HIF-1 α mRNA in rat prostate cancer cell lines and hypoxia-induced expression of HIF-1 α protein in human prostate cancer cell lines are associated with increased cell growth rates and metastatic potential. HIF-1 α mRNA was undetectable in the normal rat ventral prostate by Northern blot hybridization. HIF-1 α 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-1 α expression than cells plated at high density independent of O₂ tension. HIF-1 α 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-1 α 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-1³ both in *in vitro* and *in vivo* (4-6). HIF-1 α 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, ENO-1, pyruvate kinase, phosphofruktokinase 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-1 α 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-1 α expression in PCA has not been reported. Therefore, we tested the hypothesis that increased HIF-1 α 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 cells (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-1 α cDNA probe (593-bp *HindIII/MspI* fragment) and β -actin cDNA probe (1.8-kb; Clontech) were used. Autoradiographic signals were quantitated by Eagle eye computerized densitometry (Stratagene). The densitometric values of HIF-1 α 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

Received 9/16/98; accepted 10/16/98.

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¹ This work was supported by Prostate Cancer SPORE Grant CA-58236 from the NIH, a grant from the CaPCure Foundation, and Prostate Cancer Grant DAMD 17-98-1-8475 from the Department of Defense.

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³ The abbreviations used are: HIF-1, hypoxia-inducible factor 1; PCA, prostate cancer; LDH-A, lactate dehydrogenase A; ENO-1, enolase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCa, prostate cancer; VEGF, vascular endothelial growth factor; EMSA, electrophoretic mobility shift assay.

Table 1 Biological characteristics of Dunning rat and human prostate cancer cell lines^a

Cell lines	Doubling time (days)	Androgen sensitivity	Metastatic ability	PSA ^b	Host survival (days)
AT2.1	2.5 \pm 0.2	No	Low	NA ^b	63 \pm 3
AT6.1	4.0 \pm 0.3	No	High	NA	
AT6.3	4.0 \pm 0.3	No	High	NA	
G	4.0 \pm 0.2	Yes	Low	NA	120 \pm 10
MatLu	2.7 \pm 0.3	No	High	NA	35 \pm 1
MatLyLu	1.5 \pm 0.1	No	High	NA	26 \pm 1
PC-3	1.1	No		-	
DU-145	1.2	No		-	
TSU	1.5	No		-	
LNCaP	2.8	Yes		+	
PPC-1	1.3	No		-	

^a References 20–25.

^b PSA, prostate-specific antigen; NA, not applicable.

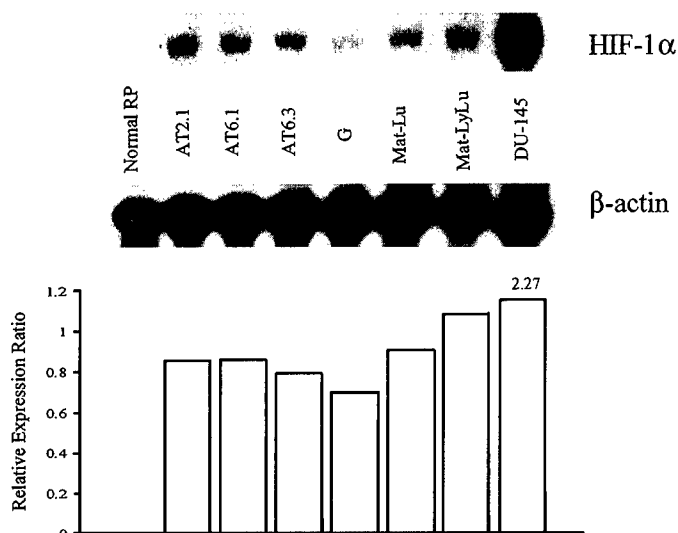


Fig. 1. HIF-1 α mRNA expression in rat prostate cancer cell lines. All cells were incubated under standard culture conditions (20% O₂ and 5% CO₂; 37°C). Total RNA (15 μ g) was isolated from normal rat prostate (Normal RP), Dunning rat prostate cancer cell lines (AT2.1, AT6.1, AT6.3, G, Mat-Lu, and Mat-LyLu), and human prostate cancer cell line (DU-145). RNA samples were blotted onto a nylon membrane and hybridized a ³²P-labeled HIF-1 α 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.

with anti-HIF-1 α (4) and reprobred with anti-topoisomerase I (Topogen). EMSA was performed using crude nuclear extract and oligonucleotide probe W18 as described previously (5).

Immunocytochemistry. PC-3 cells (0.5 \times 10⁶/ml) grown overnight on Lab-Tek chambered coverglass slides (Nunc.) were cultured for 24 h at 20 or 1% O₂, washed in cold PBS, fixed in ice-cold acetone for 5 min, washed in PBS again, and stored at 4°C for use within 1 week for staining. Immunostaining was performed using the rabbit ABC immunostain system (Santa Cruz Biotechnology), following the recommended protocol. Cells were incubated overnight with 1:100 dilution of rabbit HIF-1 α polyclonal antibody (4) in a humid chamber at 4°C. After the expected stain intensity developed, cells were counterstained with Mayer's hematoxylin.

Results

HIF-1 α mRNA Expression Is Elevated in Rat and Human Prostate Cancer Cells. HIF-1 consists of HIF-1 α and HIF-1 β subunits, and HIF-1 α is the O₂-regulated subunit (4, 5, 7). We first analyzed whether HIF-1 α 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-1 α 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-1 α mRNA was detected in the normal adult rat ventral prostate in two independent experiments (Fig. 1).

The basal levels of HIF-1 α 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-1 α 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-1 α mRNA expression ratio of Mat-LyLu cells, which have the highest metastatic ability (>90%) and the highest growth rate (1.5 \pm 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 \pm 0.2 days; Ref. 26).

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

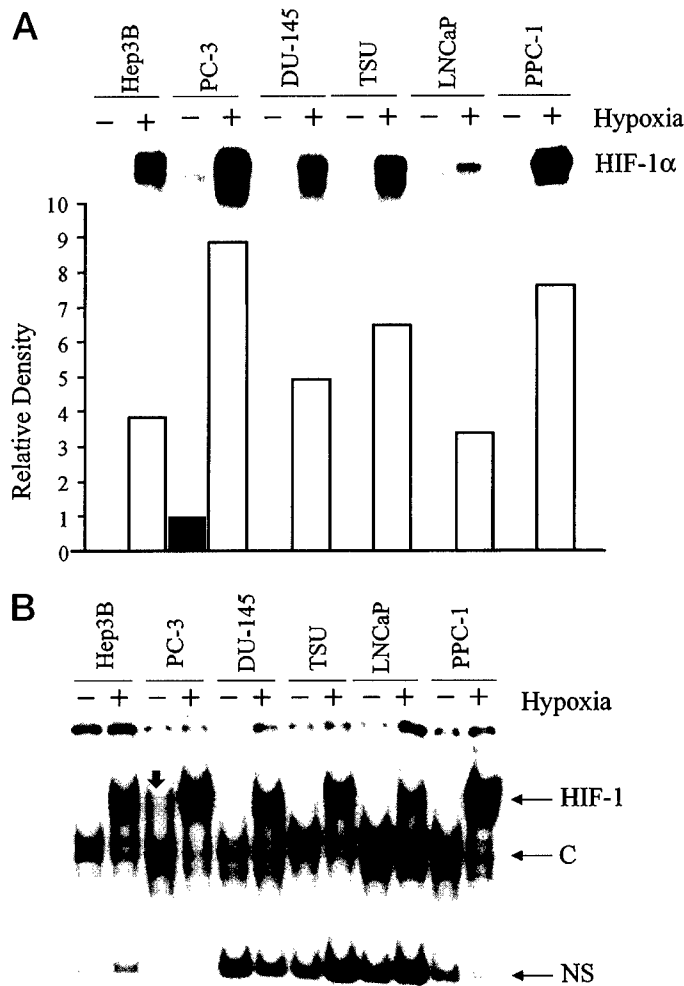


Fig. 2. Analysis of HIF-1 α protein expression and HIF-1 DNA binding activity in human prostate cancer cell lines. Cells in high density were incubated under normoxic (-) or hypoxic (+) conditions (20% and 1% O₂, respectively) at 37°C for 24 h before cell harvesting. In A, HIF-1 α was detected in nuclear 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.

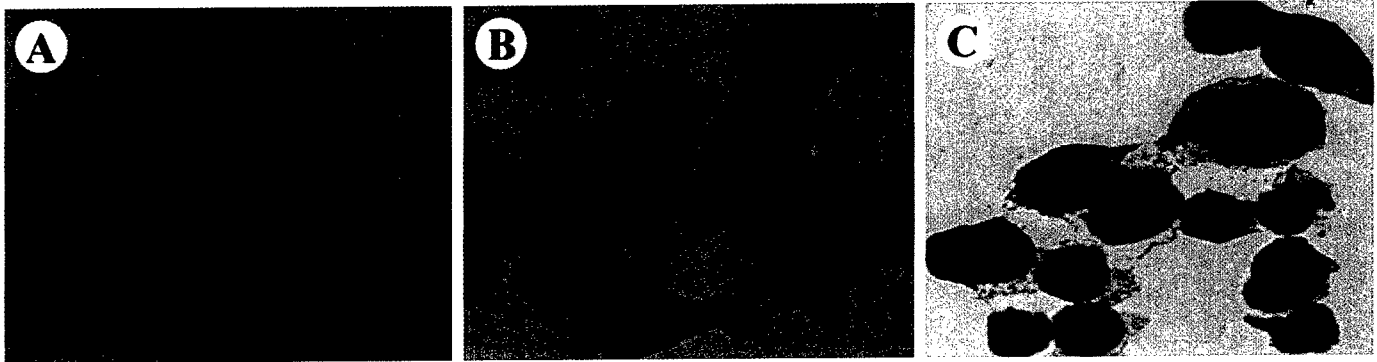


Fig. 3. HIF-1 α immunostaining in PC-3 cells. Cells were exposed to 20% (A and B) or 1% (C) O₂ for 24 h. A, negative controls with no primary antibody. Nuclear localization of HIF-1 α staining shows intense staining in nucleoli. Arrow, nucleoli staining.

shown to induce HIF-1 α 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-1 α protein at 24 h varied between the different human PCA cell lines. Hypoxia-induced HIF-1 α protein levels were highest in PC-3 cells and lowest in LNCaP cells. Thus far, detectable HIF-1 α protein in normoxic cells is only reported present in oncogene-transformed fibroblasts (12). Surprisingly, HIF-1 α protein and HIF-1 DNA binding activity were detected in PC-3 cells under normoxic conditions (20% O₂; Figs. 2 and 4). Furthermore, HIF-1 α 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-1 α expression in PCA cells: cell plating density *in vitro*. HIF-1 α 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-1 α 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-1 α and HIF-1 DNA binding activity in a panel of rat and human PCA cell lines that vary greatly in their phenotypes. Constitutive HIF-1 α 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-1 α gene expression in mRNA (G cells) or protein (LNCaP cells) levels, suggesting that HIF-1 α expression may increase in progression to androgen-refractory PCA. Our present results have shown that HIF-1 α mRNA was expressed at higher levels in those rat PCA cells characterized by fast growth and high metastatic potential. In contrast, HIF-1 α 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-1 α mRNA levels in normal prostate tissue compared with adjacent cancer tissue.⁴ Taken together, these data strongly suggest that elevated HIF-1 α 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 exces-

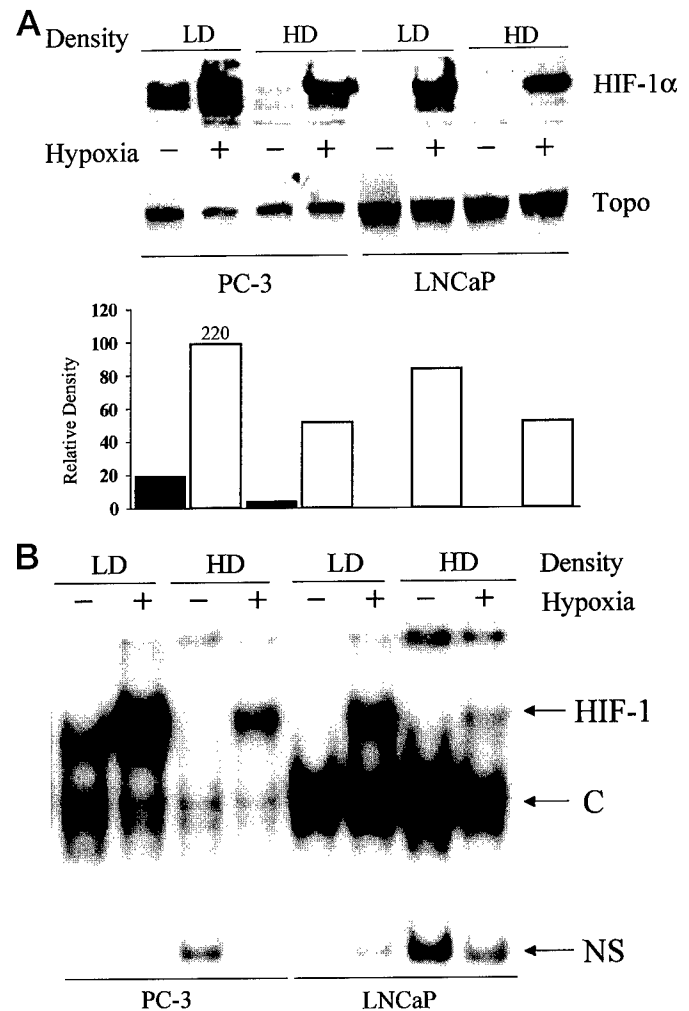


Fig. 4. HIF-1 α protein expression and HIF-1 DNA binding activity in human prostate cancer cells. Cells were incubated under normoxic (-) or hypoxic (+) conditions (20% and 1% O₂, respectively) at low density (LD) or high density (HD) culture conditions at 37°C for 24 h before cell harvesting. In A, HIF-1 α (top panel) was detected in nuclear extracts by immunoblot assay. Topoisomerase I (bottom panel) was detected after the same membrane was stripped. 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.

⁴ Unpublished data.

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 five human PCA cell lines.⁵ These results, taken together, suggest that HIF-1 α may play a critical role in mediating the Warburg effect in PCA.

Normally, HIF-1 α 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-1 α protein across the panel of biologically diverse human PCA cell lines. However, we discovered constitutive expression of HIF-1 α protein in human PCA PC-3 cells at normoxic conditions (20% O₂). To our knowledge, this is the first evidence that stable HIF-1 α 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-1 α protein in PC-3 cells. Alterations in the *HIF-1 α* 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-1 α* gene expression. For example, the v-Src oncogene product has been demonstrated to increase the expression of HIF-1 α 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-1 α 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, and tumor progression or regression in response to therapy in PCA. Coincidentally, acid phosphatase is induced in hypoxic tumor cells (31), but it is not known whether prostatic acid phosphatase gene expression is regulated by HIF-1. Prostate-specific antigen protein expression, which reflects prostate cell differentiation, is not apparently up-regulated by hypoxia in LNCaP cells (data not shown). Our demonstration of HIF-1 α protein and HIF-1 DNA binding activity in human hypoxic PCA cells provides a fundamental requirement for potential gene therapy approaches targeting hypoxic PCA cells using expression vectors containing HIF-1 binding sites (32).

We discovered greater HIF-1 α protein expression in cells plated at low density relative to high density. The positive regulation of HIF-1 α expression as a function of low cell plating density is not clear. Studies are under way to dissect the mechanisms involved. However, differences in signaling pathways involved in cell cycle kinetics and/or cell-cell contact inhibition may contribute to the differential in

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

These preliminary results have characterized *HIF-1 α* gene expression in rat and human prostate cancer cells. Unexpectedly, HIF-1 α protein expression is detected in normoxic PC-3 cells, suggesting that HIF-1 α may be a direct or indirect target of genomic alterations occurring during tumor progression. The functional consequences of increased HIF-1 α expression in PCA now require elucidation based on these studies. Characterization of the HIF-1 α -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 therapeutics directed against HIF-1 α in metastatic PCA may provide novel approaches to the management of this presently intractable malignancy.

Acknowledgments

We acknowledge the helpful comments of Dr. Donald S. Coffey and the assistance of Kimberly Cordwell in preparation of the manuscript. This work is dedicated to the memory, papers, and teachings of Efraim Racker.

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Overexpression of Hypoxia-inducible Factor 1 α in Common Human Cancers and Their Metastases¹

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ABSTRACT

Neovascularization and increased glycolysis, two universal characteristics of solid tumors, represent adaptations to a hypoxic microenvironment that are correlated with tumor invasion, metastasis, and lethality. Hypoxia-inducible factor 1 (HIF-1) activates transcription of genes encoding glucose transporters, glycolytic enzymes, and vascular endothelial growth factor. HIF-1 transcriptional activity is determined by regulated expression of the HIF-1 α subunit. In this study, HIF-1 α expression was analyzed by immunohistochemistry in 179 tumor specimens. HIF-1 α was overexpressed in 13 of 19 tumor types compared with the respective normal tissues, including colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas. HIF-1 α expression was correlated with aberrant p53 accumulation and cell proliferation. Preneoplastic lesions in breast, colon, and prostate overexpressed HIF-1 α , whereas benign tumors in breast and uterus did not. HIF-1 α overexpression was detected in only 29% of primary breast cancers but in 69% of breast cancer metastases. In brain tumors, HIF-1 α immunohistochemistry demarcated areas of angiogenesis. These results provide the first clinical data indicating that HIF-1 α may play an important role in human cancer progression.

INTRODUCTION

Altered glucose metabolism and cellular adaptation to hypoxia are fundamental to the basic biology and treatment of cancer. Four lines of evidence support this thesis: (a) clonal expansion of cancer cells depends on enhanced glucose transport and glycolysis (the Warburg effect; Refs. 1, 2); (b) tumors cannot grow beyond several mm³ without angiogenesis because of the limited diffusion of O₂, glucose, and other nutrients (3, 4). In many cancers, the degree of vascularization is inversely correlated with patient survival (5); (c) the probability of invasion, metastasis, and death are positively correlated with the degree of intratumoral hypoxia (6, 7), which is caused by an architecturally defective microcirculation such that even cells adjacent to neovessels may be hypoxic (8). Cancer cell proliferation may also outpace the rate of angiogenesis (3); and (d) tumor hypoxia is associated with resistance to chemotherapy, immunotherapy, and radiotherapy (9). Despite the critical importance of these observations, their molecular basis is not well understood. Transcription factors that regulate expression of angiogenic growth factors (such as VEGF³) or

glycolytic enzymes involved in the Warburg effect are compelling targets for interrogation. HIF-1 performs both of these functions.

HIF-1 is a bHLH-PAS transcription factor that plays an essential role in O₂ homeostasis (10-13). HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β subunits (10). Whereas HIF-1 β (also known as the aryl hydrocarbon receptor nuclear translocator) is a common subunit of multiple bHLH-PAS proteins, HIF-1 α is the unique, O₂-regulated subunit that determines HIF-1 activity (14, 15). HIF-1 transactivates genes whose protein products function either to increase O₂ availability or to allow metabolic adaptation to O₂ deprivation. Included among these are genes encoding erythropoietin, transferrin, endothelin-1, inducible nitric oxide synthase, heme oxygenase 1, VEGF, IGF-2, IGF-binding proteins -2 and -3, and 13 different glucose transporters and glycolytic enzymes (15, 16). Remarkably, most of these proteins are implicated in tumor progression (17). Analysis of isogenic tumor cell lines injected into nude mice revealed a dramatic correlation of HIF-1 expression levels with tumor growth and angiogenesis (18, 19).

Recently, we found that HIF-1 α mRNA was overexpressed in six rat PCA cell lines compared with the normal prostate, and metastatic potential was correlated with HIF-1 α mRNA levels in those cell lines (20). A human PCA cell line derived from a bone metastasis was found to overexpress HIF-1 α protein under nonhypoxic culture conditions (20). Because HIF-1 α expression was dysregulated in PCA cell lines, we tested the hypothesis that HIF-1 α is generally overexpressed in solid tumors. In this study, we screened HIF-1 α protein expression by immunohistochemistry in normal tissues and human cancers, including lung, prostate, breast, and colon carcinoma, which are the leading causes of U.S. cancer mortality.

MATERIALS AND METHODS

Production of Anti-HIF-1 α Mab H1 α 67. A human HIF-1 α cDNA fragment encoding amino acids 432-528 was cloned into pGEX2T. The GST/HIF-1 α fusion protein was purified from bacteria (14) and used to immunize BALB/c mice. Spleen cells from immunized mice were fused with P3X63-Ag8-653 myeloma cells. Hybridoma supernatants were screened by ELISA against GST and GST/HIF-1 α . Supernatant from clone 67 was affinity-purified using protein G-Sepharose (Pharmacia). The adsorbed protein was eluted with 0.1 M glycine-HCl (pH 2.7) and neutralized with 1 M Tris-HCl (pH 9.0). Nuclear extracts, prepared from human Hep3B and mouse ES cells (11), were subjected to immunoblot analysis as described previously (14) except that the primary MAb was H1 α 67 (1:500), and the secondary MAb was horseradish peroxidase-conjugated sheep antimouse immunoglobulin (1:2000).

Transient Transfection Assays. Human embryonic kidney 293 cells, growing essentially on 10-cm dishes, were transfected by calcium phosphate coprecipitation with 10 μ g of pCEP4 (Invitrogen), pCEP4/HIF-1 α (21, 22), or PL477 (23), a HIF-2 α expression vector that was generously provided by Dr. Christopher Bradfield (University of Wisconsin, Madison, WI). For reporter gene assays, the cells were cotransfected with pSV β gal and 2xWT33-luciferase, which contains two copies of a 33-bp hypoxia-response element from the human erythropoietin gene cloned upstream of a basal SV40 promoter (22).

Received 6/18/99; accepted 10/6/99.

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¹ This work was supported by NIH Grant R01-HL55338 (to G. L. S.), NIH Prostate Cancer SPORE Grant CA-58236, CaP CURE Foundation, and Department of Defense Prostate Cancer Grant DAMD 17-98-1-8475 (to J. W. S.). Under a licensing agreement between the Johns Hopkins University and Novus Biologicals, Inc., G. L. S. is entitled to a share of royalties received by the University from sales of the technology described in this publication. The terms of this arrangement are being managed by the University in accordance with its conflict of interest policies.

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³ The abbreviations used are: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; IGF, insulin-like growth factor; MAb, monoclonal antibody;

bHLH, basic helix-loop-helix; PCA, prostate cancer; GST, glutathione S-transferase; ES, embryonic stem; LI, labeling index.

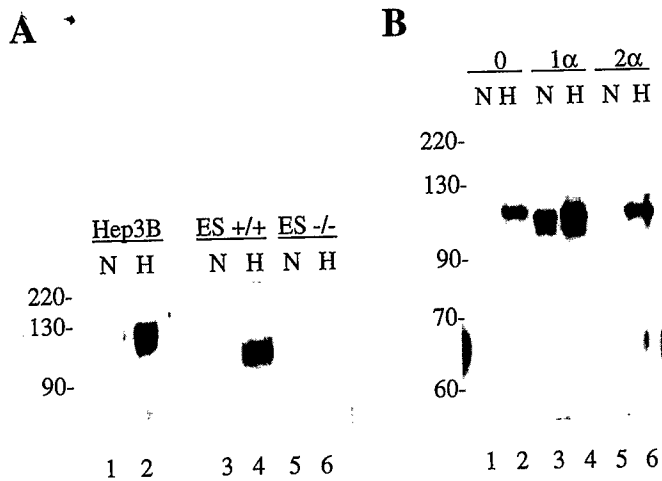


Fig. 1. Immunoblot detection of HIF-1 α by MAb H1 α 67. **A**, detection of HIF-1 α induced by hypoxia in wild-type Hep3B and ES cells but not in HIF-1 α -null ES cells. Human Hep3B cells and mouse ES cells that were either wild-type (+/+) or homozygous for a targeted mutation (11) that eliminated expression of HIF-1 α (-/-) were exposed to nonhypoxic (N; 20% O₂) or hypoxic (H; 1% O₂) culture conditions for 6 h before nuclear extract preparation and immunoblot assay using MAb H1 α 67. At left, migration of molecular weight markers (kDa). Migration of human and mouse HIF-1 α differed as previously described (34). **B**, detection of overexpressed HIF-1 α but not overexpressed HIF-2 α . Human 293 cells were transfected with an empty vector (0; Lanes 1 and 2) or expression vectors encoding human HIF-1 α (1 α ; Lanes 3 and 4) or human HIF-2 α (2 α ; Lanes 5 and 6). Transfected cells were incubated for 24 h under nonhypoxic (N) or hypoxic (H) conditions. Nuclear extracts were prepared and 30- μ g aliquots were subjected to immunoblot assay using MAb H1 α 67 at 1:500 dilution.

Table 1 HIF-1 α protein expression in normal human tissues

Tissues	N ^a	-	+	++	+++	++++	Positive cells
Adrenal	8	5		3			Cortical cells
Fetal liver	1		1				Hepatocytes
Kidney	9	6	1	2			Distal tubular epithelium
Pancreas	11	7		4			Acinar cells
Spleen	10	9		1			Proliferating B cells
Testis	7		7				Seminiferous tubules
Tonsil	3	1		1	1		Proliferating B cells
Brain	10	10					
Breast	18	18					
Heart	7	7					
Large intestine	24	24					
Liver	15	15					
Lung	10	10					
Ovary	10	10					
Pituitary	2	2					
Placenta	2	2					
Prostate	12	12					
Small intestine	1	1					
Stomach	1	1					
Thyroid	10	10					
Uterus	3	3					
Total	174	153	9	11	1	0	

^a N, number of cases analyzed; -, no staining; +, nuclear staining in less than 1% of cells; ++, in 1-10% of cells; +++, in 10-50%; +++++, greater than 50%.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue specimens were obtained and handled by standard surgical oncology procedures. Serial 4- μ m sections were prepared, and one was stained with H&E. Flanking sections were stained for HIF-1 α using Catalyzed Signal Amplification System (DAKO) which is based on streptavidin-biotin-horseradish peroxidase complex formation. In brief, after deparaffinization and rehydration, slides were treated with target retrieval solution (DAKO) at 97°C for 45 min, and the manufacturer's instructions were followed. MAb H1 α 67 (1 mg/ml) was used at a dilution of 1:1000. Nuclei were lightly counterstained with hematoxylin. Negative controls were performed using nonimmune serum or PBS instead of the MAb. A preadsorption test was also performed using GST/HIF-1 α protein. Twenty-four-well plates were coated with GST/HIF-1 α protein (2.9 mg/ml), air-dried, and incubated with H1 α 67 (1:1,000 dilution), followed by immunohistochemistry. Automated immunohistochemistry

was performed using a BioTek-Tech Mate 100 Automated Stainer (Ventana-BioTek Solutions, Inc., Tucson, AZ) with the following MABs: (a) anti-Ki67 (MAb MIB-1, Immunotech, 1:100); (b) antihuman p53 protein (MAb DO-7, DAKO, 1:250); (c) antihuman bcl-2 (MAb 124, DAKO, 1:25); (d) anticytokeratin (AE1/AE3, Boehringer Mannheim, 1:2000); and (e) anti-prostate-specific antigen (MAb 5126, Immunotech, dilution 1:50).

Three investigators (H. Z., A. M. D., and J. W. S) independently evaluated the immunohistochemistry. All of the PCA bone metastases were verified by cytokeratin and prostate-specific antigen staining. The immunohistochemical results for HIF-1 α protein were classified as follows: -, no staining; +, nuclear staining in less than 1% of cells; ++, nuclear staining in 1-10% of cells and/or with weak cytoplasmic staining; +++, nuclear staining in 10-50% of cells and/or with distinct cytoplasmic staining; +++++, nuclear staining in more than 50% of cells and/or with strong cytoplasmic staining. When independent scoring of a case differed, the case was rechecked, and the final score was determined by recounting HIF-1 α positive cells using a multiheaded microscope with all of the three reviewers simultaneously view-

Table 2 HIF-1 α protein expression in human tumors and their metastases

Tumor types	N ^a	-	+	++	+++	++++
Malignant primary tumors						
Prostate adenocarcinoma	11	2	5	1	3	
Colon adenocarcinoma	22		1	4	6	11
Breast adenocarcinoma	52	37	5	4	1	5
Lung adenocarcinoma	2					2
Lung small cell carcinoma	1					1
Renal clear cell carcinoma	1			1		
Pancreas carcinoma	5	1	2	2		
Ovarian carcinoma	2			1	1	
Gastric carcinoma	2		1			1
Brain tumors	9	4	2			3
Mesothelioma	1				1	
Melanoma	4		3	1		
Malignant fibrous histiocytoma	1		1			
Hepatocellular carcinoma	8	8				
Thyroid carcinoma	2	2				
Lymphoma	3	3				
Rhabdomyosarcoma	1	1				
Epithelioid sarcoma	1	1				
Carcinoid	3	3				
Malignant tumors in total	131	62	20	14	12	23
Metastatic tumors						
Lymph node metastases from:						
Prostate adenocarcinoma	1	1				
Breast adenocarcinoma	13	4	5	0	2	2
Colon adenocarcinoma	10	1	1	1	5	2
Bone metastases from:						
Prostate adenocarcinoma	10	6	2	1	1	
Vena caval invasion from:						
Renal clear cell carcinoma	1					1
Undifferentiated carcinoma	1					1
Metastatic tumors in total	36	12	8	2	8	6
Benign tumors						
Breast fibroadenoma	10	10				
Uterine leiomyoma	2	2				
Benign tumors in total	12	12				

^a N, number of cases analyzed; -, no staining; +, nuclear staining in less than 1% of cells; ++, in 1-10% of cells; +++, in 10-50%; +++++, greater than 50%.

Table 3 Relationship between expression of HIF-1 α and p53, bcl-2, and Ki67 in human cancers^a

	p53		bcl-2		Ki67 LI (%)			
	-	+	-	+	<5	5-15	15-50	>50
HIF-1 α (-) ^a	38	11	24	21	38	10	2	6
HIF-1 α (+/+)	3	9	8	2	9	8	7	3
HIF-1 α (+/+ +/+)	7	7	12	2	0	2	8	33
	P < 0.01 ^b		P = 0.05 ^b		P < 0.001 ^c			

^a -, no staining; +, nuclear staining in less than 1% of cells; ++, in 1-10% of cells; +++, in 10-50%; +++++, greater than 50%.

^b Kruskal-Wallis test.

^c Nonparametric Jonckhere-Terpstra test.

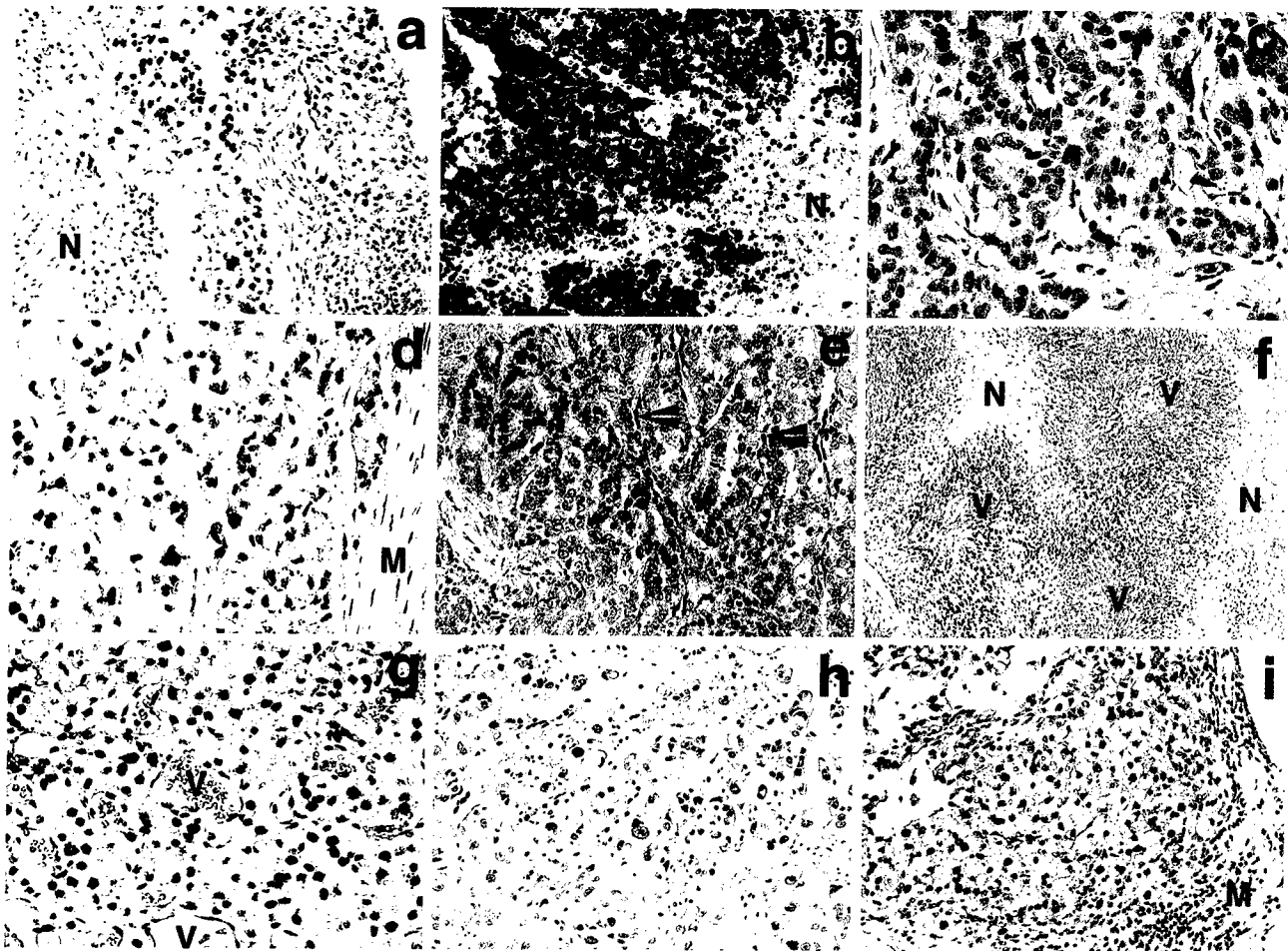


Fig. 2. Immunohistochemical analysis of HIF-1 α expression in common human cancers. Tumor sections were from: a, lung adenocarcinoma; b, lung small cell carcinoma; c, breast adenocarcinoma; d and e, colon adenocarcinoma; f, glioblastoma multiforme; g, brain hemangioblastoma; h, pancreas carcinoma; and i, renal clear cell carcinoma. N, necrosis; M, tumor margin; V, blood vessel; red arrows, stromal cells. $\times 100$ (f); $\times 200$ (a, b, h, and i); $\times 400$ (c, d, e, and g).

ing the slide. For Ki67 analysis, nuclei from approximately 1000 tumor cells from 10 randomly selected fields were counted, and the LI was determined as the percentage of positive nuclei. Bcl-2 reactivity was scored positive if $>10\%$ of tumor cells showed distinct cytoplasmic staining. Aberrant p53 accumulation was scored positive if nuclear staining was present in $>10\%$ of tumor cells.

Nonparametric statistical analyses were conducted by Dr. Steven Piantadosi, Johns Hopkins Oncology Center Biostatistics Center, using Microsoft Excel (Microsoft Corporation, Redmond, WA) and STAT-XACT, Version 4 for Windows (Cytel Software, Berkeley CA, 1998). As a singly ordered table, the Kruskal-Wallis test was used to evaluate the correlation between HIF-1 α and aberrant p53 or bcl-2 expression. As a doubly ordered table, the correlation between HIF-1 α protein expression and Ki67 LI was analyzed by Jonckheere-Terpstra test (24).

RESULTS

Characteristics of Anti-HIF-1 α MAb H1 α 67. A GST fusion protein containing amino acids 432–528 of human HIF-1 α was used as immunogen for MAb production. Five hybridoma clones were identified that reacted with GST/HIF-1 α but not with GST. Clone 67 was chosen for further characterization. MAb H1 α 67 was identified as IgG2b/ κ subtype and purified from hybridoma supernatants by protein-G affinity chromatography. Immunoblot assays demonstrated that MAb H1 α 67 recognized a hypoxia-induced protein of approximately M_r 120,000 that was identical in size to HIF-1 α , in Hep3B cells and wild-type ES cells, but not in HIF-1 α -null (11) ES cells (Fig. 1A).

MAb H1 α 67 showed reactivity against human, monkey, sheep, mouse, bovine, rat, and ferret HIF-1 α (data not shown).

MAb H1 α 67 also recognized human HIF-1 α purified 11,250-fold by anion-exchange and DNA-affinity chromatography (25) at concentrations too low to allow protein quantitation (data not shown). As a final test of its specificity, cells were transfected with expression vectors encoding no protein, HIF-1 α , or HIF-2 α (Fig. 1B). MAb H1 α 67 detected overexpressed HIF-1 α (Lanes 3–4), whereas cells overexpressing HIF-2 α (Lanes 5–6) gave the same pattern as cells transfected with the empty vector (Lanes 1–2). HIF-2 α expression in the transfected cells was confirmed by cotransfection of a reporter gene containing a hypoxia response element, which was activated 9- to 13-fold over background in cells transfected with HIF-1 α expression vector and 33- to 106-fold over background in cells transfected with the HIF-2 α expression vector (data not shown). These highly stringent tests provide convincing evidence that MAb H1 α 67 specifically recognizes HIF-1 α .

Screening of HIF-1 α Protein Expression in Normal and Malignant Human Tissues. HIF-1 α expression was extensively screened in normal tissues and human cancers resected during routine surgical oncology procedures. Twenty-one normal human tissues (174 specimens), 19 primary malignant cancers (131 specimens), and 36 metastases from 6 tumor types were interrogated (Tables 1 and 2). Most normal human tissues (14 types) showed no HIF-1 α immunoreactivity (153 of 174 clinical specimens, 88% negative). In some autopsy

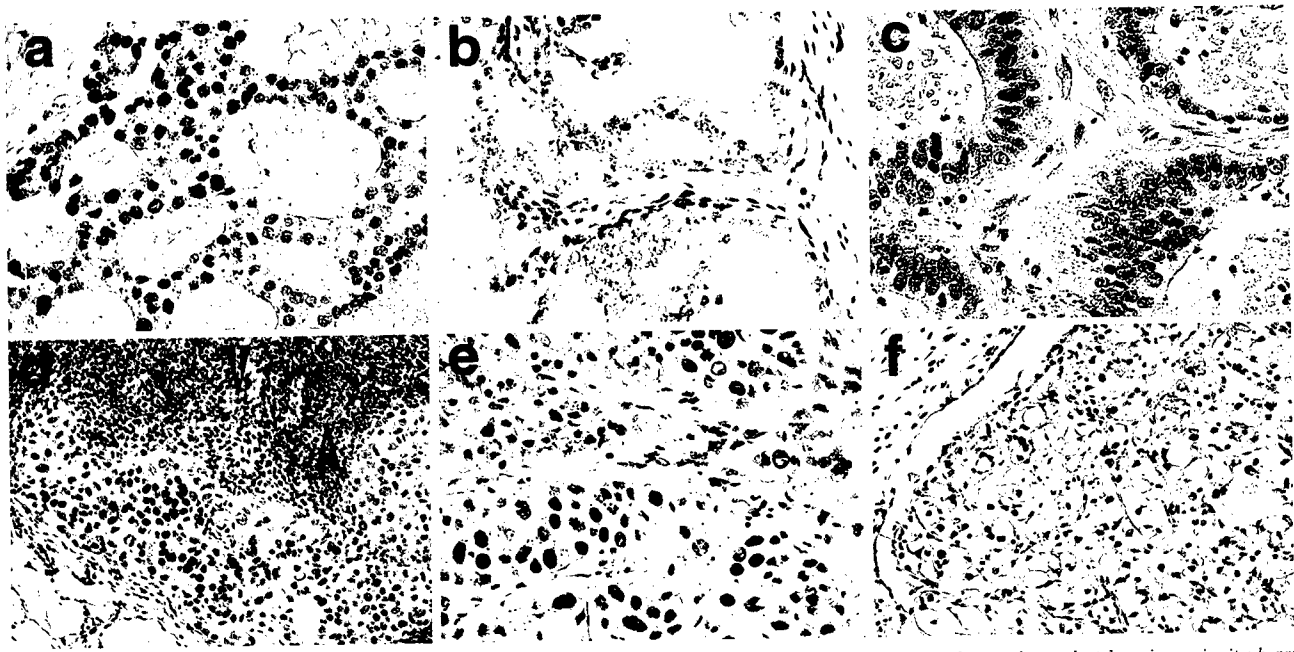


Fig. 3. Immunohistochemical analysis of HIF-1 α expression in preneoplastic lesions and cancer metastases. Analysis of sections from: a, breast ductal carcinoma *in situ*; b, prostatic intraepithelial neoplasia; c, lymph node metastasis from breast adenocarcinoma; d, lymph node metastasis from prostate adenocarcinoma; e, bone metastasis from prostate adenocarcinoma; and f, vena caval invasion by renal clear cell carcinoma. Red arrows, HIF-1 α -positive lymphocytes. $\times 200$ (d and f); $\times 400$ (a, b, c, and e).

specimens, weak staining was detected in adrenal cortical cells (3 of 8), renal distal tubular epithelium (3 of 9), pancreatic acinar cells (4 of 11), fetal hepatocytes (1 of 1), proliferating B cells from tonsil (2 of 3) and spleen (1 of 9), and seminiferous tubules of testis (7 of 7; Table 1).

Overexpression of HIF-1 α protein was found in 69 (53%) of 131 primary malignant tumors representing 13 of 19 tumor types screened (Table 2). Cases of human prostate, breast, lung, colon, pancreas, brain, gastric, ovarian, and renal cell carcinomas, mesothelioma, and melanoma were positive. Immunohistochemistry was performed on adjacent sections of vena caval invasion from a renal cell carcinoma using MAb H1 α 67 that was preadsorbed with GST/HIF-1 α protein. Whereas nonadsorbed MAb resulted in strong (+++++) staining, preadsorbed MAb resulted in no (–) staining (data not shown).

Two-thirds of all of the regional lymph node and bone metastases were also positive for HIF-1 α overexpression. HIF-1 α was overexpressed in only 29% of primary breast cancers, whereas 69% of breast metastases were positive. All four of the preneoplastic and premalignant lesions found incidentally within biopsy specimens were positive for HIF-1 α immunoreactivity, including two cases of breast comedo-type ductal carcinoma *in situ*, one case of prostatic intraepithelial neoplasia, and one case of colonic adenoma (Fig. 3, a and b). In contrast, all 12 of the benign tumors (breast fibroadenoma and uterine leiomyoma) were negative (Table 2).

HIF-1 α immunostaining was heterogeneous with signal concentrated primarily within the nucleus (Figs. 2 and 3). Cytoplasmic staining was also detected in colon (Fig. 2e), breast, pancreas, and prostate adenocarcinomas. The results were reproducible, and cytoplasmic staining was not observed in flanking normal tissue. Within tumors, clusters of HIF-1 α positive cells were most dense at the invading edge of tumor margins, the periphery of necrotic regions, and surrounding areas of neovascularization (Fig. 2, b and f). Some lymphocytes in lymph nodes containing metastatic cancer cells were positive for HIF-1 α immunostaining (Fig. 3d), but expression was not detected in nonmalignant stromal cells under the assay conditions used for this study.

To investigate whether HIF-1 α expression levels correlated with the degree of tumor angiogenesis and/or disease progression, we

evaluated nine brain tumors of different grades and degrees of neovascularization. HIF-1 α expression was strongest in glioblastomas multiforme and hemangioblastomas (Fig. 2, f and g), which are respectively the most malignant and most highly vascularized primary tumors arising in the central nervous system. In glioblastomas, the staining was especially intense in pseudopalisading tumor cells surrounding areas of necrosis.

Comparison of HIF-1 α Expression with the Expression of p53, bcl-2, and Ki67. On the basis of tissue availability, most tumor samples used for HIF-1 α staining were also stained with anti-Ki67 MAb; some tumor samples, the majority of which were colon and breast cancers, were also stained with anti-p53 and/or anti-bcl-2 MAbs. These markers were scored in a blinded manner relative to the HIF-1 α staining. Expression of HIF-1 α protein was positively correlated with aberrant p53 accumulation ($P < 0.01$), but the correlation with bcl-2 expression was of marginal statistical significance ($P = 0.05$; Table 3). Nonparametric statistical analyses demonstrated a highly significant correlation of HIF-1 α protein expression with Ki67 LI as a marker of cellular proliferation ($P < 0.001$; Table 3). HIF-1 α expression also correlated with Ki67 LI in some normal cell types. Fetal hepatocytes, proliferating B cells in tonsil and spleen, and seminiferous tubules of testis demonstrated weak HIF-1 α expression, and these cell types manifested high Ki67 LI relative to other normal tissue types.

DISCUSSION

HIF-1 α protein was overexpressed in multiple types of human cancer and in regional and distant metastases. This study has identified increased HIF-1 α expression (relative to adjacent normal tissue) in 13 tumor types including lung, prostate, breast, and colon carcinoma, which are the leading causes of U.S. cancer mortality. HIF-1 α protein was also overexpressed in preneoplastic and premalignant lesions such as colonic adenoma, breast ductal carcinoma *in situ*, and prostate intraepithelial neoplasia. These data suggest that overexpression of HIF-1 α can occur very early in carcinogenesis, before histological evidence of angiogenesis or invasion. Additional studies are

under way to assess whether HIF-1 α may represent a novel biomarker for precancerous lesions that warrant clinical surveillance or therapeutic intervention. It is provocative that every benign noninvasive tumor analyzed was negative for HIF-1 α overexpression.

HIF-1 α activates the transcription of genes encoding transferrin, VEGF, endothelin-1, and inducible nitric oxide synthase, which are implicated in vasodilation, neovascularization, and tumor metastasis (15, 17). Particularly strong HIF-1 α expression was observed in glioblastoma multiforme and hemangioblastoma. High VEGF mRNA expression has been reported in these highly malignant and vascularized brain tumors (26). HIF-1 α -positive cells were prominent at tumor margins and surrounding areas of neovascularization. In colonic adenocarcinoma, cancer cells at the leading edge of infiltrating carcinoma manifested the most intense HIF-1 α immunostaining. Comparison of tumor and flanking normal tissue allows the patient to serve as his own control and supports the hypothesis that HIF-1 α overexpression is associated with angiogenesis, invasion, and metastasis. Experimentally, xenografts of mutant mouse hepatoma cells lacking HIF-1 expression manifested significantly reduced growth rates and vascularization compared with parental and revertant cells that expressed HIF-1 (18, 19). Conversely, human colon carcinoma cells transfected with a HIF-1 α expression vector manifested significantly increased growth rates in nude mice as compared with parental cells.⁴

The patterns of immunohistochemical staining in different human cancers suggest that HIF-1 α overexpression may result from both physiological (hypoxia) and nonphysiological mechanisms. It is clear from previous studies that many human tumors have regions of significant hypoxia (6–8). This pattern was most obvious in glioblastoma multiforme in which HIF-1 α was detected in viable tumor cells that were closest to areas of necrosis and farthest from a blood vessel, as previously demonstrated for the expression of VEGF mRNA in these tumors (26, 27). In contrast, expression of HIF-1 α in hemangioblastoma could not be attributed to hypoxia because tumor cells immediately adjacent to patent blood vessels stained intensely, which indicated that factors other than hypoxia may contribute to HIF-1 α expression in human cancers.

A growing number of observations indicate that genetic alterations also affect HIF-1 α expression in cancer cells:

(a) we have correlated HIF-1 α expression with cell proliferation, both in cultured PCA cells (20) and *in vivo* (Table 3). Treatment of cultured cells with insulin, IGF-1, or IGF-2 induced expression of HIF-1 α protein, which was in turn required for expression of IGF-2 mRNA (16), suggesting the involvement of HIF-1 α in an autocrine growth factor loop. Remarkably, all of the 22 primary colon cancers analyzed overexpressed HIF-1 α , and the most highly up-regulated gene in colon cancer encodes IGF-2 (28);

(b) cells transfected with the *v-Src* oncogene overexpressed HIF-1 α , HIF-1 DNA-binding and transcriptional activity, and downstream genes including VEGF (18);

(c) HIF-1 α overexpression was associated with aberrant p53 accumulation in human tumors (Table 3). The anti-p53 MAb used in this study recognizes an epitope in the NH₂ terminus of the wild-type and mutant forms of human p53 protein. Point mutations in the *TP53* gene occur frequently in human cancers, leading to increased expression of a nonfunctional p53 protein with a prolonged half-life that is detectable by immunohistochemistry. Thus, the presence of strong nuclear staining in the majority of cancer cells is frequently observed (29). Expression of HIF-1 α protein, HIF-1 DNA-binding activity, and VEGF mRNA are increased in p53^{-/-} knockout colon carcinoma cells as compared with the parental p53^{+/+} cells⁴; and

(d) in renal clear cell carcinoma cell lines, the loss of von Hippel-Lindau tumor suppressor function results in constitutive high-level expression of HIF-1 α (30). The primary (Fig. 2*i*) and metastatic (Fig. 3*f*) renal clear cell carcinomas analyzed here represent the first demonstration of this overexpression *in vivo*. Thus, in addition to hypoxia, both oncogene activation and tumor suppressor gene inactivation are associated with increased HIF-1 α expression.

Some tumors did not stain positive for HIF-1 α in this study. These tumors may overexpress HIF-1 α but at levels that were below the limits of detection by immunohistochemistry using current methodology. Alternatively, other bHLH-PAS transcription factors that may have similar biological properties to HIF-1 α , such as HIF-2 α (also known as EPAS1, HLF, HRF, and MOP2) or HIF-3 α (23, 31–33), may also mediate hypoxic adaptation. Nevertheless, HIF-1 α was overexpressed in the majority of preneoplastic, malignant, and metastatic cancers analyzed. Given the overexpression of HIF-1 α in common human cancers relative to normal tissues and its vital importance in mediating hypoxic adaptation, additional investigations of HIF-1 α as a biomarker of metastatic potential and as a novel target for therapeutics are warranted.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Mary Ann Accavitti and the Hybridoma Core Facility (University of Alabama at Birmingham, Birmingham, AL) for MAb production; the important contributions of Drs. Josef Prchal, Christopher Bradfield, Donald S. Coffey, Frank Kujhada, William G. Nelson, and Steven Piantadosi; and the technical assistance of Jurga Sauvegot, Natalia Rioseco-Camacho, Bahar Mikhak, Colleen Hanrahan, and Kimberly Heaney.

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Direct Comparison of GAPDH, β -Actin, Cyclophilin, and 28S rRNA as Internal Standards for Quantifying RNA Levels under Hypoxia

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Received May 3, 1999

The appropriate choice of an internal standard is critical for quantitative RNA analyses. As housekeeping genes, GAPDH, β -actin, cyclophilin, and 28S rRNA are commonly employed as RNA internal standards with the assumption that their expression levels remain relatively constant in different experimental conditions. We tested this assumption under hypoxia (1% O₂, 24 hours) compared to normoxia (20% O₂, 24 hours) and compared RNA levels of these 4 housekeeping genes head to head using ribonuclease protection assays. Four biologically diverse cell lines with respect to clonal origin, neoplastic transformation, and growth rates were used in the comparison. Expression levels of 28S rRNA were constant, independent of O₂ tension, but levels of GAPDH, β -actin, and cyclophilin varied widely with hypoxia. In particular, GAPDH mRNA expression was increased by 21.2-75.1% under hypoxic conditions. Increased GAPDH transcription in hypoxia was correlated in the cancer cell lines with upregulation of the transcription factor Hypoxia Inducible Factor-1 α protein levels in identical experimental conditions. These results suggest that 28S rRNA is a reliable internal control for comparative analyses of transcription under hypoxia; GAPDH appears particularly unfavorable for this purpose either in hypoxia or other experimental conditions that upregulate HIF-1 α . © 1999 Academic Press

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, cyclophilin, and 28S rRNA are four common housekeeping genes used in quantitative RNA analyses (such as, northern blots, ribonuclease protection assays, and microarrays) to standardize for uneven loading between samples. Ideally, internal RNA con-

trols should be expressed constantly independent of experimental conditions. Matched loading based on these internal controls is critical for quantitative comparisons of gene expression among different tissue types, varying developmental stages, and experimentally treated cells. However, no one single housekeeping gene always manifests stable expression levels under all of these experimental conditions (1). Therefore, it is necessary to characterize the suitability of various housekeeping genes to serve as internal RNA controls under particular experimental conditions where transcription effects are being tested.

An emerging area of research in cancer and cardiovascular disease involves interrogating the effects of hypoxia on altered gene expression in cell types involved in the pathophysiology of these diseases (2). Microarrays and SAGE technologies allows definition of large transcription profiles of cellular responses to hypoxia, in addition to conventional single gene analyses by Northern blot or RNase protection assay (3). To identify appropriate RNA internal controls under hypoxia for quantifying single gene expression alterations and comparative microarrayed transcription profiles, GAPDH, β -actin, cyclophilin, and 28S rRNA levels were compared head to head under hypoxic and normoxic conditions *in vitro* using quantitative ribonuclease protection assays.

MATERIALS AND METHODS

Cells and tissue culture. Prostate cancer cell lines (DU145 and LnCap), human diploid umbilical vein endothelial cell line (HUVECC), and spontaneously transformed immortal human umbilical vein endothelial cell line (ECV304) were obtained from American Type Culture Collection (ATCC) and cultured according to the provided information. Hypoxia-cultured experiments (1% O₂, 24 hours) were performed as previously described (4).

Antisense probes preparation. The [α -P³²]CTP-labeled antisense probes for GAPDH, β -actin, cyclophilin, and 28S rRNA were transcribed using the appropriate antisense probe templates (Ambion), T7 polymerase, and MAXIsript *in Vitro* transcription kits (Ambion).

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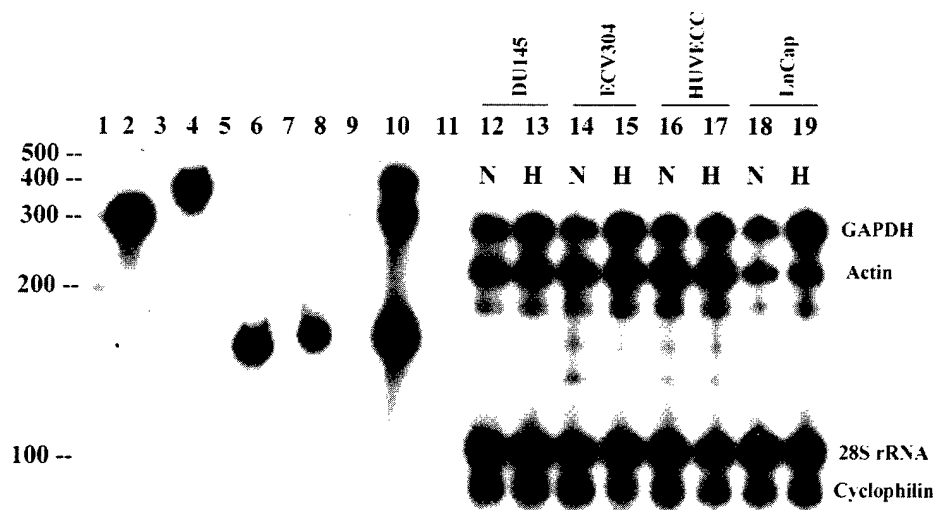


FIG. 1. Expression of common housekeeping genes (GAPDH, β -actin, cyclophilin, and 28S rRNA) in normoxic (N) and hypoxic (H) cells using a multiple probe ribonuclease protection assay. Four probes ($6-8 \times 10^4$ cpm/each probe) were hybridized with $2 \mu\text{g}$ total RNA from DU145 (lanes 12 and 13), ECV304 (lanes 14 and 15), HUVECC (lanes 16 and 17), and LnCAP (lanes 18 and 19) cells simultaneously. Lanes 2-11 are yeast RNA controls with (lanes 3, 5, 7, 9, and 11) or without (lanes 2, 4, 6, 8, and 10) RNase using β -actin (lanes 2, 3, 10, and 11), GAPDH (lanes 4, 5, 10, and 11), cyclophilin (lanes 7, 8, 10, and 11), and 28S rRNA (lanes 8, 9, 10, and 11) probes respectively. The protected bands are marked on the right and the RNA molecular weight markers are shown on the left.

The radiolabeled probes were then purified by 5% acrylamide/8 M urea gel and eluted in elution buffer (0.5 M NH_4OAc , 1mM EDTA, 0.1% SDS). The probe size/protected fragment size (nt) were 383/316 for GAPDH, 304/245 for β -actin, 165/103 for cyclophilin, and 153/115 for 28S rRNA respectively.

RNA isolation and ribonuclease protection assay. Confluent normoxic or hypoxic cells were washed with ice-cold PBS. Total RNA was prepared using TRIZOL reagent (GIBCO BRL). The $2 \mu\text{g}$ total RNA was hybridized simultaneously to the four radiolabeled anti-sense probes (approximately $6-8 \times 10^4$ cpm respectively) using RPA II kit (Ambion). Yeast tRNA was used as a negative control. The experiment repeated one time for LnCap, HUVECC and ECV304 cells, and two times for DU145 cells. The results were reproducible. Autoradiographic signals were quantitated by Eagle Eye Computerized Densitometry (Stratagene).

RESULTS AND DISCUSSION

To explore whether expression of common housekeeping genes is modulated by hypoxia, we compared the expression level of four common housekeeping genes in cells cultured under normoxic or hypoxic conditions. Under hypoxic culture conditions (1% O_2 , 24 hours), expression of 28S rRNA and mRNAs of three common housekeeping genes (GAPDH, β -actin, and cyclophilin) were either up-regulated or down-regulated at different levels among the four cell lines tested as shown in Figs. 1 and 2. GAPDH message levels were the most sensitive to modulation by hypoxia, displaying up-regulation by 21.2-75.1% relative to the observed levels under normoxic conditions. β -actin and cyclophilin displayed hypoxia-induced changes in expression of +5.6 to +27.3%, and from -22.8 to +7.5%, respectively. In contrast, 28S rRNA expression

was stable under hypoxic conditions, deviating from that observed under normoxic conditions by only -7.3 to +2.8%.

The basal expression levels under normoxic conditions of these housekeeping genes were compared among the four biologically diverse cell lines with respect to growth rate, clonal origin, normal and neoplastic karyotypes. For example, the HUVECC cell line is a normal human endothelial cells and DU-145 is an anaplastic carcinoma cell line derived from a prostate cancer brain metastasis. The basal expression levels of 28S rRNA remained stable among cell lines tested while levels of GAPDH, β -actin, and cyclophilin varied (Figs. 1 and 3).

To our knowledge, this is the first report of testing housekeeping genes as RNA internal standards head to head under hypoxic conditions. The stable expression levels of 28S rRNA relative to other housekeeping genes under a variety of experimental conditions has previously been described for some breast cell lines (1), normal human fibroblasts (5), individual rat liver tissues (6), tumorigenic and malignant mouse cell lines (7). The levels of ribosomal RNA, which make up 80% of total RNA, are thought to be less likely to vary under conditions that affect the expression of mRNAs, since they are transcribed by a distinct RNA polymerase. However, the expression levels of β -actin and cyclophilin have frequently been reported to vary in some tissues and cell lines (1, 8-11). In this study, the expression levels of β -actin and cyclophilin varied among different cell lines and as a result of hypoxia.

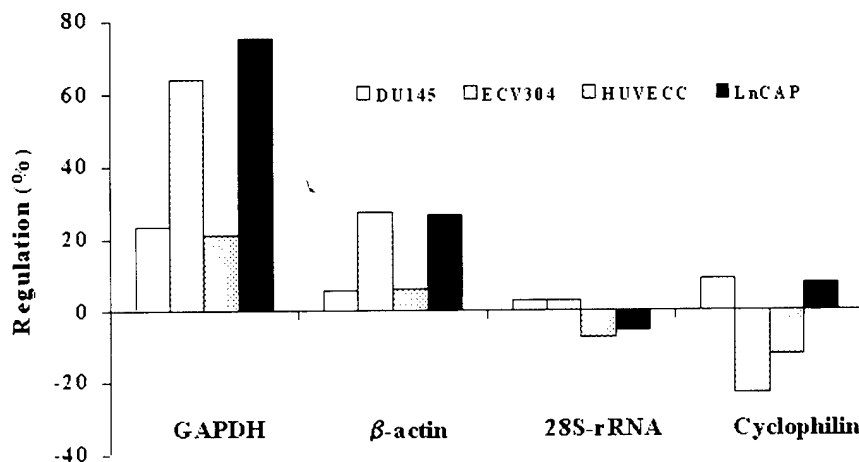


FIG. 2. Hypoxia-regulated expression levels of GAPDH, β -actin, cyclophilin and 28S rRNA compared with the levels under normoxia obtained by densitometry as a percentage difference. The data represent the mean of three independent experiments (DU145) or two independent experiments (ECV304, HUVECC, and LnCAP).

GAPDH is a key enzyme in glycolysis, which makes it an abundant RNA species for use as a potential internal RNA standard. This housekeeping gene is constitutively expressed in many tissues. However, wide variations in GAPDH expression levels have been observed in tissues at different developmental stages (12), in cells treated with insulin (13, 14), dexamethasone (12), mitogens (15), as well as in virally-transformed or oncogene-transfected fibroblasts (7). In addition, human lung cancer tissues compared with paired normal lung tissues express much higher levels of GAPDH mRNA (16). In this study, GAPDH mRNA levels were up-regulated in the presence of hypoxia in every cell line tested. The transcription of GAPDH is induced by hypoxia-inducible factor 1 (HIF-1) (17, 18). HIF-1 is a basic-helix-loop-helix (bHLH)-PAS transcription factor, consisting of α and β subunits (18). HIF-1 α protein levels, which determine HIF-1 DNA

binding activity and transcription of HIF-1-regulated genes, increase exponentially as intracellular pO_2 is reduced (19). It activates transcription of multiple genes involved in glycolysis including the gene encoding GAPDH. Under identical experimental conditions of hypoxia, we previously reported the induction of HIF-1 α protein expression and HIF-1 DNA-binding activity in DU145 and LnCAP prostate cancer cells (4), which were used in this study.

HIF-1 α protein can also be induced in cancer cells by oxygen-independent pathways involving oncoproteins. The v-Src oncogene tyrosine kinase has been demonstrated to increase the expression of HIF-1 α protein and HIF-1 α -regulated genes under both hypoxic and normoxic conditions (20). Insulin, IGF-1, IGF-2 and β -interferon also can induce HIF-1 α expression via their ligand-specific tyrosine kinase receptors (21-23). Ultimately, any of the known or unknown mechanisms

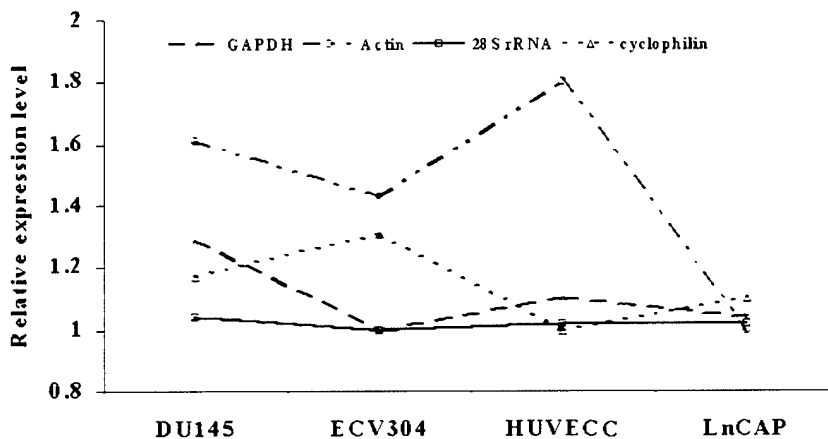


FIG. 3. Expression levels of GAPDH, β -actin, cyclophilin and 28S rRNA in relative to the lowest levels in normoxic DU145, ECV304, HUVECC, and LnCAP cells. The data represent the mean of two independent experiments.

which regulate HIF-1 α expression might eventually affect GAPDH expression given that the transcription of GAPDH is at least in part controlled by HIF-1 α protein (17). The insulin-induced increase in GAPDH mRNA levels in cultured adipocytes may result from insulin-like growth factor-1 receptor-mediated modulation of HIF-1 α and HIF-1 regulated gene expression (13). Most recently, we have found that HIF-1 α protein overexpressed in surgical specimens of 19 different types of human cancers and prostate cancer xenografts raised from cell lines including DU145 as well as LnCAP (unpublished data). Thus, factors which increase HIF-1 α protein expression may activate GAPDH transcription and account for the high levels of GAPDH mRNA expression in cancers (7, 16).

In summary, the negligible variation in both basal and hypoxia-regulated expression of 28S rRNA supports its use as an internal RNA standard for experimental conditions involving hypoxia. In contrast, GAPDH may not be suitable for use as an internal control in quantitative RNA analysis experiments performed under hypoxic conditions or other conditions under which HIF-1 α protein levels may be altered, such as in comparisons of differential gene expression between cancer and normal tissues.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Dr. Naren Ramakrishna and Kimberly Cordwell in preparation of the manuscript.

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Modulation of Hypoxia-inducible Factor 1 α Expression by the Epidermal Growth Factor/Phosphatidylinositol 3-Kinase/PTEN/AKT/FRAP Pathway in Human Prostate Cancer Cells: Implications for Tumor Angiogenesis and Therapeutics¹

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Abstract

Dysregulated signal transduction from receptor tyrosine kinases to phosphatidylinositol 3-kinase (PI3K), AKT (protein kinase B), and its effector FKBP-rapamycin-associated protein (FRAP) occurs via autocrine stimulation or inactivation of the tumor suppressor PTEN in many cancers. Here we demonstrate that in human prostate cancer cells, basal-, growth factor-, and mitogen-induced expression of hypoxia-inducible factor 1 (HIF-1) α , the regulated subunit of the transcription factor HIF-1, is blocked by LY294002 and rapamycin, inhibitors of PI3K and FRAP, respectively. HIF-1-dependent gene transcription is blocked by dominant-negative AKT or PI3K and by wild-type PTEN, whereas transcription is stimulated by constitutively active AKT or dominant-negative PTEN. LY294002 and rapamycin also inhibit growth factor- and mitogen-induced secretion of vascular endothelial growth factor, the product of a known HIF-1 target gene, thus linking the PI3K/PTEN/AKT/FRAP pathway, HIF-1, and tumor angiogenesis. These data indicate that pharmacological agents that target PI3K, AKT, or FRAP in tumor cells inhibit HIF-1 α expression and that such inhibition may contribute to therapeutic efficacy.

Introduction

Tumor progression involves the selection of cells with somatic mutations that activate oncogenes and inactivate tumor suppressor genes. These mutations have the effect of driving cells through the cell cycle in an uncontrolled manner and preventing apoptosis. Two adaptations that are universal characteristics of solid tumors, indicating that they are necessary for tumor progression, are increased glycolytic metabolism and angiogenesis (reviewed in Ref. 1). These adaptations are also driven by genetic alterations in tumor cells, but their molecular basis has remained obscure. Loss of function mutations in tumor suppressor genes or activating mutations in oncogenes have been shown to dysregulate signal transduction pathways leading from growth factors (such as EGF³) and their cognate receptor tyrosine kinases to PI3K, which catalyzes the conversion of phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-

triphosphate, respectively (reviewed in Ref. 2). These products are allosteric activators of phosphatidylinositol-dependent kinase 1, which phosphorylates and activates AKT (protein kinase B). Targets of AKT include BAD, an inhibitor of apoptosis, and FRAP, an activator of p70^{s6k}, which is required for ribosomal biogenesis and cell cycle progression (reviewed in Ref. 2). These findings have delineated mechanisms by which the PI3K/AKT pathway promotes cell proliferation and inhibits cell death. This pathway is negatively regulated by PTEN, which dephosphorylates phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate (reviewed in Ref. 2). In PCA, PTEN loss of function correlates with increased angiogenesis and appears to be critical for progression to hormone-refractory metastatic disease (3-6). However, the basis for these correlations has not been determined. The role of HIF-1 as an essential transcriptional activator of genes encoding glucose transporters, glycolytic enzymes, and VEGF is well established (reviewed in Ref. 7). In this study, we demonstrate that modulation of the EGF/PI3K/AKT/FRAP pathway alters the expression of HIF-1 α protein, HIF-1-dependent transcriptional activity, and VEGF protein in human PCA cells. These results provide a mechanism contributing to the overexpression of HIF-1 α in PCA and other solid cancers (8) and have important implications regarding cancer progression and therapy.

Materials and Methods

Tissue Culture. The human PCA cell lines DU145, PC-3, PPC-1, and TSU were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS (complete media). Cells were exposed to hypoxia as described previously (9-11).

Immunoblot Assays. Cells ($0.5-1.0 \times 10^6$) were seeded onto 150-mm tissue culture dishes (Falcon) and incubated for 36-48 h in complete media (except for AKT assays, in which cells were plated directly in media with 0.1% FBS). The cells were incubated in media with 0-0.1% FBS for 24 h and then given fresh media with 0-0.1% FBS alone or with 10% FBS, EGF (Life Technologies, Inc.), PMA, or 4 α -PMA, either alone or with LY294002, PD098059, rapamycin, or wortmannin (Alexis Corp.), for 6-8 h. For analysis of HIF-1 α expression, nuclear extracts were prepared, and aliquots were analyzed using monoclonal antibody H1 α 67 (Novus Biologicals, Inc.) as described previously (8). Blots were stripped and incubated with anti-topoisomerase I antibodies (TopoGEN). Aliquots of whole cell lysates were subjected to immunoblot assay using anti-AKT and phospho-AKT antibodies (New England Biolabs). All immunoblots were developed using enhanced chemiluminescence reagents (Amersham).

Transient Transfection Assays. DU145 cells were seeded onto 24-well culture plates at a density of 4×10^4 cells/well and incubated for 24 h in complete media. The cells were transfected with 12.5 ng of control plasmid pTK-RL (Promega) containing the herpes simplex virus thymidine kinase promoter and *Renilla reniformis* (sea pansy) luciferase coding sequences; 100 ng of reporter plasmid p2.1 containing a 68-bp hypoxia response element from the *ENO1* gene, an SV40 promoter, and *Photinus pyralis* (firefly) luciferase

Received 12/2/99; accepted 2/3/00.

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¹ Supported by NIH Prostate Cancer SPORE Grant CA-58236; NIH Grant RO1-HL55338 and Children's Brain Tumor Foundation (to G. L. S.); AEGON Gift for Accelerated Breast and Prostate Cancer Research, CaP CURE Foundation, and Department of Defense Grant DAMD 17-98-1-8475 (to J. W. S.).

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³ The abbreviations used are: EGF, epidermal growth factor; PI3K, phosphatidylinositol 3-kinase; FRAP, FKBP-rapamycin-associated protein; PCA, prostate cancer; HIF-1, hypoxia-inducible factor 1; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; IGF, insulin-like growth factor; PMA, phorbol 12-myristate 13-acetate.

coding sequences (12); and 500 ng of pCEP4 (Invitrogen) or expression vector encoding AKT-MYR, AKT (K179M), wild-type PTEN, PTEN (C124S), or p85 Δ (13–16). KD-AKT, C124S PTEN, and p85 Δ have each been shown to have dominant negative effects in cells expressing the respective wild-type protein. Cells were exposed to plasmid DNA for 8 h in 1 μ l of Fugene-6 (Boehringer Mannheim). Cells were then incubated in DMEM with 0.1% FBS for 16 h, followed by exposure to 10% FBS, 100 nM PMA, and 1% O₂ or no treatment for 24 h. Cells were lysed in 100 μ l of buffer, and Dual-Luciferase (Promega) reporter assays were performed on 20- μ l aliquots.

VEGF ELISA Assays. TSU cells were seeded onto 6-well culture plates at a density of 4 \times 10⁴ cells per well, incubated for 24 h in complete media, and then given serum-free media for 16 h, followed by fresh serum-free media, either alone or with 10% FBS, EGF, or PMA alone or with LY294002 or rapamycin, for 24 h. Conditioned media were removed for storage at -80°C, and cells were counted. VEGF protein concentration in the media was determined by ELISA using a commercial kit (R&D Systems).

Results

We demonstrated previously that human PCA lines, most notably PC-3 cells, express HIF-1 α protein and HIF-1 DNA-binding activity under nonhypoxic conditions, and expression is further increased in response to hypoxia (11). Potential clinical implications of these findings were underscored by the immunohistochemical demonstration that HIF-1 α is overexpressed (relative to adjacent normal tissue) in common human solid tumors, including PCA (8). HIF-1 α expression was also induced in transformed cells exposed to EGF, fibroblast growth factor 2, IGF-1, or IGF-2 (10). Because of the known role of EGF signaling via the PI3K pathway (reviewed in Ref. 2), we investigated whether up-regulation of this pathway contributed to increased HIF-1 α expression in PCA cells. As an initial means of modulating the activity of this pathway, we examined the effect of serum starvation and stimulation. TSU, PC-3, DU145, and PPC-1 cells were cultured at low density in serum-free medium for 24 h and then exposed to 0% or 10% FBS for 6 h. All four cell lines demonstrated some degree of HIF-1 α expression under serum-free conditions that increased in response to serum stimulation (Fig. 1A). To examine responses to specific mitogens, cells were exposed to 100 nM PMA or 20 ng/ml EGF. PMA strongly induced HIF-1 α expression in DU145, TSU, and PPC-1 cells (Fig. 1B). Exposure of TSU cells to 20 ng/ml EGF also markedly induced HIF-1 α expression, whereas the effect of EGF on DU145 and PPC-1 cells was more modest. In DU145 and TSU cells, similar levels of HIF-1 α expression were induced by exposure to PMA or hypoxia, whereas the biologically inactive 4 α -PMA had no effect (Fig. 1C).

To determine whether PI3K pathway activity was required for HIF-1 α expression, PCA cells were exposed to LY294002 or wortmannin, inhibitors of PI3K, or to rapamycin, an inhibitor of FRAP (17), a signaling molecule downstream of PI3K (Fig. 2A). PC-3 cells were cultured in 0.1% FBS in the presence of varying concentrations of LY294002 under hypoxic (1% O₂) or nonhypoxic (20% O₂) conditions. HIF-1 α expression under nonhypoxic conditions was partially inhibited by 1 μ M LY294002 and completely inhibited by 10 μ M LY294002 (Fig. 2B, top panel). In contrast, hypoxia-induced HIF-1 α expression was only partially inhibited by 10 μ M LY294002 and was more completely inhibited by 50 μ M LY294002. Wortmannin was a more potent inhibitor in nonhypoxic cells because partial inhibition and complete inhibition of HIF-1 α expression were observed in the presence of 10 and 100 nM wortmannin, respectively, in nonhypoxic cells, whereas only modest inhibition was observed with 200 nM wortmannin in hypoxic cells (Fig. 2B, middle panel). Rapamycin was the most potent drug tested; it inhibited HIF-1 α expression at concentrations of 10 and 50 nM in nonhypoxic and hypoxic cells, respectively (Fig. 2B, bottom panel). Induction of HIF-1 α expression in PC-3 or TSU cells exposed to either 10% FBS, 100 nM PMA, or 20

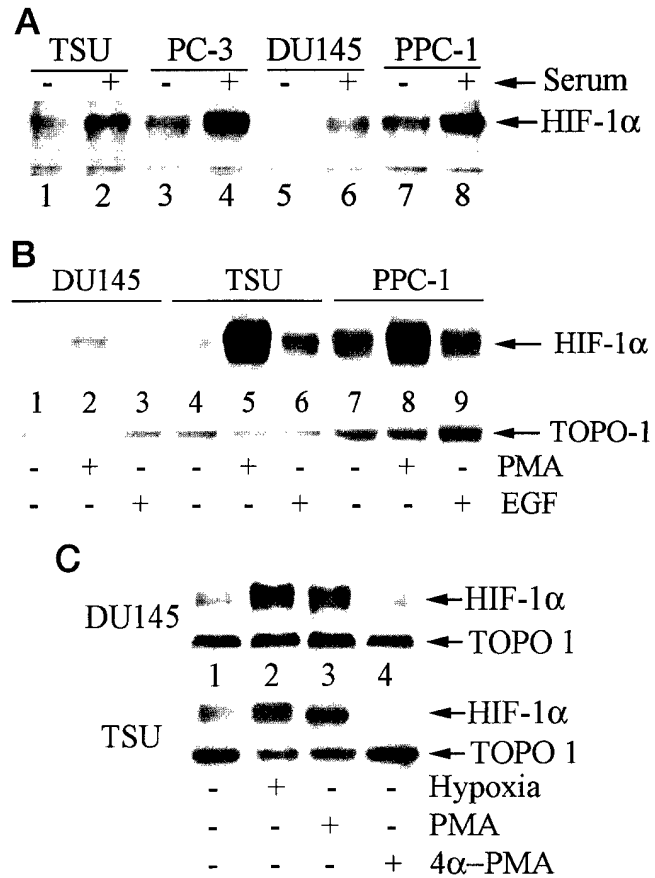


Fig. 1. Induction of HIF-1 α expression in PCA cells exposed to serum, PMA, EGF, or hypoxia. **A**, effect of serum starvation and stimulation. Cells were cultured in serum-free media for 24 h and then incubated in media containing 0% or 10% FBS for 6 h before immunoblot assay using an anti-HIF-1 α monoclonal antibody. **B**, effect of PMA and EGF stimulation. Cells cultured in media containing 0.1% FBS for 24 h were untreated (Lanes 1, 4, and 7) or exposed to 100 nM PMA (Lanes 2, 5, and 8) or 20 ng/ml EGF (Lanes 3, 6, and 9) for 8 h before immunoblot assay for HIF-1 α (top panel). The blot was then stripped and assayed for topoisomerase I (bottom panel) as a control for sample loading and transfer. **C**, effect of hypoxia and PMA. DU145 (top panels) and TSU (bottom panels) cells cultured in media containing 0.1% FBS for 20 h were untreated (Lane 1) or exposed to 1% O₂ (Lane 2), 100 nM PMA (Lane 3), or 100 nM 4 α -PMA (Lane 4) for 8 h before HIF-1 α and topoisomerase I immunoblot assays.

ng/ml EGF was completely inhibited by 50 μ M LY294002 (Fig. 2C). PMA-induced HIF-1 α expression was completely inhibited in the presence of 10 μ M LY294002 or 10 nM rapamycin (data not shown). Under the experimental conditions used, none of the inhibitors caused cell death during the study period as determined by analysis of cellular ATP concentration, morphology, or trypan blue exclusion (data not shown). Taken together, these results suggest that basal and mitogen-induced HIF-1 α expression in PCA cells is highly dependent on PI3K activity, whereas other signaling pathways stimulate hypoxia-induced expression.

AKT lies between PI3K and FRAP in this signaling pathway (Fig. 2A). In TSU cells cultured in serum-free media, a modest degree of AKT phosphorylation was detected, which increased in response to EGF stimulation (Fig. 3, top panel, Lanes 1 and 2). These data are consistent with previous reports of EGF-stimulated AKT activity in PCA cells (18, 19). Both basal and EGF-induced AKT phosphorylation were blocked by LY294002 (Lane 3). In contrast, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor PD098059 (Fig. 2A) had no inhibitory effect (Fig. 3, top panel, Lane 4), suggesting that mitogen-activated protein kinase activity is not required in these cells. In PC-3 cells, which show the highest level of HIF-1 α expression under nonhypoxic conditions (Fig.

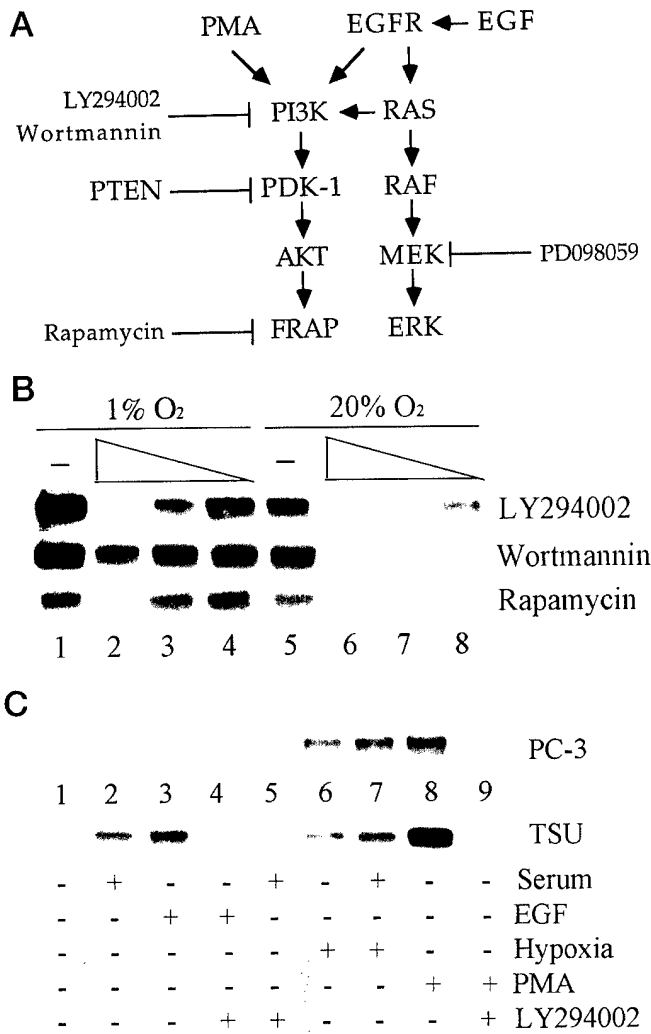


Fig. 2. Effect of pharmacologic inhibitors on HIF-1 α expression. **A**, relevant signal transduction pathways activated by EGF and PMA. **B**, effect of PI3K and FRAP inhibitors on basal and hypoxia-induced HIF-1 α expression. PC-3 cells were cultured in media containing 0.1% FBS for 20 h, followed by the addition of LY294002 (0, 50, 10, or 1 μ M), wortmannin (0, 200, 100, or 10 nM), or rapamycin (0, 50, 10, or 1 nM) and incubation in 1% (Lanes 1–4) or 20% (Lanes 5–8) O₂ for 8 h before HIF-1 α immunoblot assay. **C**, effect of PI3K inhibition on serum-, EGF-, and PMA-induced HIF-1 α expression. PC-3 and TSU cells were incubated in media containing 0.1% FBS for 24 h and then exposed to 10% FBS, 20 ng/ml EGF, 100 nM PMA, or 1% O₂ in the presence or absence of 50 μ M LY294002 for 6 h before HIF-1 α immunoblot assay.

1A; Ref. 11), the extent of AKT phosphorylation was even greater than that in TSU cells but was nevertheless completely blocked by treatment with LY294002 (Fig. 3, top panel, Lanes 5 and 6), whereas treatment with rapamycin (which inhibits the pathway downstream of AKT) or PD098059 had no inhibitory effect (Lanes 7 and 8). Hypoxia had no effect on AKT phosphorylation in PC-3 cells (Lanes 9–12). Total AKT protein levels were not affected by EGF, LY294002, PD098059, or hypoxia (Fig. 3, bottom panel).

To determine whether PI3K pathway activity affects HIF-1-mediated gene transcription, DU145 cells were cotransfected with the reporter gene p2.1 (12) containing a 68-bp hypoxia response element from the promoter of the human *ENO1* gene (which encodes the glycolytic enzyme enolase) and expression vectors encoding wild-type or mutant components of the PI3K pathway. Reporter gene activity increased 2.4-fold in response to PMA stimulation (Fig. 4A) in the presence of empty expression vector. In contrast, reporter gene activity was below basal levels in PMA-stimulated cells transfected

with vector encoding KD-AKT, a catalytically-inactive (kinase-dead) form of AKT containing a K179M missense mutation (14); wild-type PTEN; or p85 Δ , a dominant-negative form of the PI3K p85 regulatory subunit (16). Reporter activity was induced 17-fold by hypoxia, and this response was partially inhibited by KD-AKT, wild-type PTEN, or PI3K-p85 Δ (Fig. 4B). These results, which are consistent with the effects of the PI3K inhibitor LY294002 on HIF-1 α expression reported above (Fig. 2), demonstrate that PI3K and PTEN-regulated AKT activity are required for HIF-1-mediated transcription in response to PMA.

To determine whether activation of the PI3K pathway was sufficient to activate HIF-1-mediated gene transcription, DU145 cells that express wild-type PTEN (5) were cotransfected with reporter p2.1 and expression vectors encoding AKT-MYR, a myristoylated and constitutively active form of AKT (13), or a catalytically inactive form of PTEN containing a C124S missense mutation. The transfected cells were incubated in 10% FBS (Fig. 4C), 0.1% FBS (Fig. 4D), or 0.1% FBS with 100 nM PMA (Fig. 4E). Under all three conditions, both constitutively active AKT and dominant-negative PTEN induced reporter gene expression, with the greatest response observed in PMA-stimulated cells.

To demonstrate that the PI3K-mediated induction of HIF-1 transcriptional activity results in biological activity, the secretion of VEGF protein by TSU cells was analyzed by ELISA. Cells were serum-starved for 6 h and then exposed to no treatment, 10% FBS, 50 nM PMA, or 20 ng/ml EGF for 24 h. Despite the short incubation time, FBS, PMA, and EGF each increased VEGF protein levels in the tissue culture supernatant, and PMA resulted in the greatest induction (Fig. 4F), as was also observed with respect to HIF-1 α expression (Fig. 1). Treatment with low concentrations of either LY294002 (10 μ M) or rapamycin (10 nM) markedly inhibited the induction of VEGF expression by FBS, PMA, or EGF (Fig. 4F), similar to the effect of these inhibitors on mitogen-induced HIF-1 α expression (Fig. 2). Thus, both HIF-1 α -dependent gene transcription (Fig. 4, A–E) and the expression of a HIF-1-regulated gene product (Fig. 4F) are modulated by the activity of the PI3K/AKT/FRAP pathway in PCA cells.

Discussion

In this study, we demonstrate that activation of the PI3K/PTEN/AKT/FRAP pathway by EGF, PMA, serum, or autocrine stimulation results in increased expression of HIF-1 α protein, HIF-1 transcriptional activity, and VEGF protein expression in PCA cells. HIF-1 α protein expression is regulated by ubiquitination and proteasomal degradation (reviewed in Ref. 7). Additional studies are required to determine whether this process is modulated by PI3K/AKT/FRAP activity and, if so, whether such modulation involves direct phosphorylation of HIF-1 α .

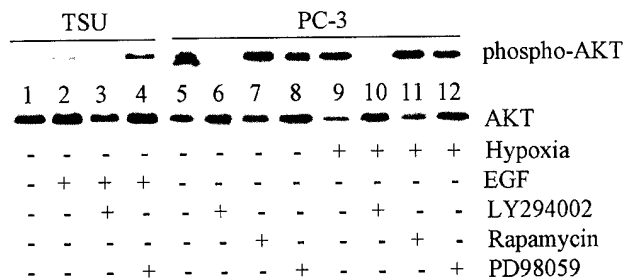


Fig. 3. Analysis of AKT phosphorylation. TSU and PC-3 cells cultured in serum-free media for 24 h were untreated or exposed to 20 ng/ml EGF, 50 μ M LY294002, 20 nM rapamycin, or 100 μ M PD098059 under nonhypoxic (20% O₂) or hypoxic (1% O₂) conditions as indicated for 8 h before immunoblot assay using antibodies against phosphorylated (top panel) or total (bottom panel) AKT protein.

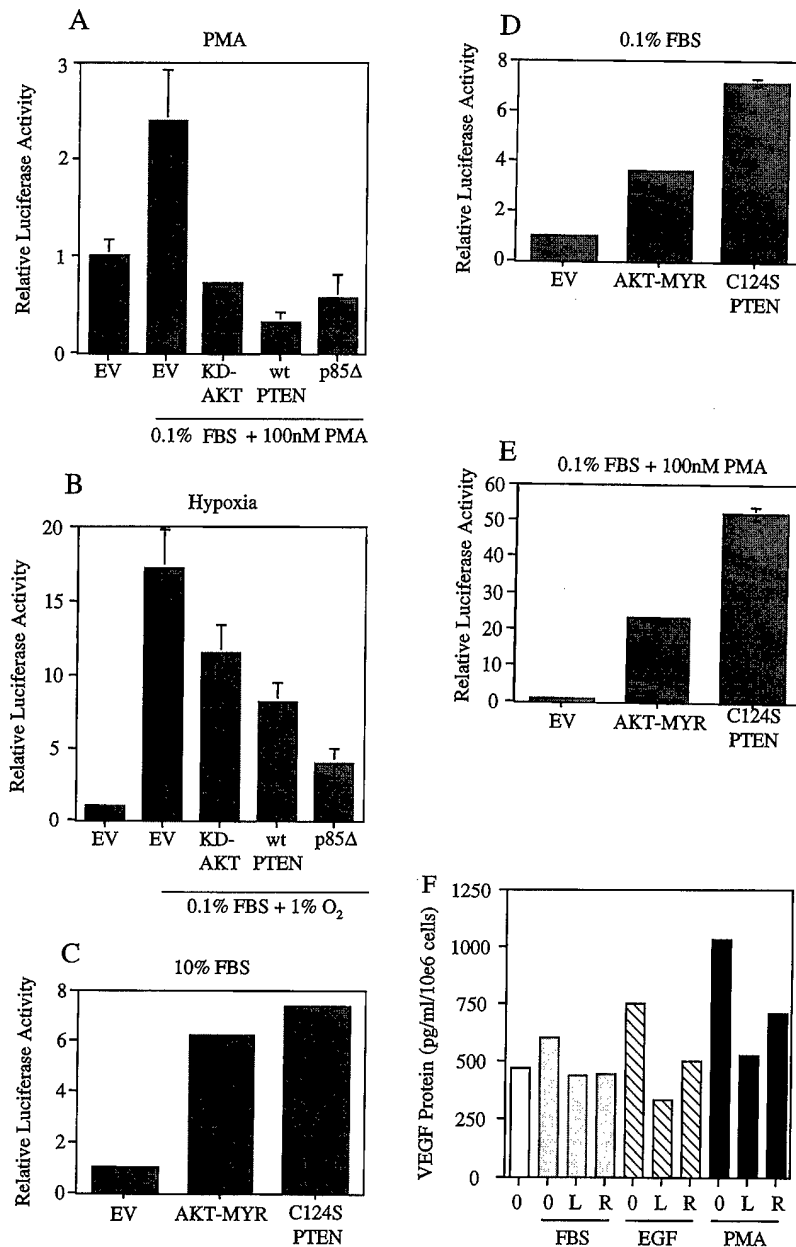


Fig. 4. Effect of altered PI3K, PTEN, or AKT activity on HIF-1-dependent gene expression. A-E, DU145 cells were cotransfected with a control plasmid containing the HSV TK promoter upstream of *Renilla* luciferase coding sequences, a reporter plasmid containing a hypoxia response element upstream of a SV40 promoter and firefly luciferase coding sequences, and expression vector containing no insert (EV) or cDNA encoding catalytically inactive (kinase-dead; KD) or constitutively active (myristoylated; MYR) AKT, wild-type (wt) or mutant (C124S) PTEN, or a deleted form of the p85 subunit of PI3K (p85 Δ). Eight h after transfection, the cells were incubated in 10% (C) or 0.1% (D) FBS for 16 h and then harvested, or serum-deprived cells were exposed to PMA (A and E) or 1% O₂ (B) for an additional 24 h. For each sample, the ratio of firefly to *Renilla* luciferase was determined and normalized to the value obtained from untreated cells transfected with empty vector (EV) to generate the relative luciferase activity. Data represent the mean and SE for three independent transfections. Note that the scale of the y axis differs between graphs. F, VEGF protein secretion by TSU cells. After 16 h of serum starvation, cells were untreated (□) or exposed to 10% FBS (■), 20 ng/ml EGF (▨), or 50 nM PMA (■) for 24 h, either alone (O) or in the presence of 10 μ M LY294002 (L) or 10 nM rapamycin (R). VEGF protein concentrations in conditioned media were determined by ELISA and corrected for cell number. Each bar represents the mean of VEGF concentrations from duplicate plates of cells, which differed by \leq 20% in all cases.

These results provide a molecular basis for the previously reported expression of HIF-1 α under nonhypoxic conditions in PCA cells (11). It is likely that, *in vivo*, increased activity of the PI3K pathway contributes to the dramatic overexpression of HIF-1 α in PCA and other human cancers (8). The tumor suppressor PTEN, which negatively regulates the PI3K pathway, is a target for mutation in PCA, breast cancer, gliomas, and other tumor types (3-6, 19-21). In PCA, inactivation of PTEN expression is associated with disease progression and angiogenesis (3, 4). It is well established that HIF-1 activates genes encoding glucose transport-

ers, glycolytic enzymes, heme oxygenase-1, IGF-2, IGF-binding proteins, inducible nitric oxide synthase, transferrin, and VEGF, all of which have been implicated in tumor progression (reviewed in Ref. 7). In particular, the association between PTEN loss of function and angiogenesis may be explained by the induction of HIF-1 α , leading to increased VEGF expression. Colon cancer cells transfected with a HIF-1 α expression vector demonstrated increased VEGF mRNA expression as well as increased growth and angiogenesis of tumor xenografts (22).

In addition to PTEN, loss of function mutations in tumor suppressor

genes encoding VHL (23) and p53 (8, 22) result in increased expression of HIF-1 α and VEGF. Gain of function mutations in oncogenes also induce HIF-1 α expression, as demonstrated for *v-src* (24) and inferred for autocrine activation of EGF and IGF-I receptors, based on the results presented above and in previous studies (9, 10). Induction of transcription via the *VEGF* gene promoter by activated H-RAS also requires PI3K/AKT activity and an intact HIF-1 binding site (16). Thus, V-SRC, H-RAS, and receptor tyrosine kinases all lead to increased activity of both the PI3K/AKT pathway (2, 18, 19, 25) and HIF-1.

Several conclusions can be drawn from the available data. First, in human tumors, increased expression of HIF-1 α is induced by genetic alterations as well as by physiological stimulation. Second, expression of HIF-1 may play a major role in promoting angiogenesis and metabolic adaptation in PCA and other common solid tumors. In addition to the data regarding the effects of increased HIF-1 α expression cited above, loss of HIF-1 expression in tumor cells is associated with decreased xenograft growth and angiogenesis (24, 26). Third, whereas genetic alterations affecting signal transduction pathways are highly variable among human tumors, increased expression of HIF-1 α may represent a common final pathway. Fourth, if HIF-1 mediated angiogenesis and metabolic adaptation play important roles in tumor progression, as suggested by previous studies (7, 8, 22–24, 26), then pharmacological inhibition of HIF-1 activity may represent a useful treatment strategy. Furthermore, the effect of PI3K/AKT/FRAP pathway inhibitors on HIF-1 α expression may provide a basis for therapeutic efficacy.

Acknowledgments

We are grateful to Naheed Ahmed, Tung Chan, and Philip Tschlis for AKT expression vectors and to Amato Giaccia for the p85 Δ expression vector. We thank Kimberly Heaney for technical assistance.

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#2181 Expression of hypoxia-inducible factor 1 α in human prostate cancer. Zhong, H., Semenza, G.L., Agani, F., Laughner, E., Isaacs, W.B., and Simons, J.W. *Johns Hopkins University School of Medicine, Baltimore, MD 21287.*

Solid tumors often have abnormal blood supply and hypoxic regions. Hypoxia-inducible factor 1 (HIF-1) is a critical transcription factor that regulates genes involved in adaptation to hypoxia. Recent studies suggest the involvement of HIF-1 in tumor progression. In the present study, HIF-1 α expression was evaluated in human prostatic cancer (PCA) cell lines and PCA tissues in the levels of mRNA and protein. Hypoxia (1% O₂) induced expression of HIF-1 α protein and HIF-1 DNA-binding activity was found in 5 human PCA cell lines tested. The transcription of HIF-1 regulated genes (LDH-A and Eno-1) were also shown in upregulated levels under hypoxia. Using ribonuclease protection assay, the mean of HIF-1 α mRNA level exhibited a moderate increase in 14 PCA samples (24.120 \pm 15.248) compared to in 10 normal prostate tissues (17.788 \pm 10.221). Five of the eight tumor/normal pairs presented increase in the amount of HIF-1 α mRNA compared to the respective normal tissues (23.818 \pm 8.237/18.999 \pm 8.836). However, HIF-1 α protein was identified in the cultured normoxic and hypoxic PC-3 cells, all 5 xenografts tested as well as surgical samples of human PCA (6/7) by immunohistochemistry but not in normal human prostate tissues (0/5). Within tumors, HIF-1 α positive cells displayed striking localization to the tumor margins, the periphery of necrotic regions, and surrounding areas of neovascularization. In most cases, the magnitude of HIF-1 α expression was consistent with the Ki67 index. This pilot data provides the first *in vivo* evidence of HIF-1 α expression in human cancer cells. The results suggest that HIF-1 α may play a critical role in tumor formation, proliferation, neovascularization, and metastatic progression.