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Chronic hypoxia promotes an aggressive phenotype in rat prostate cancer cells

OMAR ALQAWI, HONG P. WANG, MYRNA ESPIRITU, & GURMIT SINGH

Department of Pathology and Molecular Medicine, Juravinski cancer Centre, McMaster University, Hamilton, Ont., Canada

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Abstract
In general, tumors cells that are resistant to apoptosis and increase angiogenesis are a result of the hypoxic responses contributing to the malignant phenotype. In this study, we developed a chronic hypoxic cell model (HMLL), by incubating the prostate cancer MatLyLu cells in a hypoxic chamber (1% O_2) over 3 weeks. Surviving cells were selected through each cell passage and were grown in the hypoxic condition up to 8 weeks. This strategy resulted in survival of only 5% of the cells. The surviving hypoxic cells displayed a greater stimulation on hypoxic adaptive response, including a greater expression of glucose transporter1 (Glut1) and VEGF secretion. In addition, higher invasion activity was observed in the chronic hypoxic HMLL cells as compared to MatLyLu cells exposed to acute hypoxia (1% O_2, 5 h) using the matrigel assay. To further examine the role of HIF-1α in tumor progression, both MatLyLu and HMLL cells were transfected with dominant-negative form of HIF-1α (DNHIF-1α). The Matrigel invasion activity induced by chronic hypoxia was significantly attenuated by DNHIF-1α. These results suggest that signaling pathways leading to hypoxic response may be differentially regulated in chronic hypoxic cells and acute hypoxic cells. Chronic hypoxia may play a greater role than acute hypoxia in promoting the aggressive phenotype of tumor cells. This observation mimics the clinical scenario where tumor cells following treatment with radiation are subjected to hypoxic conditions. The reemergence of tumor following treatment usually results in tumor cells that are more aggressive and metastatic.

Keywords: *Chronic hypoxia, HIF-1α, NF-κB, VEGF, MMPs*

Abbreviations: *CM, conditioned medium; DNHIF-1α, dominant-negative HIF-1alpha; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; β-gal, β galactosidase; Glut1, glucose transporter 1; HIF-1, hypoxia-inducible factor 1; HMLL, continuous hypoxic MatLyLu cells; HRE, hypoxia response element; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa B; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; λ-PPase, lambda protein phosphatase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor*

Introduction

Solid tumors usually exist under acute and/or chronic hypoxic microenvironments. Tumor cells selected via sustained hypoxia exposure are observed to be more aggressive. The mechanism of this phenomenon is not well studied. Genetic and biological changes responsible for their survival have not yet been elucidated. This phenotype may occur due to a decrease in apoptosis, an increase of glycolytic metabolism and a stimulation of angiogenesis and invasion [1–4].

HIF-1 and NF-κB are two redox-sensitive transcription factors implicated in hypoxia [5]. Both are

Correspondence: G. Singh, Department of Pathology and Molecular Medicine, Research Juravinski Cancer Center, McMaster University, 699 Concession Street, Hamilton, Ont., Canada L8V 5C2. Tel: 1 905 387 9711. Ext. 67007. Fax: 1 905 575 6330.
E-mail: gurmit.singh@hrcc.on.ca

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regulated by the changes of oxygen tension, and are induced in response to low oxygen tension and elevated levels of ROS [6–9]. HIF-1 is a heterodimer consisting of an oxygen-sensitive subunit HIF-1α and a constitutively expressed HIF-1β subunit. In the presence of oxygen, a prolyl hydroxylase covalently modifies HIF-1α, resulting in a hydroxylated proline in the oxygen-dependent degradation domain (ODD) [10,11]. The hydroxylated proline is recognized by von Hippel-Lindau tumor suppressor protein (pVHL), which initiates a rapid ubiquitin-dependent proteolysis of HIF-1α [12–15]. In the absence of oxygen, the post-translational modification of HIF-1α is suppressed because the hydroxylase ceases to function [10,11]. HIF-1α then accumulates and translocates into nucleus, where it dimerizes with HIF-1β and binds to specific hypoxic-response element (HRE) and activates transcription of target genes. The DNA-binding transcription factor NF-κB is maintained inactive in the cytosol by a family of inhibitory protein κB (IκB). Upon stimulation by hypoxia/reoxygenation, IκB proteins are phosphorylated by inhibitor κB kinase (IKK) and degraded by proteasomes, which leads to exposure of nuclear translocation signals on NF-κB, and permits NF-κB nuclear import and DNA binding [16]. HIF-1α and NF-κB are both regulated by changes in oxygen concentrations. Hypoxia is known to activate HIF-1α while hyperoxia results in nuclear translocation and activation of NF-κB [5,17]. Increasing evidence indicates a crosstalk between NF-κB and HIF-1α. It has been demonstrated that, inhibition of NF-κB by pyrrolidine dithiocarbamate (PDTC) significantly blocked HIF-1-regulated erythropoietin (EPO) expression [18] and secondly, inhibition of NF-κB either pharmacologically or by transfection of an NF-κB super-repressor plasmid abrogated microtubule-depolymerizing agents (MDAs)-mediated induction of HIF-1α [19].

Several reports have suggested that HIF-1α and NF-κB play an important role in malignant progression. Overexpression of HIF-1α by *in vitro* hypoxia induces the expression of genes that stimulate angiogenesis, such as VEGF [20] and genes that regulate metabolic adaptation to hypoxia, such as glucose transporters [21], as well as genes that promote invasion, such as matrix metalloproteinase 2 (MMP-2)[4]. Blocking NF-κB activity reduces the expression of VEGF and MMP-9, thereby, decreasing angiogenesis, invasion and metastasis in human prostate cancer cells [22].

The capacity of tumor cells to invade and metastasize determines the malignant progression of the tumor. Among the many genes involved in this process are members of zinc-dependent matrix metalloproteinase (MMP) family. These matrix proteases target basement membrane constituents, such as fibronectin, laminin, collagen, proteoglycans and elastin [23,24]. There are at least 15 members in the MMP family. MMP-2 (gelatinase A/type IV collagenase) and MMP-9 (gelatinase B/type IV collagenase), two members of MMP family, play a critical role in tumor invasion [25,26]. The protein and activity levels of both enzymes are correlated with the metastatic potential of tumor cells [27]. Studies using HIF-1α +/+ and HIF-1α −/− embryonic stem (ES) cells indicate that MMP-2 is involved in hypoxia-induced Matrigel invasion activity [4]. However, it remains unclear what role these MMPs play in hypoxia-mediated tumor invasion. Despite the absence of HRE in MMP-9 promoter, multiple transcription factor binding sites are present in the promoter region of MMP-9, including NF-κB [28].

This is the first study that has established an *in vitro* chronic hypoxic murine prostate cancer cell model (HMLL). The chronic hypoxic model was developed by incubation of MatLyLu cells in 1% O_2 over 3 weeks. Surviving cells were selected through each cell passage and were continuously grown in the hypoxic condition for up to 8 weeks. The acute hypoxic model was developed by exposure of MatLyLu cells to 1% O_2 for 5 h. The aim of the present study was to compare tumor cell response to acute and chronic hypoxic conditions, and to examine the effect of these hypoxic conditions on malignant progression as a model to mimic the clinical scenario.

Materials and methods

Cell culture

Murine prostate cancer MatLyLu cell line was obtained from ATCC. Cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 and 20% O_2 in air, and referred to normoxia. The chronic hypoxic HMLL cells, derived from MatLyLu (see detail below), were maintained in a hypoxic chamber containing 1% O_2 at 37°C. Antibiotics were removed from the culture for at least two cell passages prior to an experiment.

Acute hypoxia

MatLyLu cells were replaced with fresh serum-free RPMI 1640 medium for 24 h and incubated in a hypoxia chamber (BioSpherix) containing 1% O_2, 5 CO_2 and 95% N2 for 5 h. The hypoxic chamber was maintained at 37°C.

Chronic hypoxia

MatLyLu cells were grown in the hypoxic chamber for 3 weeks. Surviving cells were selected through each cell passage and continuously maintained in the

hypoxic chamber up to 8 weeks. Twenty-four hours prior to an experiment, cells were replaced with fresh serum-free RPMI 1640 medium under hypoxic conditions.

Dominant-negative HIF-1α transfection

The dominant-negative HIF-1α, pCEP4/HIF-1αDN, was kindly provided by Dr Gregg L. Semenza (The Johns Hopkins University School of Medicine, MD, USA). The pCEP4/HIF-1αDN was stably transfected into HMLL cells using LipofectAmine (Life Technologies Inc.). The stable clones were grown in RPMI 1640 medium containing 10% FBS, 10 mM HEPES and 0.2 mg/mL hygromycin (Invitrogen). The hygromycin was removed two passages prior to an experiment to avoid the antibiotic effects. Changes of HIF-1α expression were determined by using northern blot analysis.

Immunoblot assay

Nuclear extracts were prepared using NE-PER™ nuclear and cytoplasmic reagents (PIERCE). Whole cell lysates were prepared using NP40 lysis buffer (1% Nonidet P-40, 0.4 M NaCl, 5 mM Tris pH 7.4, 0.5 mM EDTA pH 8.0, 10% protease inhibitor cocktail). Protein concentrations were quantified using Bio-Rad protein assay kit (Bio-Rad Laboratories). Nuclear proteins or whole cell lysates were denatured and separated on 12% SDS-PAGEs and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were probed with polyclonal anti-HIF-1α antibody (1:500) (Novus Biologicals), polyclonal NF-κBp65 antibody (1:2,000) (Santa Cruz), or polyclonal Glut1 antibody (1:1,000) (Santa Cruz). The membranes were stripped and reprobed with monoclonal antibodies of histone (Santa Cruz) and actin (clone C4) (ICN Biomedicals, Inc.) for equal loading control of nuclear extracts and whole cell lysates, respectively.

Dephosphorylation treatment

After hypoxic treatment, ten microgram nuclear extracts were incubated with 400 U of lambda protein phosphatase (λ-PPase) (New England BioLabs) for 2 h at 30°C. Proteins were then resolved by SDS-PAGE electrophoresis and their identity determined by immunoblotting.

Total RNA isolation and northern blot analysis

Total RNA was isolated using RNeasy mini kit (Qiagen). Ten microgram of total RNA was heat denatured and separated on a 1.5% RNA gel and transferred onto a positive charged nylon membrane (Roche Diagnostic GmbH). The membrane was hybridized overnight at 55°C with the cDNA probe labeled with [α^{32}P]dATP using a random Decalabel™ DNA labeling kit (MBI Fermentas) for HIF-1α. The probed membrane was then washed extensively and detected using a phosphor-imaging screen (Molecular Dynamics). The probe for HIF-1α was obtained by HindIII restriction cutting from the HIF-1α cDNA, which was made by RT-PCR with forward primer 5′-accgattacccatgga-3′, and reverse primer 5′-gctcagt-taacttgatccaaag-3′. The probe recognizes both the wild type and the truncated form of HIF-1α.

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from the cells using NE-PER™ nuclear and cytoplasmic reagents (PIERCE). EMSA was performed using gel shift assay systems according to the manufacturer's instructions (Promega). Briefly, double-stranded NF-κB (5′-agttgaggggactttcccaggc-3′) or HIF-1α (5′-gccctacgtgctgtctca-3′) was end-labeled with [γ^{32}P]ATP (PerkinElmer) by Klenow fragment. Equal amounts of radioactive probe were added to the DNA binding reaction mixtures containing 5 μg nuclear extract. The mixtures were incubated at room temperature for 20 min. Samples were resolved on 5% polyacrylamide gels at 250 V and imaged by autoradiography. Specific NF-κB or HIF-1α binding was verified by a competitive EMSA with 100-fold excess unlabeled double–stranded NF-κB or HIF-1α oligonucleotides in the binding reaction prior to the addition of [γ^{32}P]ATP end-labeled oligonucleotides.

Luciferase reporter assay

By use of Lipofectin reagent (Life Technologies, Inc.), cells were transiently transfected with NF-κB-Luc plasmid containing NF-κB promoter (Stratagene). After transfection cells were subjected to acute hypoxia or continuously maintained in the hypoxic condition. Forty-eight hours after the transfection, the cells were washed with PBS and lysed. The supernatant was assayed using the luciferase assay kit (Stratagene). In subsets of experiments, cells were transiently transfected with gWiz™ β-gal plasmid (Gene Therapy Systems, Inc.) and subjected to hypoxic conditions. The β-galactosidase activity was measured 48 h after the transfection according to the manufacturer's instruction (Stratagene). The relative luciferase activity was calculated by normalization of the NF-κB-Luc luciferase signal to the activity of β-galactosidase.

Conditioned medium (CM) and VEGF ELISA

Secretion of VEGF protein into CM was analyzed by ELISA using a mouse VEGF Quantikine kit (R&D

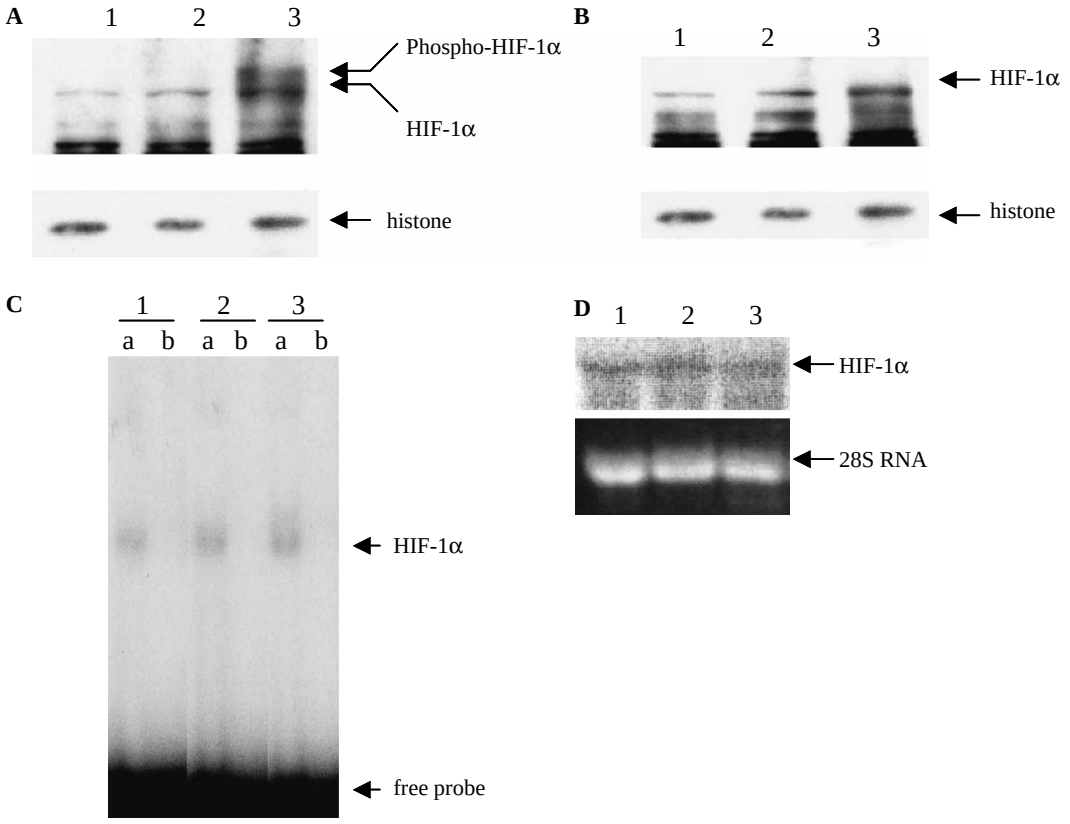

Figure 1. The expression of HIF-1α in acute and chronic hypoxic cells. MatLyLu cell were subjected to normoxia (1), acute hypoxia (1% O_2, 5 h) (2), and HMLL cells were continuously maintained in hypoxia (1% O_2) at 37°C (3). A. Nuclear extracts (10 μg) from normoxic and hypoxic conditions were denatured and separated on 10% SDS-PAGEs. HIF-1α was detected using anti-HIF-1α polyclonal antibody. B. Nuclear extracts were treated with λ-PPase prior to the immunoblot detection for HIF-1α. Histone detection was used to confirm equal loading of the nuclear protein. C. EMSA for HIF-1α DNA binding activity. The nuclear extracts (5 μg) were mixed with γ^{32}P-labeled double-stranded HIF-1α oligonucleotide (a), or with 100-fold excess of unlabeled double-stranded HIF-1α oligonucleotide prior to the addition of γ^{32}P-labeled HIF-1α (b). Samples were then resolved on a 5% polyacrylamide gel at 250 V and imaged by autoradiography. D. Northern blot for HIF-1α. Total RNA (10 μg) isolated from normoxic and hypoxic cells was separated on a RNA gel and transferred onto a nylon membrane. The membrane was hybridized with HIF-1α cDNA probe labeled with α^{32}P using DecaLabel™ DNA labeling kit. Radioactive signals were detected by autoradiography. Equal loading of RNA was confirmed by staining of 28S ribosomal bands. Representative data from three independent experiments are presented.

Systems). To generate CM, cells (5×10^5) were seeded in 6-well plates. Twenty-four hours later, the medium was replaced by fresh serum-free medium and cells were subjected to different hypoxic conditions. After hypoxic treatment CM was collected and cleared by centrifugation at 14,000 rpm at 4°C for 5 min and then stored at −80°C for ELISA. VEGF ELISA analysis was carried out according to the manufacturer's instructions. The VEGF concentrations in the CM were calculated from a standard curve derived by using recombinant mouse VEGF165 proteins.

Matrigel assay

Matrigel assay was performed as described before [29] to measure the invasiveness of the hypoxic cells. Briefly, MatLyLu and HMLL cells (2×10^4) were seeded in the Invasion Chambers coated with the Matrigel reconstituted basement membrane layer (Becton Dickinson Labware) in serum-free medium. The lower chamber was filled with medium supplied with 10% FBS. The plates were incubated at 37°C in a 5% CO_2, 20% O_2 incubator for normoxia. For hypoxic treatment, plates were placed in the hypoxic chamber containing 1% O_2, 5% CO_2 and 95% N2. After 24 h incubation, cells on the top surface of the filter were scrubbed with cotton bud, and the cells on the lower surface of the filter were fixed with methanol and stained with hemotoxyline. The same five microscopic fields were used to count the number of cells on each membrane.

Zymography analysis

Gelatinase/collagenase activity of secreted MMPs was assayed by zymography on gelatin-containing polyacrylamide gel. Both active and latent forms of the MMP-2 and MMP-9 can be visualized on the gel. Proteins in 10 μL of CM were subjected to electrophoresis under nonreducing conditions on an

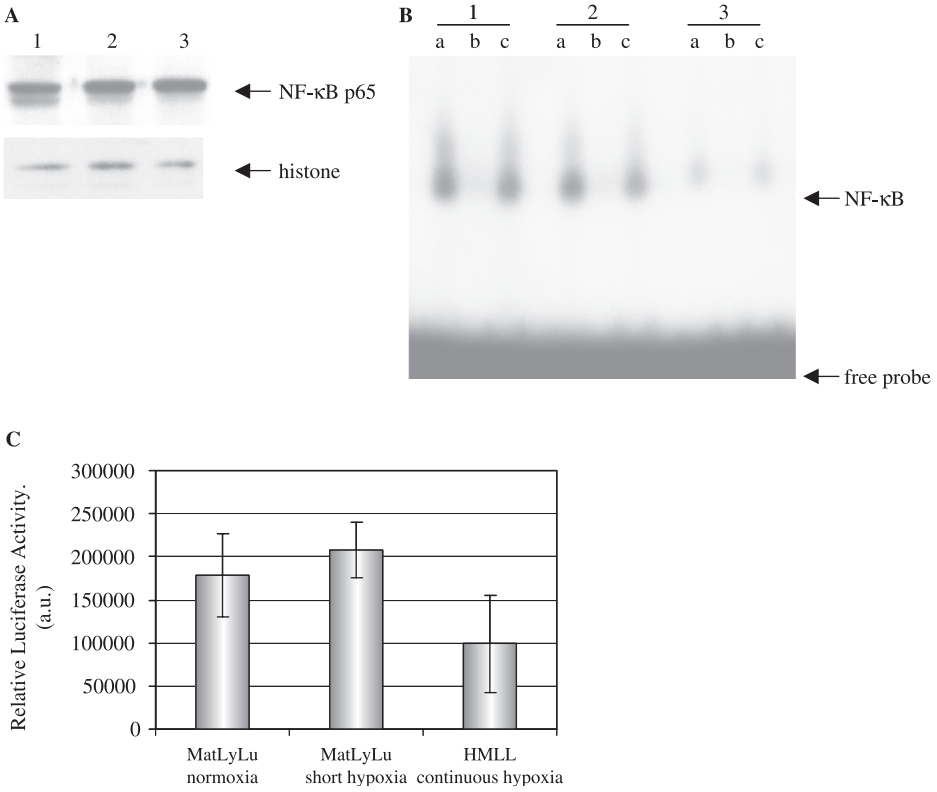

Figure 2. Analysis of NF-κB activity under acute and chronic hypoxic conditions. MatLyLu cell were subjected to normoxia (1), acute hypoxia (1% O_2, 5 h) (2), and HMLL cells were continuously maintained in hypoxia (1% O_2) at 37°C (3). A. Nuclear extracts (10 μg) from normoxic and hypoxic conditions were denatured and separated on SDS-PAGE. NF-κB was detected using anti-NF-κBp65 polyclonal antibody. Histone detection was used to confirm the equal loading of nuclear protein. Representative data from three independent experiments are presented. B. EMSA for NF-κB DNA binding activity. The nuclear extracts (5 μg) of the above cells were mixed with γ^{32}P-labeled double-stranded NF-κB oligonucleotide (a), or 100-fold excess unlabeled double-stranded NF-κB oligonucleotide prior to the addition of γ^{32}P-labeled NF-κB (b), or unlabeled Sp-1 prior to the addition of γ^{32}P-labeled double-stranded NF-κB serving as non-specific binding controls (c). After the reaction samples were resolved on a 5% acrylamide gel and imaged by autoradiography. Representative data from three independent experiments are presented. C. Promoter activity of NF-κB. Cells were transiently transfected with NF-κB-Luc and β-gal plasmids and subjected to the hypoxic treatment. The promoter activity of NF-κB was determined 48 h after the transfection. The relative luciferase activity was normalized by β-gal activity. Data are mean ± SD ($n = 4$).

8% polyacrylamide gel containing 0.3% gelatin. After electrophoresis the gel was washed in 2.5% Triton X-100 for 30 min and incubated overnight at 37°C in a solution containing 50 mM Tris–HCl, pH 8.0, 5 mM $CaCl_2$ and 0.04% NaN_3. The gel was then stained with 0.05% Coomassie Blue for 20 min and destained in a destaining solution (40% methanol, 10% acetic acid) until the achromatic bands appeared on the blue background.

Results

Chronic hypoxia induced nuclear translocation and phosphorylation of HIF-1α

To determine whether the induction of HIF-1α is differentially regulated by acute and chronic hypoxia, we analyzed the expression of HIF-1α at both transcription and translation levels. The Dunning prostate MatLyLu cells were subjected to short-term hypoxia (1% O_2 for 5 h). The chronic hypoxic HMLL cells were maintained in hypoxia chamber (1% O_2). After the hypoxia treatment, the protein expression of HIF-1α was examined in the nuclear extracts. We found there was an increase in nuclear accumulation of HIF-1α under both acute and chronic hypoxia. A more prominent elevation of HIF1α was observed in HMLL cells with a slower migrated band, compared to that in acute hypoxic cells (Figure 1A). We then treated the nuclear extracts with λ-PPase. Figure 1B shows that the slower migrated band in HMLL cells disappeared as a result of the λ-PPase treatment. Therefore, the slower migrated band was the phosphorylated form of HIF-1α. As shown in Figure 1C, a higher DNA binding activity was induced in chronic hypoxia as compared to that in acute hypoxia. We next examined whether the induction of HIF-1α protein by hypoxia resulted from an increased production of HIF-1α mRNA levels. As can be seen in Figure 1D, HIF-1α mRNA levels were not changed from normoxia to hypoxia.

These data indicate that the phosphorylation status of HIF-1α changes during chronic hypoxic conditions.

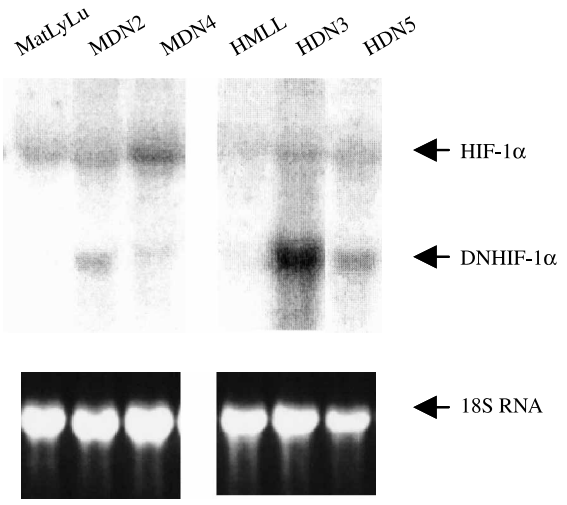

Figure 3. Expression of dominant-negative HIF-1α mRNA. MatLyLu and HMLL cells were stably transfected with dominant-negative HIF-1α plasmid, pCEP4/HIF-1αDN. Total RNA (10 μg) isolated from normoxic and hypoxic cells was separated on a RNA gel and transferred onto a nylon membrane. The membrane was hybridized with HIF-1α cDNA probe labeled with α^{32}P using DecaLabel™ DNA labeling kit. Radioactive signals were detected by autoradiography. Equal loading of RNA was confirmed by staining of 18S ribosomal bands. Representative data from three independent experiments are presented.

Cells surviving chronic hypoxia further activate HIF-1α by increasing its protein phosphorylation, and nuclear translocation as well as DNA binding function.

Effects of hypoxia on NF-κB activity

NF-κB plays a key role in the inflammatory response. Changes in oxygen concentration can also result in nuclear translocation and activation of NF-κB in several cell lines [18,30]. To determine whether NF-κB is involved in the hypoxic response in MatLyLu cells, we examined the effects of acute and chronic hypoxia on NF-κB nuclear translocation, DNA binding activity and transcriptional activity. The nuclear abundance of NF-κB was analyzed by Western blot with a NF-κB-p65 antibody. As shown in Figure 2A, MatLyLu cells consist of a constitutive level of NF-κB protein. It appears that both acute and chronic hypoxia did not alter the protein level of NF-κB in MatLyLu cells. To characterize the protein function of NF-κB, we performed EMSA and luciferase reporter assay. As can be seen in Figure 2B, EMSA revealed that a band was present in MatLyLu cells under normoxia and was reduced in the chronic hypoxic cells. This band disappeared with an unlabeled NF-κB probe but not with an unlabeled non-specific probe, suggesting that a constitutive DNA binding activity was present under normoxic condition and that chronic hypoxia inhibited DNA binding function of NF-κB. To determine the transactivation activity, MatLyLu cells were transiently transfected with NF-κB-Luc plasmid and subjected to acute and chronic hypoxia. Acute hypoxia did not alter the luciferase activities, whereas chronic hypoxia showed a minimal suppression of NF-κB luciferase activities (Figure 2C). These changes are consistent with the changes in the DNA binding activity of NF-κB. NF-κB protein was not functionally activated in acute hypoxia and was slightly suppressed in the chronic hypoxic cells.

Taken together, these data suggest that acute hypoxia and chronic hypoxia differentially regulate HIF-1α and NF-κB transcription factors. Chronic hypoxia causes activation of HIF-1α and inhibition of NF-κB activity.

Effects of dominant-negative HIF-1α on hypoxia-induced glucose metabolism

To further investigate the functional activity of HIF-1 under hypoxic conditions, we stably transfected the dominant-negative HIF-1α (DNHIF-1α) into MatLyLu cells and the chronic hypoxic HMLL cells. As determined by northern blot analysis, four clones were selected as MDN2, MDN4 from MatLyLu transfection and HDN3, HDN5 from HMLL transfection (Figure 3). We analyzed the expression

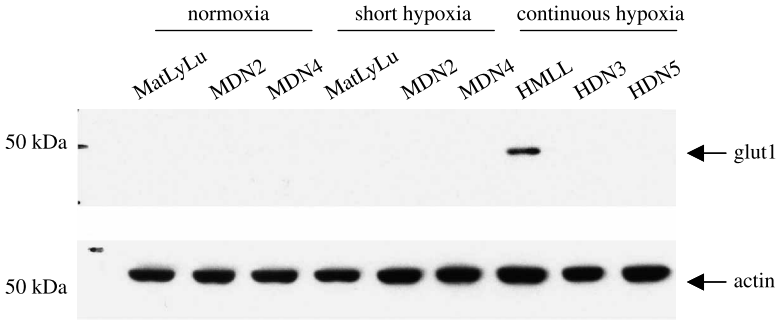

Figure 4. Effects of hypoxia and dominant-negative HIF-1α on Glut1 expression. After stable transfection, cells were subjected to normoxia or hypoxic conditions. The Glut1 expression was determined using Glut1 polyclonal antibody. Equal loading was confirmed by reprobing the membrane with β-actin monoclonal antibody. Representative data from three independent experiments are presented.

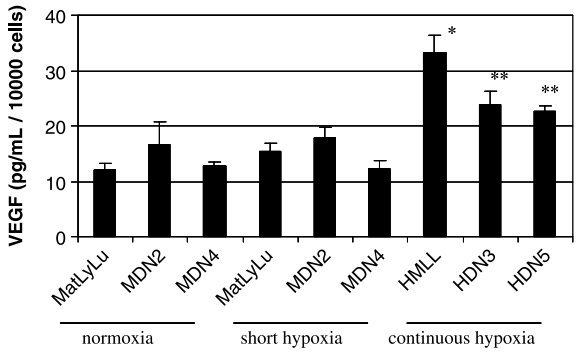

Figure 5. Effects of hypoxia and dominant-negative HIF-1α on VEGF secretion in the conditioned media. Equal number of live cells was seeded into 6 well plates and cultured in serum-free medium for 24 h. HMLL cells and their DNHIF-1α transfectants HDN3, HDN5 were continuously maintained in 1% O_2; MatLyLu cells and their DNHIF-1α transfected MDN2, MDN4 were subjected to normoxia or acute hypoxia (1% O_2, 5 h). The conditioned medium was then collected and determined using the mouse VEGF Quantikine ELISA kit. VEGF concentrations were calculated from a standard curve derived by using recombinant mouse VEGF165 proteins. Data are mean ± SD ($n = 3$), $\star p < 0.001$ compared to normoxic MatLyLu cells, $\star\star p < 0.001$ compared to HMLL cells.

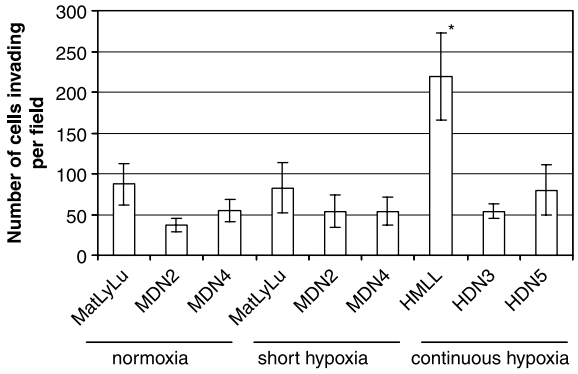

Figure 6. Effects of hypoxia and dominant-negative HIF-1α on invasion of Dunning prostate cancer cells. After hypoxic treatment, alive cells (20,000) were seeded onto Matrigel-coated filters in a Boyden chamber and incubated 24 h in 20% or 1% O_2. The number of cells on the underside of the filter was counted. Data are mean ± SD ($n = 3$), $\star p < 0.001$ compared to normoxic MatLyLu cells.

of one of the HIF-1 target genes, glucose transporter 1 (Glut1). Glut1 gene contains the consensus binding sequence for HIF-1 [31]. Glut1 is the primary glucose transporter protein expressed in most type of tissues and cell lines [2,32]. As shown in Figure 4, MatLyLu cells lack basal expression of Glut1; however chronic hypoxia induced a significant expression of Glut1 protein. Acute hypoxia did not exert the induction of Glut1 in MatLyLu cells. The induced expression of Glut1 was completely suppressed by DNHIF-1α transfection, confirming that the introduced DNHIF-1α did function in a dominant-negative manner.

Effects of dominant-negative HIF-1α on hypoxia-mediated VEGF secretion

Although VEGF is controlled by various transcription factors, the major regulator of its expression in response to hypoxia is HIF-1 [33]. HIF-1α protein is an important determinant for the activity of HIF-1. We examined the secretion of VEGF from the conditioned medium under both acute and chronic hypoxic conditions. As can be seen in Figure 5, VEGF protein levels in the conditioned medium of HMLL cells were much higher than in those of MatLyLu cells under normoxia. No significant changes in VEGF production were observed in the conditioned medium of acute hypoxic cells. The secretion of VEGF protein was significantly reduced in dominant-negative transfected clones HDN3 and HDN5, compared to the chronic hypoxic HMLL. These data again confirm the transcriptional activity of HIF-1α in hypoxic MatLyLu and HMLL cells, and the dominant-negative inhibitory function of DNHIF-1α in the hypoxic cells.

Effects of dominant-negative HIF-1α on hypoxia-mediated cell invasion

Due to disruption of HIF-1 pathway causing attenuation in the expression of both glucose metabolism- and angiogenesis-associated genes under hypoxia, we hypothesize that the disruption of HIF-1 pathway may also interrupted in the Dunning prostate tumor cell invasion. To investigate the role of HIF-1α on invasion, MatLyLu and HMLL cells as well as DNHIF-1α transfected cells were seeded onto a filter that was coated with Matrigel, an experimental basement membrane, and were exposed to normoxia, acute hypoxia and chronic hypoxia. The number of cells that digested Matrigel and migrated through 8 μm pores in the filter was counted 24 h later. The invasiveness of HMLL cells was significantly increased in the chronic hypoxic cells (Figure 6). The acute hypoxia, however, did not result in an increase in the invasiveness, compared to the normoxic controls

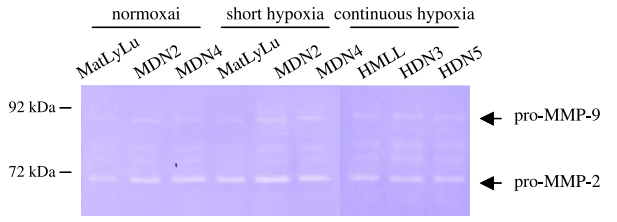

Figure 7. Effects of hypoxia and dominant-negative HIF-1α on the expression of MMP-2 and MMP-9. Equal number of live cells was seeded and grown in serum-free medium 24 h before the experiments. After acute and chronic hypoxia, aliquots of conditioned medium were analyzed by gelatin zymography as described in Materials and Methods. Representative data from three independent experiments are presented.

(Figure 6). DNHIF-1α transfectants, HDN3 and HDN5, showed a significant decrease in cell invasion under chronic hypoxia. These data suggest that HIF-1α activity plays an important role in basement membrane invasion by HMLL cells under chronic hypoxic conditions.

Effects of hypoxia on the production of MMP-2 and MMP-9

To analyze MMP production, MatLyLu cells and HMLL cells were incubated in serum-free medium for 24 h and were then subjected to hypoxia. Gelatin zymography was performed to determine the levels of MMP-2 and MMP-9 in the conditioned medium (Figure 7). Two bands with strong gelatinolytic activity at molecular weight of approximately 72 and 92 kDa were observed. The levels of pro-MMP-2 and pro-MMP-9 remained unchanged in both acute hypoxic cells and chronic hypoxic cells compared to normoxic MatLyLu cells. DNHIF-1α had no effects on the expressions of MMPs. These data suggest that MMP-2 and MMP-9 expression is not controlled by hypoxia in MatLyLu cells. Hence the regulation of MMP-2 and MMP-9 is not directly mediated by HIF-1α.

Discussion

Hypoxia, which activates the hypoxia inducible factor-1 alpha (HIF-1α) pathway, is a common feature in malignant tumor cells and has been linked with tumor cell survival and resistance to radiation therapy. Cells cope by turning host genes that can counteract the effect of low oxygen levels, or hypoxia. The expression of HIF-1 in prostate cancer cells is one of the mechanisms that activate survival genes. HIF-1's target genes include those involved in controlling cell growth, division, survival and mobility. Activation of HIF-1α by hypoxia is regulated at multiple levels, including protein stabilization, nuclear translocation, DNA binding activity and transcription activation function [34]. In this study, we found that chronic hypoxia has a profound induction of HIF-1α activity in Dunning prostate MatLyLu tumor cells. Furthermore, stabilized HIF-1α was observed in the nucleus of the chronic hypoxic HMLL cells. HIF-1α-mediated gene expressions of Glut1 and VEGF were significantly higher in HMLL cells compared to acute hypoxic MatLyLu cells. Also, chronic hypoxia stimulates HIF-1α protein phosphorylation in HMLL cells. Despite a constitutive binding activity in MatLyLu cells, a higher HIF-1α DNA binding activity is found in the chronic hypoxic cells, compared to that in the acute hypoxic cells. These findings provide more mechanistic data to the recent report that hypoxia induced a biphasic effect on HIF-1α stabilization with accumulation in early hypoxia (5 h) and reduction during prolonged hypoxia (16 h), which is due to the activation and inactivation of PI3K/Akt/GSK3β, respectively [35]. It should be noted that the chronic hypoxic HMLL cells were established by growing MatLyLu cells continuously under hypoxic condition. Surviving cells were selected through each cell passage and may have developed resistance to hypoxia. Thus, HMLL cells may respond differently from cells that are exposed to prolonged hypoxia. The phosphorylation status of HIF-1α in the acute and chronic hypoxic cells indicates that the signaling pathways leading to HIF-1α function under these two conditions are differentially regulated. The involvement of several kinases, such as PI3K/Akt, MAPK and ERK, in the activation of HIF-1α upon hypoxia still remains largely controversial [35–38]. It appears that activation of these kinases is cell type specific as well as hypoxic condition dependent. Further studies are clearly required to examine the pathways leading to the activation of HIF-1α in the hypoxic resistant cell model vs. those in the cells exposed to acute hypoxia. We used dominant-negative HIF-1α [39] to verify the role of HIF-1α in chronic hypoxic cells. Glut1 expression was completely suppressed by DNHIF-1α during hypoxic conditions, confirming the inhibitory effectiveness of the dominant-negative construct. However DNHIF-1α did not completely block the induction of VEGF proteins under chronic hypoxia, suggesting that HIF-1α is not the only transcription factor contributing to their expression. Multiple transcription regulatory mechanisms are present in VEGF promoter, including Sp-1, AP-1, AP-2 [32,40,41]. NF-κB has been proposed as a potential candidate for the expression of VEGF although the binding sites for NF-κB in the 5′- and/or 3′-regulatory region(s) are not identified yet [22]. The nuclear translocation of NF-κB remained unchanged upon acute and chronic hypoxia. Furthermore, NF-κB activity remained stable in acute hypoxia but attenuated in chronic hypoxic cells as determined by DNA binding activity and luciferase reporter assay. These findings indicate that the nuclear translocation and DNA binding of NF-κB are differentially regulated by hypoxic stress. Redox regulation has been suggested as a mechanism for NF-κB activation. ROS appear to be a requisite component for this transcription factor but a reducing condition is needed in the nucleus for DNA binding and transcriptional activity [5,42]. The involvement of ROS in HIF-1α stabilization and activation remains controversial [5,43]. Data from our study demonstrate that the oxygen and redox-sensing transcription factors HIF-1α and NF-κB are oppositely regulated in the chronic hypoxic cells.

In addition to the regulation of angiogenesis and metabolism adaptation, HIF-1α activity is also associated with tumor progression. The constitutive expression of HIF-1α protein and the DNA the

elevated cell growth rate and increased metastatic potential [44–45]. Krishnamachary et al. [4] showed that binding activity in MatLyLu cells is associated with the malignant tumor phenotype, including data from embryonic stem cell line demonstrating the hypoxia-mediated upregulation of a group of genes that play important roles in the pathophysiology of invasion, including MMP-2, cathepsin D, urokinase plasminogen activator receptor (uPAR), vimentin and transforming growth factor α (TGF-α). Our data extend this observation that chronic hypoxia stimulates a higher degree of invasion potential in rat prostate tumor cells. Dominant-negative HIF-1α efficiently blocks this process, indicating the important role of HIF-1α in the invasive characteristics of cancer cell phenotype. The expressions of MMP-2 and MMP-9 are not affected upon exposure to hypoxia in MatLyLu and HMLL cells. Furthermore, disruption of HIF-1 pathway by dominant-negative HIF-1α did not alter MMP-2 and MMP-9 levels. This is in line with previous report that the *in vitro* expression of MMP-2 and MMP-9 in several neuroblastoma cell lines remains constant in the hypoxic environment [46]. The pericellular environment where the proteolytic processes occur is important for tumor cell migration and invasion. It is regulated by plasminogen activator system, consisting of urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (t-PA), their inhibitors, and receptors, and the MMP system, consisting various MMPs and their inhibitors. It can be speculated that these metastasis-associated genes and their interactions might be also affected by the chronic hypoxia, which may change the proteolytic balance and potentiate the invasive phenotype.

Chronic hypoxia is often subjected to tumor tissue *in vivo*. This is the first study charactering *in vitro* chronic hypoxic response in HMLL cells. HIF-1α is strongly activated whereas NF-κB activity is suppressed under chronic hypoxic condition. Tumor cell invasion ability is highly activated by chronic hypoxia. Disruption of HIF-1α significantly inhibits the invasive potential of HMLL cells. These results suggest that signaling pathways utilized by hypoxic stress are involved in the chronic hypoxia-mediated tumor progression. Identification of these downstream targets will provide a mechanism for invasiveness and a potential therapeutic target in preventing chronic hypoxia-mediated tumor aggressiveness.

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