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Chronic hypoxia induces androgen-independent and invasive behavior in LNCaP human prostate cancer cells

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Abstract

Purpose: Tumor hypoxia is a common feature of any cancer, including prostate cancer (CaP), and associated with tumor cell aggressiveness. Although some reports are available on acute hypoxia-response in CaP cells aggressiveness, little is known about chronic hypoxia-response. We investigated the effects of chronic hypoxia on human CaP cells.

Materials and methods: The human androgen-dependent CaP cell line LNCaP was cultured under normoxia (21% O_2), acute hypoxia (1% O_2), or chronic hypoxia (1% O_2 for over 6 months). The cell growth, cell cycle and cell behavior of these cells were analyzed by cell count, flow cytometric analysis and in vitro cell migration and invasion assay, respectively. The expression of matrix metalloproteinases and intracellular signaling pathways were tested by real time reverse transcriptase-polymerase chain reaction and Western blotting.

Results: Chronic hypoxia-conditioned LNCaP cells grew in an androgen-independent manner with acceleration of G1 to S phase cell cycle progression. Chronic hypoxia, but not acute hypoxia, accelerated cell migration and invasion. The expressions of matrix metalloproteinase-7, -9, -14, and -15 were significantly up-regulated in LNCaP cells under chronic hypoxia, but not under acute hypoxia. In addition, PI3K/Akt, JAK/STAT, and HIF-1 pathways were activated in chronic hypoxia-conditioned LNCaP cells.

Conclusions: These results suggested that chronic hypoxia plays an important role in enhancement of malignant potential during androgen-independent CaP progression.

Keywords: Chronic hypoxia; Prostate cancer; Cell growth; Cell invasion

1. Introduction

Prostate cancer (CaP) is one of the most common malignancies diagnosed and treated in men in the Western countries [1]. Androgen ablation therapy plays a central role in the treatment of patients with advanced CaP. However, approximately 15% of patients do not respond to hormone manipulation, and the majority relapses within 2–3 years from treatment. This treatment-resistant CaP, called castration-resistant CaP [2], remains incurable.

Cancer cells often encounter a hypoxic environment during tumor growth and progression. This tumor hypoxia is a common feature of several cancers and is associated with tumor progression, therapeutic resistance, and poor outcome. Tumor cells undergo a variety of biological responses when placed under hypoxic conditions, including activation of signaling pathways and drastic changes in gene expression patterns that enable them to better survive in a suboptimal O_2 environment and even increase their potential aggression [3,4].

Also in CaP, tumor hypoxia exists and is correlated with a poor clinical outcome [5–8]. In addition, androgen ablation therapy induces hypoxic environment via reduction of blood flow to CaP tissue [9]. Although the molecular basis of CaP progression is increasingly well documented, the potential role of tumor hypoxia in this process remains poorly understood. Some reports are available on acute hypoxia-response in CaP cell aggressiveness [10–12], but little is known about chronic hypoxia-response.

Therefore, we hypothesized that CaP cells, which were led into chronic hypoxia condition during tumor growth, progression, and androgen ablation therapy, acquired androgen-independent and invasive cell behavior. In this study, we established chronic hypoxia-conditioned LNCaP cells by culturing LNCaP, human androgen-dependent CaP cell

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line, at 1% O_2 for over 6 months, and examined the effect of chronic hypoxia on androgen-independent growth and invasion in CaP cells.

2. Materials and methods

2.1. Cell line and culture conditions

The human androgen-dependent CaP cell line, LNCaP, was purchased from the American Type Culture Collection (ATCC, Rockville, MD). LNCaP cells were cultured in a humidified incubator at 37°C containing 5% CO_2 and 95% air (normoxia) or 5% CO_2, 94% N_2, and 1% O_2 (hypoxia). Chronic hypoxia-conditioned LNCaP cells were established by culturing LNCaP cells under hypoxia (1% O_2) for over 6 months. These cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 15% fetal bovine serum (Sigma-Aldrich), 50 μg/ml streptomycin and 50 IU/ml penicillin (Gibco, Grand Island, NY). For androgen (dihydrotestosterone: DHT) ablation, these cells were cultured in phenol red free RPMI-1640 (Sigma-Aldrich) supplemented with 15% charcoal/dextran treated fetal bovine serum (HyClone, Logan, UT), 50 μg/ml streptomycin and 50 IU/ml penicillin.

2.2. Cell growth analysis

The cells were seeded onto 6-well plates at a density of 3×10^5 cells/well. The cells were trypsinized, collected, and counted at 24, 48, 72, and 96 h. The average number of cells was calculated from 3 independent experiments, repeated in duplicate.

2.3. Flow cytometric analysis

The cells were seeded onto 6-well plates at a density of 3×10^5 cells/well. The plates were incubated in each condition for 48 hours. The cells were collected by trypsinization, washed with PBS, fixed with 70% methanol, and stored at –20°C for over 4 hours. The fixed cells were incubated with 10 μg/ml RNase A (Sigma-Aldrich) for 30 minutes at room temperature and stained with 50 μg/ml propidium iodide (Sigma-Aldrich) for 30 minutes at room temperature. Relative DNA content was determined with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The cell cycle distribution was calculated using CellQuest software (Becton Dickinson).

2.4. Migration and invasion assays

Cell migration was assessed using 24-well BioCoat Control Insert Chambers (Becton Dickinson) with polycarbonate filters containing 8-μm pores. Cells were plated at 5×10^4 cells/well in 0.5 ml of serum-free medium. The outer chambers were filled with 0.5 ml of media containing 15% fetal bovine serum. After 48 hours, cells migrating to the undersurface of the filters were counted. The top surface of the membrane was gently scrubbed with a cotton bud, and cells on the undersurface were fixed in 100% methanol and stained with 1% toluidine blue before undergoing a series of washes. The same 5 microscopic fields were used to count the number of cells passing to the undersurface of each filter. For invasion assays, the control insert chambers were replaced with BioCoat Matrigel Invasion Chambers (Becton Dickinson) treated with Matrigel Matrix reconstituted basement membrane layer.

2.5. Real time RT-PCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN, Germantown, MD) according to the manufacturer's instructions. Total RNA (1 μg) was synthesized into cDNA using ThermoScript RT-PCR System (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Each cDNA sample (2 μl) was amplified using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) and QuantiTect Primer Assays (QIAGEN GmbH, Hilden, Germany) on a LightCycler (Roche Diagnostics Corporation, Indianapolis, IN). Each cycle consisted of denaturation at 95°C for 15 seconds, annealing at 55°C for 5 seconds, and polymerization at 72°C for 10 seconds. The primers were as follows: MMP2 (Hs_MMP2_1_SG QuantiTect Primer Assay; QT00088396), MMP7 (Hs_MMP7_1_SG QuantiTect Primer Assay; QT00001456), MMP9 (Hs_MMP9_1_SG QuantiTect Primer Assay; QT00040040), MMP14 (Hs_MMP14_1_SG QuantiTect Primer Assay; QT00001533), MMP15 (Hs_MMP15_1_SG QuantiTect Primer Assay; QT00014063), β-actin (Hs_ACTB_1_SG QuantiTect Primer Assay; QT00095431). β-Actin was used as an endogenous control to normalize each sample. The experiment was performed by 3 independent experiments in duplicate.

2.6. Protein extraction and Western blot analysis

Whole cell extracts were obtained with M-PER Mammalian Protein Extraction Reagent containing Halt protease inhibitor cocktail and 1 mM Na_3VO_4 (Pierce, Rockford, IL). Protein concentration was determined with the Coomassie Plus Protein Assay Reagent Kit (Pierce). Cell proteins were electrophoresed on 4%–20% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore Corporation, Bedford, MA). Following transfer, the membranes were blocked in 0.1% Tween-20 and 0.1 mol/l phosphate-buffered saline (T-PBS) containing 5% skim milk for 1 hour at room temperature and incubated overnight at 4°C in each primary antibody. After washing with T-PBS, the membranes were incubated with the corresponding secondary antibodies that were conjugated with HRP for 1 hour at room temperature. Immunoreactive bands were visualized with the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ).

2.7. Antibodies and inhibitors

Mouse monoclonal antibodies against cyclinD1 (1:2000 dilution), CDK4 (1:1000 dilution), CDK6 (1:2000 dilution), p21Cip1 (1:2000 dilution), Rb (1:2000 dilution), phospho-STAT-3 (Tyr 705; 1:1000 dilution), rabbit polyclonal antibodies against p27Kip1 (1:1000 dilution), phosphors-Rb (Ser 780; 1:1000 dilution), Akt (1:1000 dilution), phospho-Akt (Ser 473; 1:1000 dilution), and rabbit monoclonal antibody against STAT-3 (1:1000 dilution) were obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-AR (1:250 dilution), mouse monoclonal anti-PSA (1:500 dilution), and rabbit monoclonal anti-VEGF (1:200 dilution) antibodies were obtained from Upstate (Lake Placid, NY), DAKO Cytomation (Carpinteria, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Mouse monoclonal anti-HIF-1alfa (1:1000 dilution) and anti-β-tubulin (1:10000 dilution) antibodies were obtained from Chemicon International (Temecula, CA). Mouse monoclonal anti-Bcl-2 (1:1000 dilution) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The inhibiters of PI3-Kinase (LY294002), JAK (P6), and HIF-1 (YC-1) were obtained from Calbiochem (Darmstadt, Germany).

2.8. Statistical evaluation

Values were expressed as means ± SD. Statistical analysis was performed using the Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of chronic hypoxia on cell growth and cell cycle

To assess the effect of chronic hypoxia on CaP cells proliferation in normal or androgen (dihydrotestosterone: DHT) ablated condition, we first analyzed the cell growth and cell cycle of LNCaP cells under normoxia, acute hypoxia, and chronic hypoxia by cell count and flow cytometry, respectively. Though, in normal medium, cell growth of LNCaP under acute hypoxia was restricted compared with under normoxia, cell growth of LNCaP under chronic hypoxia was significantly ($P < 0.05$) accelerated compared with under acute hypoxia (Fig. 1A, left). In androgen ablated condition, chronic hypoxia-conditioned LNCaP cells grew faster significantly ($P < 0.05$) than LNCaP under normoxia and acute hypoxia (Fig. 1A, right). In normal medium, both of the increase of G1 phase and the decrease of S phase were observed in LNCaP under acute hypoxia, but not changed in LNCaP under chronic hypoxia compared with LNCaP under normoxia (Fig. 1B, upper). The androgen ablation-induced G1 cell cycle arrest in LNCaP under normoxia and acute hypoxia, but not in LNCaP under chronic hypoxia (Fig. 1B, lower).

Next, we evaluated the change of cell cycle regulatory proteins by Western blotting (Fig. 1C). The expression of cyclin D1 and the phosphorylation levels of Rb protein, accelerators of G1 to S phase cell cycle progression, decreased in LNCaP under acute hypoxia but increased on LNCaP under chronic hypoxia. Although the androgen ablation-induced de-phosphorylation of Rb in LNCaP under normoxia and acute hypoxia, the level of phosphorylated Rb protein was invariable on LNCaP under chronic hypoxia. The expression of p27Kip1, G1 to S phase cell cycle progression inhibitory protein, was decreased in chronic hypoxia-conditioned LNCaP.

These results demonstrated that chronic hypoxia-conditioned LNCaP cells grew androgen-independent and hypoxia-resistant manner with acceleration of G1 to S phase cell cycle progression.

3.2. Effect of chronic hypoxia on cell migration and invasion

To assess the effect of chronic hypoxia on CaP cells invasiveness, we analyzed the cell migration and invasion in LNCaP under normoxia, acute hypoxia, and chronic hypoxia by in vitro cell migration and invasion assay (Fig. 2). The cell migration and invasion in LNCaP under chronic hypoxia was significantly ($P < 0.05$) increased about 1.5 and 2.5 times, respectively, compared with LNCaP under normoxia. In LNCaP under acute hypoxia, the cell migration was decreased significantly ($P < 0.05$) and the cell invasion was not changed against LNCaP under normoxia.

3.3. Matrix metalloproteinase expressions in chronic hypoxia-conditioned LNCaP cells

To clarify the mechanism of chronic hypoxia-induced CaP cells invasiveness, we analyzed the expression of MMPs by real time RT-PCR (Fig. 3). MMP-2 was not detected in LNCaP cells (data not shown). The expressions of MMP-7, MMP-9, membrane type-1 MMP (MT1-MMP; MMP-14), and membrane type-2 MMP (MT2-MMP; MMP-15) were significantly up-regulated ($P < 0.05$) in LNCaP under chronic hypoxia compared with LNCaP under normoxia.

3.4. Intra-cellular signaling pathways in chronic hypoxia-conditioned LNCaP cells

To clarify the chronic hypoxia-induced intra-cellular signaling pathways, we analyzed PI3K/Akt, JAK/STAT, HIF-1, and AR pathways and the expression of bcl-2 by Western blotting (Fig. 4A and B). Akt phosphorylation at Ser 473, a site required for Akt activation, was strongly induced in LNCaP under chronic hypoxia, but not in LNCaP under acute hypoxia compared with LNCaP under normoxia. Akt phosphor-

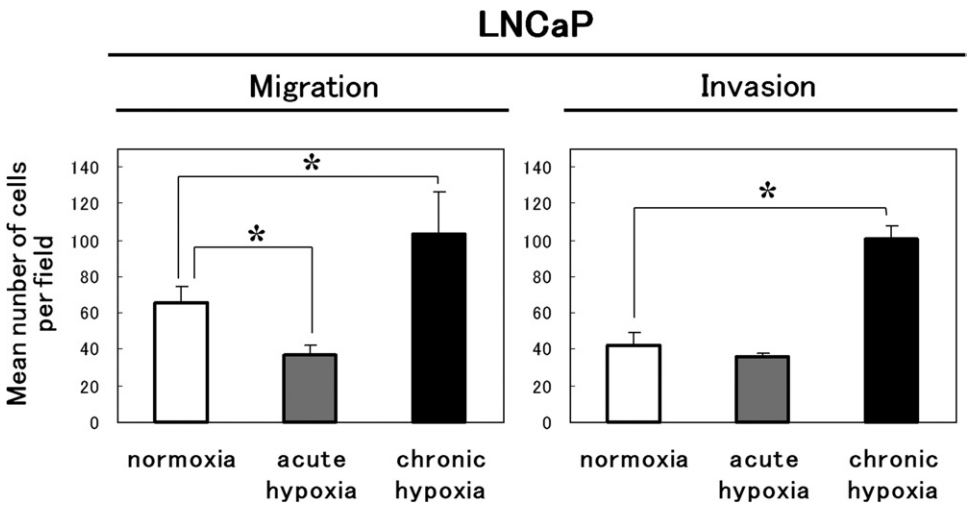

Fig. 2. Activation of cell invasiveness by chronic hypoxia. The cell migration and invasion in LNCaP cells under hypoxia by Matrigel invasion assay. Each bar is the mean ± SD from 3 independent experiments in duplicate. *$P < 0.05$.

ylation was also induced by androgen ablation in LNCaP under all conditions. STAT-3 phosphorylation at Tyr 705, a site required for STAT-3 activation, was only induced in LNCaP under chronic hypoxia. HIF-1α, a well-known transcription factor that accumulates under hypoxia, was increased moderately in LNCaP under acute hypoxia and strongly in LNCaP under chronic hypoxia compared with LNCaP under normoxia. Under acute and chronic hypoxia, the expression of VEGF, a target gene of HIF-1α, was up-regulated against normoxia. Although the expression of AR was not changed under hypoxia, the expression of PSA, a target gene of AR, was up-regulated in LNCaP under acute hypoxia and not changed in LNCaP under chronic hypoxia compared with LNCaP under normoxia. Androgen ablation decreased PSA expression under all conditions. Bcl-2 was up-regulated by chronic hypoxia.

These results demonstrated that PI3K/Akt, JAK/STAT, and HIF-1 pathways were activated in chronic hypoxia-conditioned LNCaP cells and anti-apoptotic status via overexpression of Bcl-2 also presented in chronic hypoxia-conditioned LNCaP cells.

3.5. Effect of cell signaling inhibitors on chronic hypoxia-conditioned LNCaP cells proliferation

To confirm the effect of PI3K/Akt, JAK/STAT, and HIF-1 pathways on chronic hypoxia-conditioned LNCaP cell proliferation, each specific inhibitor, LY294002, P6, and YC-1, was used. Although LY294002 and YC-1 significantly ($P < 0.05$) inhibited cell growth, cell growth inhibition by P6 was slight (Fig. 5, lower). These results demonstrated that activation of PI3K/Akt and HIF-1 pathways were mainly associated with the proliferation of LNCaP cells under chronic hypoxia.

4. Discussion

Tumor hypoxia is present in human CaP and is associated with the cancer cells aggressiveness [5–12]; however, only limited data have been reported on the effect of chronic hypoxia in CaP cells aggressiveness. We cultured LNCaP cells under hypoxia and found that chronic hypoxia-conditioned (for over 6 months) LNCaP cells grew hypoxia-resistant manner. This hypoxia-resistant cell growth was not observed in LNCaP cells under hypoxia for 1 month or 3 months (data not shown). Therefore, we did further analysis using this chronic hypoxia-conditioned (for over 6 months) LNCaP cells. In this report, we demonstrated that chronic hypoxia-conditioned LNCaP cells acquired androgen-independent cell growth and farther invasiveness.

In this study, chronic hypoxia-conditioned LNCaP cells grew androgen-independently. The molecular basis of hormone refractory CaP is well documented, and it is well known that long-term androgen deprivation induces androgen independency in LNCaP [13]. However, little has been reported on acquisition of androgen independency by hypoxia. We established androgen-independent LNCaP by cul-

Fig. 1. (A)–(C) Androgen-independent and hypoxia-resistant growth in chronic hypoxia-conditioned LNCaP cells. DHT = dihydrotestosterone. (A) LNCaP cells proliferation under hypoxia and androgen (DHT) ablated condition was analyzed by cell count. Each bar is the mean ± SD from 3 independent experiments in duplicate. Asterisk indicates $P < 0.05$ vs. normoxia. Dagger indicates $P < 0.05$ vs. acute hypoxia. (B) The acceleration of G1 to S phase cell cycle progression under chronic hypoxia. The cell cycle of LNCaP cells under hypoxia and DHT ablated condition was analyzed by flow cytometry. (C) The change of cell cycle regulatory proteins expression was determined by Western blot analysis. (Color version of figure is available online.)

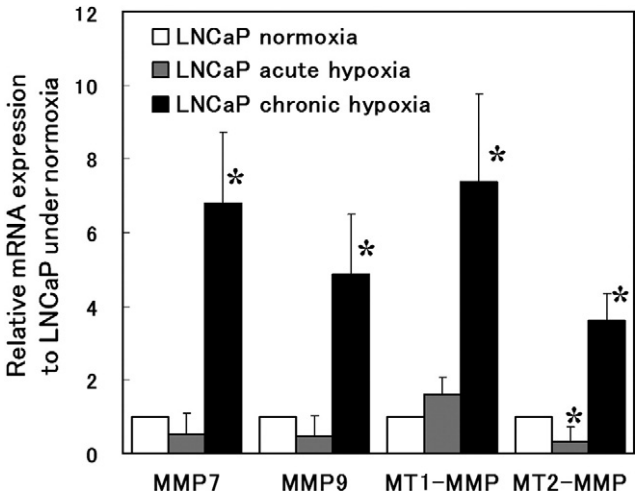

Fig. 3. Overexpression of matrix metalloproteinases under chronic hypoxia. The expression of matrix metalloproteinases was analyzed by real time RT-RCR. Each bar is the mean ± SD from 3 independent experiments in duplicate. Asterisk indicates $P < 0.05$ vs. normoxia.

turing LNCaP under hypoxia (1% O_2) for over 6 months. In LNCaP, it is well known that a large number of passages induce androgen-independent phenotype. Therefore, in a control experiment, we used LNCaP that cultured the same number of passages as against chronic hypoxia-conditioned LNCaP. Our finding is in line with Butterworth et al.'s report that LNCaP-H1 cell line, obtained by repeated long-term exposure to hypoxia (0.1% O_2 for 48 h/wk for 6 weeks, then 0.1% O_2 for 72 h/wk for a further 18 weeks), showed androgen-independent growth [14]. The activation of cell proliferation is an important factor in hormone refractory CaP. Cyclin D1, belonging to a family of cell-cycle regulators called cyclins, functions in the G1 phase of cell cycle and activates the kinase activities of G1 cyclin-dependent kinases (cdk). The active cyclin/cdk complexes phosphorylate proteins, which are important in cell-cycle control. One putative substrate of these cyclin D1-activated kinases is the retinoblastoma protein (Rb). The hypo-phosphorylated form of Rb prevents cells from entering the S phase. When Rb is hyper-phosphorylated, it can no longer prevent the cells from entering S phase. The activities of cyclin/cdk can be inhibited by several negative cell-cycle regulatory proteins (for example p27 Kip1). In our study, the expression of cyclin D1 and the phosphorylation levels of Rb protein decreased in LNCaP on acute hypoxia but re-increased on chronic hypoxia. Although androgen ablation induced dephosphorylation of Rb in LNCaP on both normoxia and acute hypoxia, the level of phosphorylated Rb protein was invariable on chronic hypoxia. The expression of p27Kip1 was decreased in LNCaP on chronic hypoxia. Recently, it has been reported that cyclin D1 overexpression might be related to the evolution of androgen-independent metastatic disease in CaP [15,16]. These results suggest that chronic hypoxia-induced acceleration of G1 to S phase cell cycle progression is a cause of hormone refractory CaP.

Metastasis is the critical factor in the lethality of CaP. The movement of CaP cells from the acinar epithelium through the basement membrane and interstitial stroma into blood or lymph vessels, with subsequent malignant cell migration and colonization of distant tissue sites, is facilitated by degradation of cell adhesion and extracellular matrix proteins. Matrix metalloproteinases (MMPs) have been regarded as major critical molecules assisting tumor cells during metastasis [17–20]. We showed that cell migration, cell invasion, and expressions of MMPs were activated under chronic hypoxia, but not under acute hypoxia. These results suggest that chronic hypoxia plays an important role in CaP cells metastasis via overexpression of MMPs. In contrast, Muñoz-Nájar et al. reported that acute hypoxia (1.5% O_2 for 24 hours) stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation [21].

We showed the change of intra-cellular signaling pathways by hypoxia. The expression of PSA was up-regulated by acute hypoxia. This result concurs with Park's report that acute hypoxia stimulates the activity of AR and its promoter occupancy [12]. On the other hand, the expression of PSA

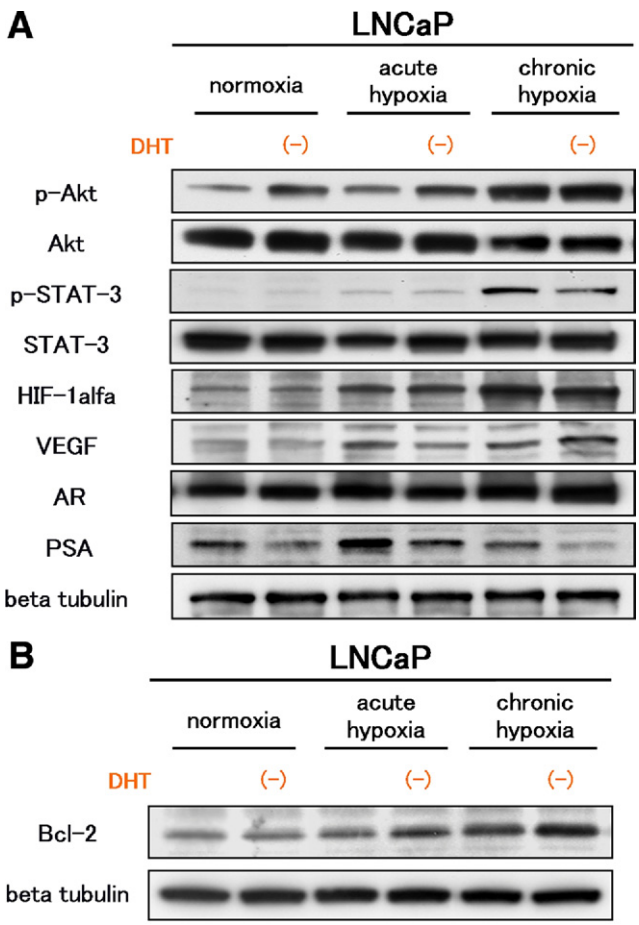

Fig. 4. (A)–(B) Intra-cellular signaling pathways in chronic hypoxia-conditioned LNCaP cells were analyzed by Western blotting. DHT = dihydrotestosterone. (A) PI3K/Akt, JAK/STAT, HIF-1, and AR pathways. (B) The expression of bcl-2. (Color version of figure is available online.)

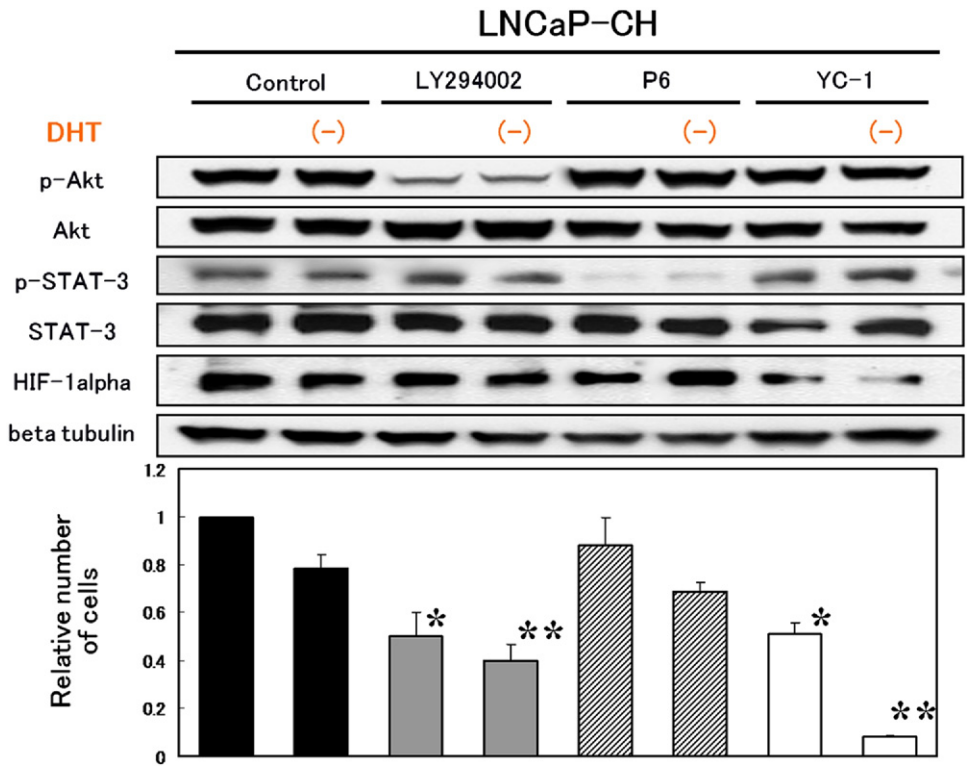

Fig. 5. Cell growth inhibition by cell signaling inhibitor in chronic hypoxia-conditioned LNCaP cells. PI3K/Akt, JAK/STAT, and HIF-1 pathways were analyzed using each specific inhibitor, LY294002, P6 and YC-1. DHT = dihydrotestosterone. Each bar is the mean ± SD from 3 independent experiments in duplicate. *$P < 0.05$ vs. control. **$P < 0.05$ vs. control DHT(–). (Color version of figure is available online.)

was not changed by chronic hypoxia. These results may indicate that AR pathway is not correlated with chronic hypoxia-induced androgen-independent and invasive behavior in LNCaP cells. In our study, PI3K/Akt, JAK/STAT, and HIF-1 pathways were activated in chronic hypoxia-conditioned LNCaP cells and each specific inhibiter, LY294002, P6, and YC-1, inhibited LNCaP cells growth, respectively. The cell growth inhibition by P6 was slight. These results suggest that activation of PI3K/Akt and HIF-1 pathways were mainly associated with enhanced chronic hypoxia-conditioned LNCaP cells proliferation. Bcl-2, which plays important role in acquisition of androgen independency of CaP [13], was up-regulated by chronic hypoxia. This result may indicate that Bcl-2 is one of the elements of chronic hypoxia-induced androgen independency in LNCaP cells.

Recently, many anti-angiogenic agents were developed and work well for several cancers, including CaP. These anti-angiogenic agents demonstrate their ability as an anti-cancer drug via induction of hypoxic environment in cancer tissue. Our data may indicate that long-term use of anti-angiogenic agents for androgen-dependent CaP causes chronic hypoxia condition in cancer tissue and induces androgen-independent and invasive behavior. Further studies are needed to clarify the effect of chronic hypoxia on CaP progression.

5. Conclusions

In conclusion, the effect of chronic hypoxia on CaP cells behavior is distinct from that of acute hypoxia. Chronic hypoxia may play an important role on androgen-independent CaP progression.

The degree to which these results in vitro apply to in vivo models is unclear. Studies are in progress to clarify whether chronic hypoxia-conditioned CaP cells proliferate in aggressive and castration-resistant manner in vivo.

Acknowledgments

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