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Urologic Oncology: Seminars and Original Investigations 31 (2013) 1124–1131

UROLOGIC ONCOLOGY

Original article

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

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Received 17 October 2011; received in revised form 8 December 2011; accepted 8 December 2011

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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1078-1439/$ – see front matter
http://dx.doi.org/10.1016/j.urolonc.2011.12.007

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).