Effect of overexpression of hypoxia-inducible factor-1α induced by hyperoxia in vivo in LNCaP tumors on tumor growth rate

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ABSTRACT

Objective: To study effect of overexpression of hypoxia-inducible factor-1α induced by hyperoxia in vivo in LNCaP tumors on tumor growth rate.

Methods: The prostate cancer LNCaP cells were inoculated in the abdomen of mice. All the mice were randomly placed in the gas chamber with different oxygen content. The groups were divided as follows: twelve mice in hypoxia group, sixteen mice in normoxia group, ten mice in hyperoxia group. After 28 d of treatment, the mice were weighed, the blood samples were taken from the left ventricle, and the tumor was isolated and weighed. Tumor growth, angiogenesis and vascularization, HIF-1α expression and intracellular signal transduction molecules expression in each group of xenografts were detected and analyzed by using Western blotting and immunofluorescence and determination of hemoglobin.

Results: Comparison of the growth of xenografts in each group showed that, the xenografts growth of hypoxia group was more quickly than that of normoxia group. The difference was statistically significant (P = 0.004). The difference in xenografts growth between hyperoxia group compared and normoxia group was not statistically significant (P > 0.05). The expressions of HIF-1α, VEGF and VEGF-R of xenografts in hyperoxia group were significantly higher than those of normoxia group (P < 0.05). The expression of HIF-1α of xenografts in hypoxia group and normoxia group were similar. The blood growth rate of xenografts in hypoxia group (170%) was significantly higher than that of normoxia group (40%) (P < 0.05). The expression of Nrf2 of xenografts in hyperoxia group was significantly higher than that of normoxia group (P < 0.05).

Conclusions: When hyperoxia induces the overexpression of HIF-1α in LNCaP tumor, it will not affect tumor growth. It provides a new ideas and theoretical basis for the clinical treatment of prostate cancer.

1. Introduction

Hypoxia is the main cause of malignant solid tumor chemotherapy and radiotherapy resistance [1]. When the speed of tumor proliferation was faster than the speed of oxygen supply by capillary network, a hypoxia microenvironment probably formed in the tumor cell, therefore activate the self-adaptive mechanism to have more resistance to antagonistic environment, which probably transfer to the apoptosis process of a rapid cell growth inhibition [2-5]. In-vitro studies have shown that hypoxia-inducible factor-1α (HIF-1α) overexpression to regulate and adapt to hypoxia was based on the activation of oxygen sensor of downstream gene, which made the hypoxia microenvironment survive [6-8]. By decrease of oxygen inhale to increase the extent of tumor cell hypoxia will induce moderate systematic hypoxia, which will result the growth rate of prostate xenografts LNCaP increased nearly doubled [9]. But when tumor is lack of
oxygen and HIF-1α is no longer overexpression, which indicated xenografts grew in the alternative pathway of non-HIF-1α centered [10]. When the value of oxygen in prolyl hydroxylase reaction was in expression of PO2 between 40 and 150 mmHg, HIF-1α ubiquitination was nearly activated [11]. Some researchers proposed that whether hyperoxia can used as a powerful method to prevent the derivative change of HIF-1α protein expression or not [12]. Although controversy existed, hyperoxia did play a role in overcoming the primary and secondary onset of certain types of tumors [13–15]. These factors urge us to compare the different effects on tumor growth and HIF-1α expression of prostate cancer (LNCaP) cell, sensitive to androgen, under hypoxia and hyperoxia environments by in vivo test in mice. The present study aimed to provide a new direction for clinical treatment of prostate cancer and a theoretical base for the research of antitumor drugs.

2. Materials and methods

2.1. Cells

LNCaP cells (80%–90% confluence) were cultured in RPMI-1640 medium with 10% (V/V) heat inactivated fetal bovine serum and l-glutamine, and cultured in 5% CO2.

2.2. Mice

A total of 38 Foxn 1 mice, 7 wk old, about 27–30 g, were fed and used according to the Guide for Laboratory Animals. Animal processing, training agreement and euthanasia method were approved by Animal Processing Committee of Milan University. Drinking water and paddling were sterilized under high temperature, and feed was by irradiation at 60°C. The mice were kept in a 12/12 h light/dark photoperiod, and can access to water and feed until 24 h before they were sacrificed.

2.3. Processing

On first day, the LNCaP cells were re-suspended in the final concentration of 3 × 10⁶/0.1 mL 0.9% agar (1:1), and inoculated in the abdomen of mice by 26G insulin syringe. Then the mice were transferred to the gas chamber. All of the gas (balance N₂). According to the oxygen content the mice were divided into: Containing 10% O₂ as Hypoxia Group (n = 12), containing 21% O₂ as Normoxia Group (n = 16) containing 30% O₂ as Hyperoxia Group (n = 10). All groups were treated for 28 d.

2.4. In vivo measurement

The weight of mice and the volume of xenografts were measured three times a week. Before measurement, the mice in hypoxia and hyperoxia groups were transferred to complemental gas chamber for anaerobic compensation.

2.5. Euthanasia

On 28th day, the mice were transferred to complemental gas chamber, anesthetized by injected sodium pentothal with heparin (500 IU) at the dose of 10 mg/100 g body weight, and then sacrificed by cervical dislocation. The mice were removed from the gas chamber and thoracotomy was performed, then the blood sample was collected from the left ventricle. The xenografts were isolated, weighted, and stored in liquid nitrogen at −80°C.

2.6. Western blot

Prepare each biopsy extraction at 4°C. Frozen tissue (50–80 mg) and the mixture of 10 mm HEPES, 1.5 mm magnesium chloride, 0.5 mm DTT, 0.2 mm PMSF, 10 mm KC1 and proteinase inhibitor at the ratio of 1:3 (w/v) were homogenized in glass, pH 7.9, centrifuged at 14 000 r/min for 20 min and obtained particles suspension were centrifuged at 14 000 r/min for 10 min. The supernatant was obtained from the centrifugal pool.

Coomassie brilliant blue kit was used to determine the protein concentration of extracting. Each electrophoresis tank, 70 g protein was added in and separated by SDS-PAGE (6%–8% acrylamide gels). Then, the protein was blotted on the nitrocellulose membrane immersed in the normal saline with 5% skim milk powder and 0.1% Tween Tris buffer (room temperature, 1 h). At 4°C, the membrane was incubated with primary antibody, following with secondary antibody incubation marked by horse radish peroxidase (room temperature, 1 h). Primary antibodies and diluents adopted were: rabbit polyclonal anti-HIF-1α (1:300), rabbit polyclonal anti-bad vessel endothelium (1:400), mouse monoclonal anti-actin (1:10 000), rabbit polyclonal anti-Akt (1:10 000), rabbit polyclonal anti-Phosphorylated protein kinase AKT antibody-Ser473 (1:1000), rabbit polyclone anti-NF2(1:1000). Secondary antibody was anti-mouse IgG (1:10 000) or anti-rabbit IgG (1:10 000) marked by horse radish peroxidase. LiteAblot chemiluminescence substrate was used to soften incubation membrane, and then chemiluminescence was detected under the X-ray film exposure. Gel Doc was used for quantization of blotting intensity and the result images were collected.

2.7. Immunofluorescence

Frozen samples were embedded in OCT, and sliced continuously into 5 μm thickness section with freezing microtome and placed on the silanization glass slide. The sections were dried at room temperature for 3 min, fixed with 4% formalin for 45 min, rinsed twice with PBS for 5 min. Then the sections were fixed with acetic acid 2:1 (v/v) for 5 min, rinsed twice with PBS for 5 min, boiled with 10 mmol/L pH 6.0 citrate buffer for 10 min, washed once in distilled water, three times in PBS, at last in immunofluorescence.

Immunofluorescence technique was employed by binding HIF-1α, Ki67 and CD31 on one immunofluorescence marked antibody. Then mild stirred with 10% goat serum for 1 h. The sections were processed with rabbit polyclonal anti-HIF-1α or rabbit polyclonal anti-Ki67 (1:100), or rabbit polyclonal anti-CD31, washed with PBS at 4°C. At room temperature using goat anti-rabbit IgG fluorescent conjugated secondary antibody treated for 45 min, washed four times with PBS, placed in pH 8.5 PBS containing 0.1% of phenylene diamine as anti- quenching agent and glycerin (10:1). The test sample in
control group was treated with 1.5% normal goat serum substituted of anti-HIF-1α or anti-Ki67 antibody. The inverted slide was detected under the fluorescent microscope and fluorescein filter at the magnification of 40×, and the random images were captured by CCD vidicon and stored in the computer.

2.8. Determination of hemoglobin

Hemoglobin (Hb) concentration was determined as following: 10 μL blood and 1 mL Drabkin's reagent were diluted, stirred, and cultured at room temperature for 30 min, using 1 mL test tube measured the absorbance at λ = 540 nm, calculated the concentration supposed ε = 11.05 cm M⁻¹. Hb content in xenografts and reference organ (left kidney) tissue was determined by diluting 10 μL extracting solution and 1 mL Drabkin's reagent, the following procedures as described previously.

2.9. Statistical analysis

All data were expressed as mean ± SD. One-way ANOVA and Dunnet multiple comparison test were used to analyze the significant difference among hypoxia, normoxia and hyperoxia. The significant level was P = 0.05 (two tailed).

3. Results

3.1. General conditions of mice

In hypoxia group and hyperoxia group, each had a mouse death before euthanasia for unknown cause. Therefore, the left number of mice in each group was: hypoxia group 11 mice, normoxia group 16 mice, hyperoxia group 9 mice. The increase or inhibition of weight in each group was: hypoxia group (0.1 ± 0.4) g, normoxia group (3.6 ± 0.7) g, hyperoxia group (2.3 ± 0.4) g, the difference was not statistical significant (P > 0.05). The success rate of transplantation in each group was: hypoxia group 68%, normoxia group 90%, hyperoxia group 61%, the difference was not statistical significant (P > 0.05).

3.2. Growth of xenografts

Figure 1A shows the variation of average volume/weight of xenografts in three groups at different time points. Xenografts growth in hypoxia group was faster than that in normoxia, the difference was statistically significant (P = 0.004), while compared with normoxia group the xenografts growth in hyperoxia group determined at any time point showed no statistically significant difference (P > 0.05). Xenografts
growth in hypoxia group was twice as fast as that in normoxia and hyperoxia groups, and compared with normoxia group, the difference was statistical significant (\(P = 0.001\)) (Figure 1B, left). The weight of xenografts in hyperoxia group, compared with the normoxia group (Figure 1B, right), the difference was not statistical significant (\(P > 0.05\)); while the weight of the xenografts in hypoxia group was significantly higher than that in normoxia group (\(P = 0.005\)).

Figure 2. Comparison of the expressions of VEGF and VEGF-R2 with xenografts growth in the three groups. Note: compared with normoxia group, \(^{#}P < 0.05\).

Figure 3. Comparison of the Hb content and vascularization of xenografts growth in the three groups. Note: compared with normoxia group, \(^{#}P < 0.05\).
3.3. Angiogenesis and vascularization

VEGF and VEGF expressions in xenografts in hyperoxia group were significantly higher than in normaxia group (Figure 2) \((P < 0.05)\), but the difference between hypoxia group and normaxia group was not statistically significant difference \((P > 0.05)\). Hb content of xenografts in hypoxia group was significantly higher than that in normaxia and hyperoxia groups (Figure 3A, left) \((P < 0.05)\). Hb growth rate (170%) of xenografts in hypoxia group was significantly higher than that in hyperoxia group (40%) (Figure 3A, medium) \((P < 0.05)\). Compared with the Hb content of the left kidney, Hb content of xenografts tissue in hypoxia group was increased by 110% (Figure 3A, right). Through the qualitative estimation of anti-CD31 immune response of endothelial cells (Figure 3B), vascularization degree of xenografts tissues in nomaxia group and hypoxia group was quite similar, while vascularization in hyperoxia was slightly less.

3.4. Hypoxia-inducible factors and intracellular signal transduction

Western blot test (Figure 4A) and immunofluorescence (Figure 4B) were used to determine HIF-1α. Results showed that the expression of HIF-1α of xenografts in hyperoxia group was significant increased compared with that in normaxia group \((P < 0.05)\). The expression of HIF-1α of xenografts in hypoxia group and normaxia group was similar (Figure 4C). Expression of Nrf2 of xenografts in hypoxia group was significantly higher than that of normoxia group (Figure 5A and C left) \((P < 0.05)\). Akt activity in hypoxia group was higher than that of normaxia group and hyperoxia group, but the total expression of Akt remained unchanged (Figure 5C, right). Fluorescent staining based on immunohistochemical method was used to evaluate the expression level of Ki67 (Figure 5B). Figure 5D showed that the total number of pixel intensity, the immunoreactions of hypoxia group was higher than that of normaxia group and hyperoxia group.
4. Discussion

In the present study, the growth of \textit{in vivo} xenografts LNCaP cells of mice in hypoxia group was twice as fast as that in normoxia group, while the xenografts growth in hyperoxia group and normoxia group was similar. The expression level of HIF-1\textalpha in hyperoxia group was higher than that of hypoxia group. It suggested that \textit{in vivo} hyperoxia can induce the growth of LNCaP cell xenografts but did not affect the speed of xenografts growth. Studies have reported that hyperoxia can reduce tumor growth of rat breast tumor and gliomas by increasing cell apoptosis and reduce vascular density \cite{16}, hyperbaric oxygen therapy can also induce multi-differentiation and transcription of epithelial mesenchymal cells of low invasive tumor, thus weakened the tumor growth \cite{17-19}. Hyperoxia had no effect on mice inoculated PC3 prostate cancer cells \cite{20}. The expressions of HIF-1\textalpha and VEGF of xenografts in hyperoxia group increased, probably because hyperoxia induced blood vessel contraction and blood flow volume decreased, thus caused regional hypoxia.

4.1. Hypoxia-inducible factor

HIF-1\textalpha plays a key role in the development of malignant tumors including prostate cancer, and the up-regulation of HIF-1\textalpha manifests early prostate cancer and cancer development promoting angiogenesis \cite{21}. Researches had shown that in

\begin{figure}[h]
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\caption{Comparison of the expression of cell signaling factors in the three groups. Note: compared with normoxia group, "\#P < 0.05.}
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clinical trials the digoxin and topotecan as HIF-1α inhibitors failed to show the significant antitumor effect [22]; when LNCaP cells were acute hypoxia, HIF-1α had a high expression, with time course, HIF-1α returned to normal or even low expression level [23]. Therefore, antitumor therapy only counted on HIF-1α inhibitors may be invalid. In the present study the growth rate of xenografts is inversely proportional to the increase of HIF-1α expression, which suggested that the expression of HIF-1α doesn’t play a decisive role in the growth mode of xenografts. The hyperoxia test provides a possibility to study the related mechanism of HIF-1α in the signal pathway of tumor growth.

4.2. Angiogenesis

VEGF/VEGF-R2 signal pathway was recognized as the most prominent way for tumor promotion [24], Hypoxia can slightly induced VEGF-R2, while hyperoxia can adjust VEGF and VEGF-R2 at a larger extent through stimulating the expression of HIF-1α, thus increase the pathway of activated VEGF/VEGF-R2 [25]. However, the increased activity of VEGF/VEGF-R2 signal pathway has no relation with the increased Hb content of xenografts. Therefore, in the results of present study VEGF and VEGF-R2 of xenografts in hyperoxia group has high expressions, while the Hb content is lower than that of hypoxia group. Slight vascularization in hyperoxia group observed may caused by hyperoxia induced vasoconstriction, thus reduce angiogenesis.

4.3. Signal pathway

Akt activation is the sign of cells survival under hypoxic environment [26]. The activity of Akt in hypoxia group is higher than that of normoxia group and hyperoxia group. It suggested that the faster growth of hypoxia-induced xenografts in prostate cancer cell has a certain relation with the increased activation of Akt. Nrf2 protein is a transcription factor which can activate antioxidant reaction [27], and regarded as a tumor suppressor with its ability to remove cellular environmental toxicity [28]. In the results, the expression level of Nrf2 in hyperoxia was significantly higher which showed hyperoxia cells has a strong antioxidant response ability, and the suggested hyperoxia make the in vivo redox reequilibration out of balance.

Hyperoxia in pulmonary epithelial cells can stimulate Nrf2 transcriptional response by PI3K/Akt signal [29]. In the preset study, the overexpression of Nrf2 of xenografts in hyperoxia group is not consistent with the high expression of Akt in xenografts in hypoxia group. This may caused by the role of ROS at two different phases [30], i.e. ROS as Akt activation in low expression (such as hypoxia), while in high expression the toxic effects turn into advantages (such as hyperoxia), leading to Akt inactivation.

In conclusion, hyperoxia can induce the overexpression of HIF-1α in LNCaP cells without affecting the xenografts growth rate, and diminish in vivo hypoxia and angiogenic signaling by unbalancing in vivo hyperoxia redox reaction and the complex interaction of cell proliferation pathways. The present study provides a new idea for the clinical treatment of prostate cancer and the future research on the basic features of hyperoxia affecting the process of tumor will be of great significance.

Conflict of interest statement

We declare that we have no conflict of interest.

References


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