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Research Paper

Over-expression of hypoxia-inducible factor 1 alpha increases angiogenesis of LNCaP cells

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Abstract

Objective: To evaluate the effect of HIF-1 α over-expression on angiogenesis in human prostate cancer cells. **Methods:** LNCaP cells (a human prostate cancer cell line) were transfected with the recombinant plasmid pcDNA3.1(-)-HIF-1α with Lipofectamine 2000 system. The positive clones were selected by G418 being further confirmed by Western blot and immunofluorescence. The expression levels of VEGF, iNOS and Ang-Ⅱ were determined. **Results:** The expression of HIF-1α in the LNCaP/HIF1α cells was significantly increased in transfected cells, which induced the up-regulation of VEGF, iNOS, whereas Ang-Ⅱ expression remained un- changed. **Conclusion:** Over-expression of HIF-1α can induce angiogenesis proteins and may improve the angiogenesis potency of prostate cancer.

Keywords: prostate cancer; angiogenesis; HIF-1α

INTRODUCTION

Hypoxia is a common phenomenon in malignant solid tumors, and can trigger a series of emergent protective reactions, making tumors adapt to hypoxic environment assisting the tumors likelihood of survival and progress. Hypoxia inducible factor-1α, a nuclear transcriptional factor, can be activated by hypoxia to promote the transcription and translation of several target genes, which relate to angiogenesis, invasion, and metastasis of a tumor. This can improve the angiogenesis and metabolism of tumors, making them suitable to micro environment which may bring changes to their biologic behaviors[1].

In this study, LNCaP cells (a human prostate cancer cell line) were transfected with HIF-1α by using the Lipofectamine 2000 system, and then the expression of VEGF, iNOS and Ang-Ⅱ were determined to investigate the effect of HIF-1α on tumor angiogenesis *in vitro*.

MATERIALS AND METHODS

Establishment of HIF-1α over-expressed cells

The human prostate cancer LNCaP cells were cultured in RPMI1640 medium with 10% FBS. Recombinant plasmid pcDNA3.1(-)/HIF-1α (provided by Leland WK Chung, Emory University) was constructed and transfected into LNCaP cells with Lipofectamine 2000 system (Life Technologies Inc, USA). Cells were then cultured in medium containing G418 (600 μg/ml) for 4 weeks to get positive clones. The LNCaP cells successfully transfected with pcDNA3.1 (-)/HIF-1α expression vector and were designated as LNCaP/HIF-1α.

Confirmation of HIF-1α expression

After culture, cells were fixed in 2% paraformaldehyde for 30 minutes and blocked with goat serum for 30 minutes. Cells were then incubated

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at 37℃ for 1 hour with rabbit anti-human HIF-1α polyclonal antibody(Santa Cruz, USA) at a dilution of 1:200. After being washed 3 times with PBS, the cells were incubated with a fluorescence isothiocyanate (FITC) conjugated goat anti-rabbit antibody at 37℃ for 1 hour. The fluorescence staining intensity and intracellular localization were examined under a fluorescence microscope.

Total protein was isolated from 10^7 of cells by using 200 μl of ice cold lysis buffer containing 1% NP-40,50 mmol/L Tris (pH 7.4),150 mmol/L NaCl, 0.1%SDS,0.5% deoxycholate,200 μg/ml PMSF and 50μg/ml aprotinin. Insoluble materials were removed by centrifugation at 15,000 g for 15 minutes at 4℃. The concentration of the extracted protein was measured spectrophotometrically with Coomassie G-250.

Aliquots of 80 μg protein were separated by 10% SDS-PAGE and electro-transferred to nitrocellulose membranes. The membranes were initially blocked with 5% nonfat dry milk in TBS for 2 hours, then incubated with rabbit anti-human HIF-1α polyclonal antibody (Santa Cruz, USA) at a 1:100 dilution in 5% nonfat dry milk/TBS at 4℃ for overnight. After washing three times with TBS, the membranes were incubated with the secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody for 2 hours at room temperature followed by TBS wash for three cycles, Chemiluminescence Immunoassay (Amresco,USA) was used to detect immunopositive protein bands.

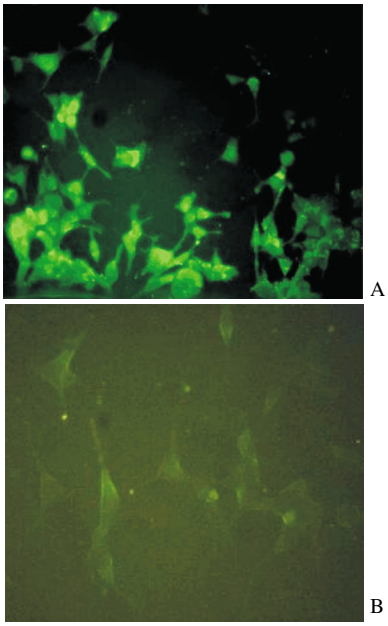
Detection of angiogenesis associated proteins

Aliquots of 80 μg protein from the LNCaP/HIF-1α and LNCaP were separated by 10% SDS-PAGE and electro-transferred to nitrocellulose membranes. The membranes were initially blocked with 5% nonfat dry milk in TBS for 2 hours and then incubated with the primary antibodies (rabbit anti-VEGF, rabbit anti-iNOS, and rabbit anti-Ang-Ⅱ, Boshide Inc, China) all at a 1:100 dilution in 5% nonfat dry milk/TBS at 4℃ for overnight. The secondary horseradish peroxidase (HRP)- conjugated goat anti-rabbit antibody for VEGF, iNOS, Ang-Ⅱ (Boshide Inc,China) were diluted to 1:200 in 5% nonfat dry milk/TBS (0.1% Tween-20). After washing three times with TBS, the membranes were incubated with the secondary antibodies for 2 hours at room temperature, they were then washed with TBS again for three times, Chemiluminescence Immunoassay (Amresco,USA) was used to detect immunopositive protein bands.

RESULTS

Establishment of HIF-1α over-expressed prostate cancer cells

We established HIF-1α over-expression cancer cells, LNCaP/HIF-1α as described above. Immunostaining results showed stronger signals of HIF-1α observed in LNCaP/HIF-1α cells when compared to LNCaP cells (***Fig 1***). Furthermore, Western blotting showed significant increased expression of HIF-1 in transfected cells (***Fig 2***).



A:Staining of HIF-1α in LNCaP/HIF-1α cells (400 ×); B:Staining of HIF-1α in LNCaP cells(400 ×).

Fig 1 Immunofluorescence staining of HIF-1α in LNCaP/HIF-1α cells and LNCaP cells.

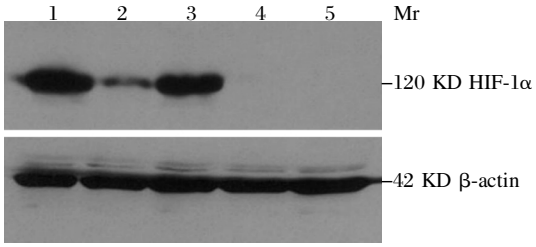


Fig 2 Western blotting of HIF-1α expression in LNCaP/HIF-1α (Label 1,2 and 3) cells and LNCaP cells (Label 4,5)

Effects of HIF-1α over-expression on angiogenesis associated proteins

Western blotting indicated that the expression of VEGF and iNOS, was up-regulated in LNCaP/HIF-1α when compared to LNCaP cells. This suggested that HIF-1 may increase the angiogenesis by up-regulation of VEGF and iNOS. The expression of Ang-Ⅱ showed no difference between LNCaP/HIF-1α cells and LNCaP cells(***Fig 3***).

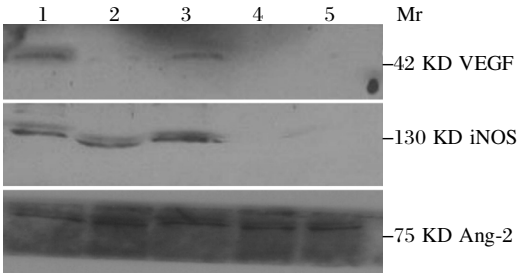


Fig 3 Western blotting of expression of angiogenesis-associated proteins. Expression of VEGF and iNOS were up-regulated in LNCaP/HIF1α,but Ang-Ⅱ expression remained un-change between LNCaP/HIF1α cells and LNCaP cells.

DISCUSSION

In hypoxic conditions, cells receive the hypoxic signals by an oxygenic inductor in the cell membrane, to induce the genes expression in order to adapt to hypoxia. HIF-1 is an important regulatory transcriptional factor in the oxygenic signal pathway.

Hypoxia-inducible factor-1 (HIF-1), is a heterodimeric transcription complex, composed of HIF-1α(120 KD) and HIF-1β(91~94 KD) subunits. HIF-1α is an O_2-regulated subunit that determines HIF-1 activity. HIF-1β is the structural subunit, and can not be regulated by oxygen [2]. The half life of HIF-1α is less than 1 min, and this can be degraded rapidly by ubiquitin-protease after generation in the normoxia, however this is inhibited in hypoxic conditions[3]. HIF-1 is activated and binds to hypoxic reaction element(HRE) after translocation into the nucleus and formation of a heterodimer with HIF-1β, stimulating transcription of its target genes[4].

In the progression of tumors, tumors grow faster than blood vessels, which make for a hypoxic environment in the tumors, promoting the expression of HIF-1α. Zhong *et al* found overexpression of HIF-1α in 13 of 19 human common malignant tumor types by Immunohistochemical Assay. The expression levels were associated with hypoxia, whereas there was no expression of HIF-1 in the benign and normal tissues. They considered that neovascularization and increased glycolysis, two universal characteristics of solid tumors, represent adaptations to a hypoxic micro-environment, that are correlated with tumor invasion and metastasis. HIF-1 may play an central role in human cancer progression[5].

Recent studies have shown that HIF-1α plays an important role in angiogenesis in hypoxia [6], and it can directly participate in the process of angiogenesis by regulating the expression of associated proteins. (1)At the initial phase of angiogenesis,HIF-1α stimulates vasodilatation and improves vascular permeability by up-regulation of NO VEGF and its ligands. (2)At the progressive phase, the extracellular matrix is degraded by metalloproteinase which is activated by HIF-1α, then vascular endothelial cells migrate and hyperplasia occurs. Vascular buds form by co-operating with angiopioetin-2. (3)At the formative phase, a vascular cavity appears in the vascular buds, and anastomose each other, forming a vascular net by the action of VEGF, angiopiontin-1 and integrin. (4)At the shaped phase, the integrated vascular wall form by PDGF and angiopiontin-1[7-9].

HIF-1α can up-regulate VEGF and iNOS to improve the angiogenesis in tumors. It has been reported that HIF-1α up-regulates VEGF by enhancing the transcriptional activity of VEGF and stabilizing VEGF mRNA [10]. A series of evidence has shown that HRE exists in the enhancer at the 5th end of VEGF. When HIF-1 is activated, it binds to HRE and enhances the transcription of VEGF [11]. iNOS is also a target gene of HIF-1. Blagoskonny *et al* [12] showed that HIF-1 is critical in hypoxia induced up-regulation of iNOS, but the mechanism is not known yet. It is reported that HRE is located in the promotor of iNOS in rats, but not in human beings[13].

Up-regulation of VEGF and iNOS by HIF-1 play a critical role in the angiogenesis of tumors. VEGF is a key and direct factor of stimulation of angiogenesis in tumors. HiF-1-induced iNOS up-regulation can increase NO production which may enhance blood flow and oxygen supply in tumor tissues. Furthermore, iNOS and VEGF have synergistic action since NO contributes to binding of VEGF to its receptor which stimulates endothelial cells to elongate and spread, resulting in formation of blood vessels and mitosis [14,15]. Moreover, even though VEGF is the downstream gene of HIF-1, its expression level can also be regulated by iNOS. iNOS possibly co-operates with HIF-1 to regulate expression of VEGF[16].

In this study, we noticed that Ang-Ⅱ expression did not change in HIF-1 transfected cells. The result is similar to Midori's report showing that HIF-1 overexpression did not change the Ang-Ⅱ expression levels in the renal cancer cell line or tubular epithelial cell line [17]. The current result may be explained partially by the biological characteristics of Ang-Ⅱ. It is reported that the role of Ang-Ⅱ plays in angiogenesis is determined by the levels of VEGF. When the level of VEGF is low, Ang-Ⅱ inhibits the interaction of the endothelial cells with their circumambient cells, damaging the stability of blood vessels and accelerating the degeneration of blood vessels. On

the other hand, when VEGF exists, Ang-Ⅱ promotes the process of angiogenesis. Therefore, function of Ang-Ⅱ in angiogenesis is mainly determined by intracellular levels of VEGF[18].

In conclusion, this study examined the expression levels of angiogenesis-associated proteins (VEGF、iNOS、Ang-Ⅱ) in LNcap cells transfected with HIF-1. This is the first report discussing the effect of HIF-1α on angiogenesis in human prostatic caner cells. Further research on the angiogenesis mechanism of the tumor, is this most likely to provide a new strategy in tumor therapy.

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