BCL-xL IS A TARGET GENE REGULATED BY HYPOXIA-INDUCIBLE FACTOR-1-ALPHA

Ni Chen1,2, Xueqin Chen1,2, Rui Huang1,2, Hao Zeng1,3, Jing Gong1,2, Wentong Meng4, Yiping Lu3, Fang Zhao5, Lin Wang6, and Qiao Zhou1,2

From Laboratory of Pathology, State Key Laboratory of Biotherapy1, Departments of Pathology2 and Urology3, Laboratory of Stem Cell Research, State Key Laboratory of Biotherapy4, West China Hospital, West China Medical School, Sichuan University, Chengdu, China 610041, Department of Pathology, Haartman Institute, University of Helsinki, Helsinki5, Haartmaninkatu 3, PL 21, Finland, 00014, and Department of Pediatrics, The University of Texas M.D. Anderson Cancer Center6, 1515 Holcombe Blvd, Houston, TX, USA 77030

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Address correspondence to: Qiao Zhou, MD, PhD, Department of Pathology, West China Hospital, Chengdu, China 610041. Fax: 86-28-85164027, Email: zhouqiao@mcwcums.com

The transcription factor hypoxia-inducible factor-1α (HIF-1α) plays pivotal roles in physiology and pathophysiology. Constitutive or hypoxia-induced HIF-1α over-expression is observed in many types of cancers including prostate adenocarcinoma, in which it is associated with resistance to apoptosis and therapeutic agents. BCL-xL, a hypoxia-responsive, anti-apoptotic protein of the Bcl-2 family, is also over-expressed in prostate carcinoma and many other cancers. Despite this connection, whether BCL-xL expression is directly regulated by HIF-1α is not known. We used prostate cancer PC-3 cell with constitutive high HIF-1α level as a model to address this important question. We first generated prostate cancer PC-3 cells in which HIF-1α was stably knocked-down (HIF-KD) by using small interference RNA. BCL-xL was dramatically decreased in HIF-KD PC-3 cells, in parallel with sensitization to apoptosis with caspase-3 activation as well as decreased cell proliferation. We then demonstrated that HIF-1α directly regulated BCL-xL transcription by binding to a hypoxia responsive element in the BCL-xL promoter (-865 to -847) by reporter gene assay, chromatin immunoprecipitation, and electrophoretic mobility shift and supershift assays. HIF-1α-dependent BCL-xL over-expression may be an important mechanism by which HIF-1α protects prostate cancer cells from apoptosis and leads to treatment resistance.

The transcription factor hypoxia-inducible factor-1α (HIF-1α) plays major roles in cellular response to hypoxia as well as in disease processes including carcinogenesis (1-4). Many genes have been identified as HIF-1 targets (3,4), including GLUT-1, GAPDH, and VEGF, which are involved in such biological processes as energy metabolism, cell survival, and angiogenesis. Hypoxia inhibits proteasome-dependent degradation of HIF-1α, resulting in HIF-1α stabilization, which dimerizes with HIF-1β and activates target genes by binding to hypoxia responsive element (HRE) within their promoters. Hypoxia and HIF-1α over-expression are implicated in the pathogenesis of many cancers, including prostate carcinoma (3-5), in which it is...
associated with advanced clinical stage and treatment failure (6). HIF-1α over-expression has been identified in both prostate adenocarcinoma tissue (5,7) and cell lines (8).

Although acute hypoxia may lead to cell death, prolonged hypoxia results in resistance to apoptosis as well as to radiotherapy and chemotherapy (4,9,10), the mechanism of which is not well-understood. Only recently have a few apoptosis regulators been identified as HIF-1α target genes, most notably the anti-apoptotic Mcl-1 (11) and BIRC5/survivin (12). Although pro-apoptotic molecules BNIP3, NIX (13,14) and Noxa (15) are also responsive to HIF-1α, hypoxia-induced apoptosis-resistant phenotype eventually predominates.

BCL-xL (BCL2-like 1, BCL2L1), a major anti-apoptotic protein of the Bcl-2 family, is also over-expressed in prostate carcinoma and many other cancers. BCL-xL over-expression is associated with the hormone-refractory phenotype and renders prostate cancer cells apoptosis-resistant, while BCL-xL knocking-down increases sensitivity to chemotherapeutic agents (16,17). Despite the correlation of BCL-xL over-expression with HIF-1α in some tumors (18), and the observation that BCL-xL is a key molecule underlying hypoxia-driven cell death resistance (10), the mechanism by which hypoxia induces BCL-xL expression is unclear, as it has not been elucidated if HIF-1α directly regulates BCL-xL.

BCL-xL gene is regulated by several transcription factor families, including STATs (19), NF-κB (20), Ets (21), GATA (22), PAX3 (and the PAX3/FKHR fusion) (23), and POU (Brn-3a) (24). These regulators, however, are not closely related to hypoxia as HIF-1α.

We tested the hypothesis that BCL-xL is under HIF-1α regulation, using prostate cancer PC-3 cell as a model, in which HIF-1α level is constitutively high. We show that stable knocking down of HIF-1α by small interference RNA (siRNA) results in dramatic decrease of BCL-xL with consequent increase in apoptosis, and most importantly, BCL-xL is transcriptionally regulated by HIF-1α.

**EXPERIMENTAL PROCEDURES**

**Cells, tissues, and general reagents-** Human prostate cancer cell lines LNCaP, DU145 and PC-3 were maintained in RPMI1640 with 10% FCS (GIBCO, Rockville, MD). Prostate adenocarcinoma tissue and normal prostate tissue (from prostatectomy specimens of non-prostate diseases) were snap-frozen, in accordance with institutional guidelines. Normal prostate epithelial cells were collected by laser capture microdissection with the Leica AS LMD system (Leica Microsystems, Wetzler, Germany). The phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 was from Sigma (St. Louis, MO). Tris base, Tween 20, dithiothreitol (DTT) and EDTA were from Amresco (Solon, OH). PMSF, leupeptin, pepstatin and aprotinin were from Roche Diagnostics (Mannheim, Germany).

**RT-PCR and real-time quantitative PCR-** Total RNA was extracted by using the TRIZol reagent (Invitrogen Corp, Carlsbad, CA). Revertra Ace reverse transcriptase (ToYoBo, Osaka, Japan) was used for reverse transcription (RT). PCR primers were designed according to cDNA sequences (GenBank) as follows: HIF-1α (5'-CCT ATG ACC TGC TTG GTG CTG-3', 5'-CTG GCT CAT ATC CCA TCA ATT CG-3', product length 157bp), BNIP3 (5'-ACC AAC AGG GCT TCT GAA AC-3', 5'-GAG GGT GGC CGT GCG C-3', 202bp), GLUT-1 (5'-GCA AGT CCT TTG AGA TGC TGA TT-3', 5'-GCC GAC TCT CTT CCT TCA TCT CC-3', 402bp), GAPDH (5'-CCA CCC ATG GCA AAT TCC ATG GCA-3', 5'-TCA AGA CGG CAG GTC AGG TCC ACC-3', 597bp), BCL-xL/BCL-xS (5'-GCA GGC GAC GAG TTT TTT
GAA CT-3', 5'-CTC GGC TGC TGC ATT GTT C-3', BCL-xL 330bp, BCL-xS, 141bp), BIK (5'-GAG GTT CTG TTC CAG AAG CAC CAC C-3', 5'-GAT ATG ACG GAG GAA GGA CG-3', 178bp), BAX (5'-GCT TCA GGG TTT CAT CCA GG-3', 5'-CCA GTT GAA GTT GCC GTC AG-3', 244bp), BIRC5/survivin (5'-GCA GTT TGA AGA ATT AAC CCT TG-3', 5'-CAC TTT CTC CGC AGT TTC CTC-3', 121bp), CIAP1 (5'-TTG TCA ACT TCA GAT ACC ACT GGA G-3',5'-CAA GGC AGA TTT AAC CAC AGG TG-3', 123bp), CIAP2 (5'-AGG GAA GAG GAG AGA GAA AGA GC-3', 5'-CGG CAG TTA GTA GAC TAT CCA GG-3', 133bp), XIAP (5'-GGG TTC AGT TTC AAG GAC ATT AAG-3', 5'-CGC CTT AGC TGC TCT TCA GTA C-3', 182bp), CASP3 (5'-GTT TGT GTG CTT CTG AGC CAT G-3', 5'-CCA CTG TCT GTC TCA ATG CCA C-3', 188bp), CASP6 (5'-TCA GAC AGA GAG GAG GAG GAG C-3', 5'-CGG CAG TTA GTA GAC TAT CCA GG-3', 133bp), XIAP (5'-GGG TTC AGT TTC AAG GAC ATT AAG-3', 5'-CGC CTT AGC TGC TCT TCA GTA C-3', 182bp), XIAP (5'-GGG TTC AGT TTC AAG GAC ATT AAG-3', 5'-CGC CTT AGC TGC TCT TCA GTA C-3', 182bp), XIAP (5'-GGG TTC AGT TTC AAG GAC ATT AAG-3', 5'-CGC CTT AGC TGC TCT TCA GTA C-3', 182bp), XIAP (5'-GGG TTC AGT TTC AAG GAC ATT AAG-3', 5'-CGC CTT AGC TGC TCT TCA GTA C-3', 182bp), XIAP (5'-GGG TTC AGT TTC AAG GAC ATT AAG-3', 5'-CGC CTT AGC TGC TCT TCA GTA C-3', 182bp). Standard PCR protocols were used and products resolved by 2% agarose gel electrophoresis and visualized by staining with the fluorescent dye Goldview™ (SBS, Beijing, China).

The Real-time PCR Master Mix containing SYBR Green (ToYoBo) was used for real-time PCR on Light Cycler 2.0 (Roche) and data were recorded and analyzed by the Light Cycler software 4.05. Copy number of target genes (relative to β-actin) was defined by $2^{-\Delta\Delta C_{t}}$, where $\Delta\Delta C_{t}=\Delta C_{t_{\text{HIF-KD-target}}}-\Delta C_{t_{\text{HIF-KD-actin}}}=(C_{t_{\text{HIF-KD-target}}}-C_{t_{\text{HIF-KD-actin}}})-(C_{t_{\text{HIF-CON-target}}}-C_{t_{\text{HIF-CON-actin}}})$.

**Inhibition of PI3K in PC-3 cells**- PC-3 cells were cultured in 6-well plates in FCS-free media, and treated with 0μM, 10μM, 20μM and 50 μM of PI3K inhibitor LY294002 for 1h, respectively. Cells were collected for Western analysis of HIF-1α, BCL-xL, AKT1/2 and phosphorylated AKT (pAKT).

**Hypoxia mimetic treatment of PC-3 cells**- PC-3 cells were cultured in 6-well plates in FCS-free media, and treated with 0μM, 200μM, 400 μM of CoCl2 for 4h, respectively. Cells were collected for Western analysis of HIF-1α and BCL-xL.

**HIF-1α RNA interference**- The expression vector pRNAT-U6.1/Neo (GenScript Corp. Piscataway, NJ) was used to construct HIF-1α siRNA plasmids by inserting siRNA-coding sequences under U6 promoter for siRNA expression. Two (HIF-siRNA1 and 2) expressing vectors were constructed with the following siRNA sequences: HIF-siRNA1, GCCACATCATCACCATATA, (nt1960-1978, NM_001530); HIF-siRNA2, CTAACTGGACACAGTGTGT (nt379-397). Corresponding control siRNAs with scrambled sequences were also designed and prepared as Scrumbled 1, GACCTACAACTACCTATCA, and Scrumbled 2, GTGGACACCCGATAAGTTT. These sequences were checked to ensure non-homology with known human mRNA sequences. Co-expression of green fluorescence protein (GFP) from the plasmid was used for checking transfection efficiency.

PC-3 cells were transfected by using Lipofectamine 2000 (Invitrogen). To obtain HIF knock-down cells (HIF-KD) with stable transfection of HIF-1α-siRNA1 and HIF-1α-siRNA2 (designated as HIF-KD1 and HIF-KD2, respectively), and the control plasmids (RNAi-CON1 and RNAi-CON2, respectively), cells were selected by G418 at 500 μg/mL for 2 weeks (starting at 48h after transfection), and
maintained in growth medium supplemented with G418 (200 μg/mL).

Western blot analysis- The primary antibodies used were: HIF-1α (mouse monoclonal, 1:1500) from Chemicon Inc. Pittsburgh, PA; BCL-xL (rabbit polyclonal, 1:1,000;); and pAKT (rabbit polyclonal, 1:600) from Cell Signaling Technology Inc., Danvers, MA; BNIP3 (mouse monoclonal, 1:3000) from Sigma, St. Louis, MO; BIRC5/survivin (rabbit polyclonal, 1:1000) from R and D Systems Inc., Minneapolis, MN; BAX (mouse monoclonal, 1:800), CIAP1 (rabbit polyclonal, 1:800), CIAP2 (rabbit polyclonal, 1:800), CASP3 (rabbit polyclonal, 1:800), CASP9 (rabbit polyclonal, 1:600), and AKT1/2 (goat polyclonal, 1:800) from Santa Cruz Biotechnology, Santa Cruz, CA, GAPDH (mouse monoclonal, 1:10,000) from Kangcheng, Shanghai, China, and β-tubulin (mouse monoclonal, 1:1,000) from Huatesheng, Shenzhen, China. Horseradish peroxidase-labelled secondary antibodies were from Zymed.

Total proteins resolved by SDS polyacrylamide (Sigma) gel electrophoresis were electroblotted to PVDF membrane (Amersham Biosciences Ltd., Little Chalfont, U.K.), blocked with 5% nonfat milk and 0.1% Tween 20, incubated primary and secondary antibodies at room temperature for 2h and 1.5h respectively. Signals were detected by exposure to x-ray films after treatment with the SuperSignal enhanced chemiluminescence kit (Pierce Biotechnology Inc.).

Ultraviolet (UV) irradiation induced cell death- Cells in culture plates were briefly exposed to UV irradiation in a UV cross-linker (UVC-500, Hoefer, San Francisco, CA, USA) at 120mJ/cm² for 30s. Cells were then cultured as appropriate for subsequent assays.

Immunocytochemistry- Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, incubated overnight at 4C with anti-human Ki67/MIB-1 antibody (mouse monoclonal, 1:100, DakoCytomation, Glostrup, Denmark), anti-human cleaved caspase-3 antibody (rabbit polyclonal, 1:200, Cell Signaling Technology) or anti-human BCL-xL antibody (1:200). Standard labeled streptavidin-biotin protocol was used for staining with 3'-diaminobenzidine (DAB) as chromogen and hematoxylin as counterstain.

Cell viability assay- Cells were cultured in 96-well plates and measured by tetrazolium-based MTT (Sigma) cell proliferation assay. The working concentration of MTT was 1mg/ml.

Caspase-3 activity assay- Cultured cells were lyzed with lysis buffer containing 50mM Hepes (pH 7.4), 100 mM NaCl, 0.1 % CHAPS, 1mM EDTA, 10% glycerol and 10mM DTT. The soluble fraction of the cell lysate was used for colorimetric caspase-3 activity assay using acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-PNA) (Calbiochem, Darmstadt, Germany) as substrate on FL600 plate reader (BIO-TEK, Winooski, VT, USA).

Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling (TUNEL)- TUNEL was performed by using in situ cell death detection kit (Roche Diagnostics). Cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton-100/0.1% sodium citrate, incubated with TUNEL reaction mixture, then with alkaline phosphatase-conjugated anti-fluorescein antibody, stained with NBT/BCIP and counterstained with methyl green. Reaction without terminal transferase was used as negative control. Apoptotic index was represented as TUNEL(+) cells /total number of cells (%).

Overexpression of BCL-xL in HIF-KD1 cells- Full length cDNA of BCL-xL coding sequence was cloned into TA vector pMD18-T (TaKaRa, Dalian, China) and subcloned into pDsRed vector.
(Clontech, Palo Alto, CA). The primers used for cloning were: 5'-AGA TCT AAT GTC TCA GAG CAA CCG GGA-3' and 5'-GTC GAC CGT TTC CGA CTG AAG AGT GAG-3'. HIF-KD1 cells were transfected with BCL-xL expression plasmid (HIF-KD1-xL) or pDsRed control vector (HIF-KD1-DsRed) using Lipofectamine 2000. Transfected HIF-KD1-xL and HIF-KD1-DsRed cells were maintained in RPMI1640.

**Reporter gene assay for HIF-1α-dependent BCL-xL promoter activity**- The basic pGL3 luciferase reporter vector (Promega, Madison, WI) was used to construct reporter plasmids with various lengths of the BCL-xL promoter. Four plasmids were constructed in which the BCL-xL promoter spanning from -1075 to +617 (relative to the transcription start site) or truncated fragments of which were inserted upstream of the luciferase gene. The reporter constructs were designated as pGL1642 (-1075 to +617), pGL1281 (-664 to +617), pGL828 (-211 to +617), and pGL621 (-4 to +617), respectively. Two additional plasmids with HRE1 and HRE2 site-specific mutation were constructed: pGL828-MUT (-211 to +617, with CGTG at -78 to -75 of HRE1 mutated to TCGG), and pGL1642-MUT (-1075 to +617, with CGTG at -858 to -855 of HRE2 mutated to TCGG). Each reporter construct and the pRL-CMV plasmid (Promega) containing the Renilla luciferase gene as internal control were used in dual reporter gene assay for studying HIF-1α-dependent gene expression. Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen). Four hours after transfection, the medium was replaced by fresh medium. Thirty-six hours after transfection, cells were treated with 400 μM CoCl₂ for 12h and luciferase activity was determined by using Luminometer TD-20/20 (Turner Designs, Sunnyvale, CA, USA).

**Chromatin immunoprecipitation (ChIP)**- Cells were lysed and nuclei were pelleted. The extract was sonicated and supernatants collected, treated with sheared salmon sperm DNA (Invitrogen) and protein A/G-sepharose (Santa Cruz). Immunoprecipitation was performed overnight at 4°C with 3 μg HIF-1α monoclonal antibody, or the control isotype IgG2b (Lab Vision Corp., Fremont, CA), or no antibody, and then with protein A/G-Sepharose and salmon sperm DNA. Precipitates were washed, extracted with 1% SDS, 0.1 M NaHCO₃. Eluates were pooled and heated. DNA fragments were purified and used as template for PCR. The promoter-specific primers used were: BCL-xL, 5'-CGA GCA GTC AGC CAG GTA G-3' and 5'-GAC GGC GAA GGC TCC TAT TG-3'; VEGF (as positive control): 5'-GTG CCC TGG CAA CAT CTG G-3' and 5'-GAC ATC AAA GTG AGC GGC AG-3'.

**Electrophoretic mobility shift assay (EMSA) and supershift assay**- The sequences of the two BCL-xL promoter oligonucleotide probes were: BCL-xL-Pro1, 5'-GAGCCAAGGGGCCTGCAGAGAGAGG-3' (-89 to -64), and BCL-xL-Pro2, 5'-CCCTGTGCGTCAGAGCCGT-3' (-865 to -847), respectively. The VEGF promoter probe 5'-CAGTGCATACGGCGCTCCA-3' (-989 to -970) was used as positive control. Three corresponding probes with HRE mutation were prepared: BCL-xL-Pro1-MUT, 5'-GAGCCAAGGGGTCCCAAGAGAGG-3', BCL-xL-Pro2-MUT, 5'-CCCTGTGGTGAGAGCGCTCCA-3', VEGF-Pro-MUT, 5'-CAGTGCATACGGCGCTCCA-3', VEGF-Pro-MUT. Labeled wild-type probes were prepared by biotinylation (Invitrogen). Unlabeled wild-type and mutant probes were used for competition experiments. For each probe, complementary strands were synthesized and equimolar concentrations of complementary strands were annealed for use in EMSA. PC-3 cells were harvested after 6h incubation with
400μM CoCl₂. Nuclear extract was prepared and 10 μg was incubated with 100 pmol of biotinylated probe in 10 μl reaction mixture (with 0.5 μg of poly [dl-dC]) for 30min at room temperature. For competition assays, a 50-fold excess of unlabeled wild-type probe or mutant probe was used. For supershift assays, 1.0 μg of monoclonal anti-HIF-1α antibody was added to the reaction mixture, incubated at 4°C overnight. The mixture was electrophoresed at 4°C on 6% PAGE for 3h, transferred to nylon membranes (Roche Diagnostics) by electroblotting. After baking and blocking, horseradish peroxidase-labelled streptavidin (1:1000, Zymed) was added and incubated at room temperature for 2h. Signals were detected by exposure to x-ray films after treatment with the SuperSignal enhanced chemiluminescence kit (Pierce Biotechnology Inc.).

Statistical analysis- Statistical analysis was performed by using the SPSS 10 software package (Chicago, IL)

RESULTS

HIF-1α siRNA significantly decreased expression of HIF-1α and its target genes. HIF-1α mRNA and protein over-expression in prostate cancer cells and primary prostate adenocarcinoma tissues was validated by conventional RT-PCR (Fig. 1A) and Western blot (Fig. 1B) analysis, respectively. In normal prostate epithelium, HIF-1α mRNA and protein were undetectable (Fig. 1A and B).

The two HIF-siRNA constructs showed comparable interference efficiency, with HIF-siRNA1 being more potent (Fig. 1C, D, E). In contrast, the control constructs with scrambled sequences had no effect on HIF-1α expression.

The G418-selected PC-3 cells with stably transfected HIF-siRNA1 and HIF-siRNA2 (HIF-KD1 and HIF-KD2 cells, respectively) and the control plasmids (RNAi-CON1 and RNAi-CON2 cells, respectively) were largely homogeneous, as shown by the co-expression of green fluorescence protein (Fig. 1F).

HIF-1α mRNA (Fig. 1G) and protein (Fig. 1H) were significantly reduced in the HIF-KD cells, with consequent down-regulation of known HIF-1α target genes BNIP3, GLUT-1, and GAPDH (Fig. 1G and H). The control constructs had no effect on mRNA and protein expression of HIF-1α or its target genes (Fig. 1G and H). Quantitative PCR analysis of mRNA of HIF-1α and its target gene GLUT-1 further validated the interference effect of HIF-1α siRNA, as both of which were significantly reduced (not shown).

HIF-1α siRNA inhibited PC-3 cell proliferation. HIF-KD cells also showed significantly reduced cell growth (Fig. 2A and B). Decreased cell proliferation was further demonstrated by immunocytochemistry of the proliferative antigen Ki67/MIB-1, which showed much lower Ki-67-labeling index (50%) in HIF-KD cells than control (90%) (Fig. 2A).

HIF-KD cells were more sensitive to ultraviolet (UV) irradiation and flutamide treatment. HIF-KD cells showed significantly higher cell death rate (Fig. 2C and D) after UV-irradiation. TUNEL assays demonstrated the spontaneous apoptotic index (AI) in HIF-KD1 and HIF-KD2 cells were 1.0% (±0.2%) and 0.9% (±0.1%), respectively; and in RNAi-CON1 and RNAi-CON2 cells were 0.2% (±0.01%) and 0.2% (±0.01%), respectively (Fig. 2D). Upon UV-irradiation, the apoptotic index in HIF-KD1 and HIF-KD2 cells increased significantly to 17.3% (±1.7%) and 12.7% (±1.3%), respectively (Fig. 2D), but in RNAi-CON1 and RNAi-CON2 cells, the apoptotic index only increased slightly to 1.7% (±0.2%) and 1.5% (±0.1%), respectively.

Immunocytochemistry staining of cleaved caspase-3 (as manifestation of caspase-3 activation)
showed prominent activation of caspase-3 in HIF-KD cells, but not in RNAi-CON cells (Fig. 2E). Colorimetric caspase-3 activity assay further demonstrated higher caspase-3 activity in HIF-KD cells after UV-irradiation (Fig. 2F).

HIF-siRNA also sensitized PC-3 cell to the anti-androgen drug flutamide, the treatment by which further inhibited growth rate of HIF-KD cells (Fig. 2B). In contrast, RNAi-CON cell growth was exuberant and essentially remained the same with or without flutamide treatment.

HIF-1α siRNA dramatically decreased BCL-xL expression. To elucidate the mechanisms by which HIF-1α siRNA inhibited cell proliferation and promoted apoptosis in PC-3 cells, we examined the effect of HIF-1α siRNA on major apoptosis regulators: the Bcl-2 family, the IAP family, and the caspase family.

Most prominently, BCL-xL expression was significantly down-regulated by HIF-1α siRNA (Fig. 3A-D), while the other examined members of the Bcl-2 family showed little change.

BCL-xS, which is encoded by the same gene locus, also showed decrease in expression upon HIF-1α siRNA. As the base level of BCL-xS was extremely low, the effect appeared less dramatic than BCL-xL (Fig. 3A).

As expected, HIF-1α siRNA also induced significant decrease of survivin, a documented HIF-1α target (Fig. 3A and B). Other members of the IAP family, including CIAP1, CIAP2, and XIAP, were not down-regulated. Moreover, HIF-1α siRNA had no effect on expression level of the examined caspases (Fig. 3A and B).

Over expression of BCL-xL in HIF-KD cells promoted cell growth and inhibited cell death. To further demonstrate the importance of HIF-induced BCL-xL expression in apoptosis resistance, BCL-xL was artificially overexpressed in HIF-KD1 cells (HIF-KD1-xL, Fig. 4A-E), which counteracted the effects of HIF-1α siRNA, resulting in enhanced cell growth (Fig. 4C) and inhibition of UV-irradiation induced cell death and caspase 3 activity (Fig. 4D and E).

Inhibition of PI3K/Akt pathway resulted in down-regulation of both HIF-1α and BCL-xL. The down-regulation of BCL-xL by HIF-1α siRNA was dramatic, given the constitutive high BCL-xL mRNA (Fig. 5A) and protein (Fig. 5B) level in prostate cancer cell lines. Since PI3K/Akt is a major signaling pathway that controls HIF-1α level, we tested if PI3K inhibitor LY294002 could lead to decrease of BCL-xL. As shown in Fig. 5C, both HIF-1α and BCL-xL were simultaneously reduced upon PI3K/Akt inhibition in dose-dependent manner.

Hypoxia mimetic CoCl2 boosted concomitant BCL-xL and HIF-1α expression in PC-3 cells. Treatment of PC-3 cells with the hypoxia mimetic CoCl2 resulted in additional increase of BCL-xL and HIF-1α simultaneously, further supporting the potential dependence of BCL-xL on HIF-1α in response to hypoxia (Fig. 5D).

Potential HREs on BCL-xL promoter. The above experiments provided important clues to relationship between HIF-1α and BCL-xL. Since BCL-xL is a gene regulated by NF-κB, which could potentially be activated by PI3K/Akt signaling, reduction of BCL-xL expression by PI3K/Akt inhibition might be the effect of either HIF-1α or NF-κB inhibition, or both. This prompted us to investigate whether BCL-xL was directly regulated by HIF-1α.

Potential HRE was searched in the human BCL-xL promoter region. Four short HRE consensus motifs were identified within the ~1,000 bp region preceding the transcriptional start site. Two of them, starting at positions -78 and -858 (Fig. 6A), respectively, fit the extended consensus and were highly conserved across species (Fig. 6B).

Reporter gene assay of putative HRE activity.
The reporter constructs were shown in Fig. 6C. The pGL1642 (-1075 to +617) contained both HREs (HRE2 and HRE1) flanking two NF-κB binding sites, while pGL1281 (-664 to +617) differed by lacking HRE2. The pGL828 (-211 to +617) contained HRE1 only, and pGL621 (-4 to +617) contained no HRE. Two constructs were prepared with site-specific mutation of respective HRE: pGL828-MUT and pGL1642-MUT.

PC-3 cells were transiently transfected with one of the six constructs, with PRL-CMV co-transfection as internal control. The hypoxia mimic CoCl2 was used to simulate hypoxia and to further boost HIF-1α, as a means of showing hypoxia-induced gene transcription in the reporter gene assay.

The experiment showed significantly higher luciferase activity of the HRE-bearing constructs (Fig. 6C) than baseline (pGL3). The effects were more dramatic with the HRE2-bearing construct upon CoCl2 treatment, while mutation of HRE (notably HRE2) core sequence resulted in significant reduction of transcriptional activity. These experiments indicated that the reporter gene transcription was under control of BCL-xL promoter containing HRE, particularly HRE2, which responded to the hypoxia mimic.

ChIP assay displayed HIF-1α interaction with BCL-xL promoter. To show HIF-1α physically bind to BCL-xL promoter, we first used ChIP assay of PC-3 cells treated by CoCl2. Using the chromatin fraction pulled down by anti-HIF-1α antibody as template, a PCR fragment corresponding to -1025 to -821 (containing HRE2) of BCL-xL promoter was detected (Fig. 6D) and verified by sequencing. This fragment was not detected when isotype control IgG2b or no antibody was used for the pull-down (Fig. 6D).

EMSA and super shift assay demonstrated HIF-1α binding to the promoter region of BCL-xL. To further confirm binding of HIF-1α to the putative HRE in human BCL-xL promoter, we performed EMSA with two oligonucleotide probes, each containing one of the extended HRE consensus sequence, designated BCL-xL-Pro1 (-89 to -64) and BCL-xL-Pro2 (-865 to -847), respectively. Two probes with HRE core sequence mutation were also prepared for competition experiments and were designated as BCL-xL-Pro1-MUT and BCL-xL-Pro2-MUT, respectively. A known HIF-1α binding oligonucleotide derived from the VEGF receptor gene promoter (VEGF-Pro) was used as positive control (Fig. 6E), together with a corresponding HRE mutation probe, VEGF-Pro-MUT, for competition assays.

The experiments showed that the labeled BCL-xL-Pro2 (Fig. 6E, lanes 1 and 2), but not BCL-xL-Pro1 (not shown), caused gel mobility shift when incubated with nuclear proteins from PC-3 cells. The shift could be suppressed in the competition experiments with excess unlabeled wild-type probe BCL-xL-Pro2 (Fig. 6E, lane 3), but not with the HRE-mutated probe BCL-xL-Pro2-MUT (Fig. 6E, lane 4). When HIF-1α monoclonal antibody was included in the binding reaction, a supershift band was observed as well with BCL-xL-Pro2 (Fig. 6E, lane 5), but not BCL-xL-Pro1 (not shown). Gel mobility shift and supershift were also shown with the VEGF-Pro positive control (Fig. 6E, lanes 6 and 7), together with competition and supershift assays (Fig. 6E, lanes 8-10). These results further demonstrated HIF-1α binding to the HRE2 in the -865 to -847 region of BCL-xL promoter.

**DISCUSSION**

We generated prostate cancer PC-3 cells in which HIF-1α was stably knocked-down by using siRNAs, which resulted in significant decrease of the anti-apoptotic molecule BCL-xL. We then
showed that HIF-1α directly regulated BCL-xL gene transcription. These novel findings point to HIF-1α-dependent BCL-xL over-expression as an important mechanism by which HIF-1α protects prostate cancer cells from apoptosis and leads to treatment failure.

Hypoxia is common in solid tumors (4), including prostate carcinoma (3), in which the extent of hypoxia is correlated with clinical stage and treatment failure (6). Hypoxia-mediated increase in HIF-1α plays critical roles in tumorigenesis and progression of many cancers, through HIF-1α-dependent activation of genes that promote cancer cell survival, proliferation, spreading, and angiogenesis. Over-expression of HIF-1α and its target genes has been observed in a variety of solid tumors, for example, tumors of the brain (25), kidney and the urinary tract (26), lung (27), as well as prostate (5,7).

High level of HIF-1α has been observed in prostate cancer tissue and cell lines (5,7,8). Up-regulation of HIF-1α might be an early event in prostate carcinogenesis, since high-grade prostate intraepithelial neoplasia showed higher HIF-1α level than benign epithelium (5). It is noteworthy that, although HIF-1α over-expression is often hypoxia-dependent, prostate cancer cells have constitutively high HIF-1α level, which could be further increased by hypoxia (8). HIF-1α gene amplification (28) and P582S polymorphism or mutation in the oxygen-dependent domain (29) might contribute to over-expression of HIF-1α at normoxic conditions.

Hypoxia and HIF-1α over-expression contribute to resistance to radiotherapy and chemotherapy (4). For example, the multi-drug resistance 1 gene has been observed to be hypoxia-responsive and is regulated by HIF-1α (30). Treatment of LNCaP cells with the androgen receptor antagonist Casodex results in up-regulation of a subset of hypoxia-related genes, including membrane metallo-endopeptidase and cyclin G2, which might be involved in development of the androgen-independent phenotype (31).

Knocking down of HIF-1α by siRNA or anti-sense techniques inhibits cell growth, proliferation, or migration, and promotes apoptosis. The effects have been observed in human respiratory epithelium (32) and umbilical vascular endothelial cells (33), as well as in a variety of tumors, including glioma (34), non-small cell lung cancer (35), hepatocellular carcinoma (11), pancreatic cancer (36), pituitary adenoma (37), squamous cell carcinoma (38), and prostate cancer (39).

Of more clinical interest is that silencing of HIF-1α gene results in sensitization of cancer cells to therapeutic agents. For example, HIF-1α knocking down increases sensitivity to 5-fluorouracil, doxorubicin, and gemcitabine in pancreatic cancer cell (36). Our finding that HIF-1α knocking down renders the androgen-independent PC-3 cells more sensitive to UV or flutamide treatment also supports that HIF-1α is a potential therapeutic target in androgen-independent prostate cancer.

The effects of such inhibition are mainly mediated by down-regulation of HIF transcriptional targets involved in diverse biological processes as cell proliferation, metabolism, and angiogenesis. These targets include, for example, phosphoglycerate kinase (35), GLUT-1 (34), chemokine receptors CXCR1 and CXCR2 (39) or CRCX4 (40), and VEGF (35).

Promotion of apoptosis is a recurrent theme of HIF-1α knocking-down (11,32-40). However, the underlying mechanisms have been less clear. Caspases could be increased or activated upon HIF-1α siRNA, but it most probably reflects the activation of caspase-dependent pathways rather than transactivation. It was only recently that two
anti-apoptotic genes, BIRC5/survivin (12) and Mcl-1 (11,32), were identified as HIF-1α targets. Despite responsiveness to HIF-1α by pro-apoptotic genes BNIP3, NIX, and Noxa (13-15), most experiments have shown that knocking-down of HIF-1α promotes cell death and inhibits cell proliferation (32-35,37,38), apparently as the end result of a complex regulatory circuit.

Although BCL-xL has been found to be a key molecule involved in hypoxia-induced resistance to cell death (10), and BCL-xL over-expression has been associated with increased HIF-1α in tumors such as non-small cell lung cancer (18), the mechanism by which hypoxia induces BCL-xL up-regulation, and the relationship between HIF-1α and BCL-xL, has not been known.

Our study thus provides the first evidence that HIF-1α directly regulates BCL-xL transcription by interacting with HRE in the BCL-xL promoter. It has been shown that in the androgen-independent PC-3 cell, BCL-xL is more responsible for apoptosis-resistance than the prototypic Bcl-2 (16). Our data therefore indicate that HIF-1α-dependent over-expression of BCL-xL in PC-3 cells is one of the major mechanisms by which prostate cancer cells, particularly androgen-independent cells, resist apoptosis and chemotherapy. Recently, the IAP family member survivin has been identified as a transcriptional target of HIF-1α (12). Being upregulated in many cancers, survivin is reported to be involved in the regulation of both apoptosis and cell division. Thus, HIF-1α overexpression (either constitutive or hypoxia-induced) may promote tumorigenesis by exerting double effects on key members of major gene families controlling cell death and proliferation: the inhibition of cell death by upregulating BCL-xL and survivin, and promotion of cell proliferation by upregulation of survivin. Elucidation of HIF-1α-dependent BCL-xL expression may provide a new dimension for understanding BCL-xL regulation.

REFERENCES

FOOTNOTES

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The abbreviations used are: BCL-xL, BCL2L1, BCL2-like 1; BNIP3, BCL2/adenovirus E1B 19kDa interacting protein 3; CASP, caspase; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; GLUT-1, glucose transporter-1; HIF-1α, hypoxia-inducible factor 1; HIF-KD, HIF-1α knock-down PC-3 cells; hypoxia HRE, hypoxia-responsive element; IAP, inhibitor of apoptosis protein; Mcl-1, myeloid cell factor-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-Indolyl phosphate; NIX, BCL2/adenovirus E1B 19kDa interacting protein 3-like; RNAi, RNA interference; siRNA, small interference RNA; STAT, the signal transducer and activator of transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP digoxigenin nick end labeling; VEGF, vascular endothelial growth factor.

FIGURE LEGENDS

**Fig. 1.** HIF-1α siRNA significantly decreased HIF-1α overexpression in prostate cancer cells. HIF-1α mRNA(A) and protein (B) were overexpressed in prostate cancer cells PC-3, LNCaP, and DU-145, as were in primary prostate cancer tissue (PCA), but were undetectable in normal prostate epithelium (NP) collected by laser capture microdissection. β-actin and tubulin were used as control for the RT-PCR and Western blot analysis, respectively. The two HIF-1α siRNA constructs (HIF-1α-siRNA1 and 2) significantly knocked down HIF-1α level, as measured by conventional (C) or real-time quantitative RT-PCR (D) and Western blot analysis (E). The respective scrambled siRNA (Scrambled 1 and 2) had no effects. G418-selected stable HIF-1α knockdown cells (HIF-KD1 and HIF-KD2) and control cells (RNAi-CON1 and RNAi-CON2) were homogeneously GFP-expressing (F). Knocking-down of HIF-1α resulted in significant down-regulation of HIF-1α target genes BNIP3, GLUT-1 and GAPDH.
in HIF-KD cells in comparison to RNAi-CON cells, as shown by RT-PCR (G) and Western blot analysis (H).

Fig. 2. Effects of HIF-1α knock-down on cell proliferation and apoptosis. Cell growth was significantly reduced in HIF-KD cells as compared to RNAi-CON cells, as demonstrated by immunocytochemical staining of the proliferative antigen (Ki67/MIB-1), which showed much lower proliferation index (~50%) in HIF-KD cells than control (~90%) (A). The dark brown-stained nuclei were Ki67/MIB-1-positive proliferating cells. MTT assay further showed the decreased cell growth of HIF-KD as compared to RNAi-CON cells (mean ± SD of three independent experiments, *P* < 0.01) (B). HIF-1α siRNA also sensitized HIF-KD cells to anti-androgen drug flutamide (10^{-6} M), which further inhibited cell growth of HIF-KD cells, but was ineffective on RNAi-CON cells (B). HIF-KD cells were rendered more sensitive to UV-irradiation-induced cell death (C). TUNEL assays demonstrated significant increase of apoptotic index (AI) upon UV-irradiation in HIF-KD cells but not in RNAi-CON cells (mean ± SD of three independent experiments, *P* < 0.01) (D), concomitant with increased level of cleaved caspase-3 (E, immunocytochemistry, cytoplasmic brown-staining, as a manifestation of caspase-3 activation) and increased caspase-3 activity (F, colorimetric assay of cell extract, mean ± SD of three independent experiments, *P* < 0.01).

Fig. 3. Effects of HIF-1α knock-down on members of Bcl-2 family, IAP family, and caspase family. Expression of BCL-xL was significantly reduced after HIF-1α knock-down, as shown by conventional RT-PCR (A), Western blot analysis (B), real-time RT-PCR (C, *P* < 0.01 between HIF-KD and RNAi-CON cells), and immunocytochemistry (with cytoplasmic brown staining representing BCL-xL protein) (D). BCL-xS, which was expressed at extremely low level, was also decreased (A). The mRNA and protein level of survivin of the IAP family was also significantly down-regulated by siRNA of HIF-1α (B). Other members of the Bcl-2 family and IAP family, as well as the caspases, did not show significant changes.

Fig. 4. Artificial overexpression of BCL-xL in HIF-KD cells counteracted the effects of HIF-1α knock-down. HIF-KD1 cells (A, left panel, with GFP expression) transfected with BCL-xL expression vector (HIF-KD1-xL cells) resulted in overexpression of BCL-xL (A, middle panel, red fluorescence; right panel, merged image of left and middle panels) as compared to the control cells (HIF-KD1-DsRed), which was confirmed by RT-PCR (B, left panel) and Western blot analysis (B, right panel). Overexpression of BCL-xL in HIF-KD1-xL cells resulted in significant promotion of cell growth (C, MTT assay, mean ± SD of three independent experiments, *P* < 0.01), reduced apoptosis (D, TUNEL assay) and caspase-3 activity upon UV-irradiation (E, colorimetric assay of cell extract, mean ± SD of three independent experiments, *P* < 0.01), as compared to HIF-KD1-DsRed control cells.

Fig. 5. BCL-xL overexpression in prostate cancer cells could be inhibited by the PI3K inhibitor LY294002, but boosted by the hypoxia mimetic CoCl₂. BCL-xL mRNA (A) and protein (B) were overexpressed in prostate cancer cells PC-3, LNCaP, and DU-145, and in primary prostate cancer tissue (PCa), but were not detected in normal prostate epithelium (NP), as shown by RT-PCR and Western blot.
analysis, respectively. Inhibition of PI3K/Akt signaling pathway by LY294002 resulted in concomitant down-regulation of HIF-1α and BCL-xL in PC-3 cells (C) as phosphorylated Akt (p-Akt) was decreased, while Akt1/2 remained unchanged. Cell lysates were harvested 1 hour after LY294002 treatment at indicated concentrations. In contrast, PC-3 cells treated with the hypoxia mimetic CoCl2 showed further elevation of BCL-xL as well as HIF-1α level (D, Western blot). The protein loading was reduced for gels shown in D to better appreciate this change.

Fig. 6. Hypoxia-responsive element (HRE) in BCL-xL promoter: binding to and transcriptional regulation by HIF-1α. Two potential HRE sites (Site 1 and Site 2, starting at position -78 and -858 of human BCL-xL promoter, respectively) identified by sequence analysis (A) were conserved across species (B).

Dual reporter gene assays were performed with expression constructs carrying various lengths of BCL-xL promoter (range relative to transcription start site) (C). The promoter regions were inserted into respective reporter gene (firefly luciferase) constructs, and the upper panel shows restriction analysis of the inserts (which have also been verified by sequencing). pGL1642 (-1075 to +617) contained HRE2 and HRE1 flanking two NF-κB binding sites. pGL1281 (-664 to +617) lacked HRE2. pGL828 (-211 to +617) contained HRE1 only, and pGL621 (-4 to +617) contained no HRE. In pGL1642-MUT and pGL828-MUT, the respective HRE was mutated (red cross). PC-3 cells were co-transfected with one of the six constructs together with pRL-CMV (which carried the Renilla luciferase gene controlled by CMV promoter, as internal control). CoCl2 was used to simulate hypoxia and to boost HIF-1α level. The reporter gene activity, represented by relative luciferase activity (firefly/Renilla), was significantly increased over the baseline when HRE2 was present and the cells were stimulated with CoCl2. (C). In contrast, pGL1642-MUT significantly reduced the reporter gene activity. Constructs with HRE1 only (pGL828) resulted in slightly higher reporter gene activity over pGL621, but the difference was not significant, and mutation of HRE1 (pGL828-MUT) had little effect, indicating HRE1 was not effectively regulated by HIF-1α. Also notice that presence of the NF-κB binding site resulted in significantly higher reporter gene activity over constructs lacking NF-κB binding site, indicating significant contribution of this binding site to BCL-xL promoter activity.

ChIP (chromatin immunoprecipitation) assay was used to show HIF-1α binding to the BCL-xL promoter (D). PCR using chromatin (input) pulled down by anti-HIF-1α antibody (HIF-Ab) as template yielded the BCL-xL-Pro fragment (-1025 to -821 containing HRE2) of the BCL-xL promoter, which was verified by sequencing. When isotype control IgG2b or no antibody (No Ab) was used for the pull-down, no PCR product was observed. PCR of VEGF promoter (VEGF-Pro) was used as positive control.

EMSA (electrophoretic mobility shift assay) was performed to further confirm HIF-1α binding to HRE (E). Oligonucleotide probes BCL-xL-Pro1 (-89 to -64) and BCL-xL-Pro2 (-865 to -847) contained HRE1 and HRE2, respectively. The HRE core sequence was mutated in the corresponding BCL-xL-Pro1-MUT and BCL-xL-Pro2-MUT probes. A known HIF-1α binding oligonucleotide derived from the VEGF receptor gene promoter (VEGF-Pro) was used as positive control, together with a corresponding HRE mutation probe, VEGF-Pro-MUT. The biotin-labeled BCL-xL-Pro2 (E, lanes 1 and 2), but not BCL-xL-Pro1 (not shown), caused gel mobility shift when incubated with nuclear proteins from PC-3 cells. The shift was suppressed by competition with excess unlabeled wild-type probe.
BCL-xL-Pro2 (E, lane 3), but not with the HRE-mutated probe BCL-xL-Pro2-MUT (E, lane 4). When HIF-1α monoclonal antibody (HIF-Ab) was included in the binding reaction, a supershift band was observed with BCL-xL-Pro2 (E, lane 5), but not BCL-xL-Pro1 (not shown). Gel mobility shift and supershift were also shown with the VEGF-Pro positive control (E, lanes 6 and 7), together with competition and supershift assays (E, lanes 8-10).
Figure 1

A

PC-3  LNCaP  DU145  PCA  NP
HIF-1α
ACTIN

B

PC-3  LNCaP  DU145  PCA  NP
HIF-1α
TUBULIN

C

HIF-siRNA1  HIF-siRNA2  Scrambled 1  Scrambled 2
HIF-1α
ACTIN

D

HIF-siRNA1  HIF-siRNA2  Scrambled 1  Scrambled 2
Relative mRNA level

E

HIF-siRNA1  HIF-siRNA2  Scrambled 1  Scrambled 2
HIF-1α
TUBULIN

F

HIF-KD1  HIF-KD2
RNAi-CON1  RNAi-CON2

G

HIF-KD1  HIF-KD2  RNAi-CON1  RNAi-CON2
HIF-1α
BNIP3
GLUT1
GAPDH
ACTIN

H

HIF-KD1  HIF-KD2  RNAi-CON1  RNAi-CON2
HIF-1α
BNIP3
GAPDH
TUBULIN
Figure 2

A

HIF-KD1

HIF-KD2

RNAi-CON1

RNAi-CON2

B

Cell growth

0 24 48 72 96 120 (hours)

1. RNAi-CON2
2. RNAi-CON1
3. RNAi-CON2 treated by flutamide
4. RNAi-CON1-treated by flutamide
5. HIF-KD2
6. HIF-KD1
7. HIF-KD2 treated by flutamide
8. HIF-KD1 treated by flutamide

C

HIF-KD1

HIF-KD2

RNAi-CON1

RNAi-CON2

D

HIF-KD1

HIF-KD2

RNAi-CON1

RNAi-CON2

Blank

Al

E

HIF-KD1

HIF-KD2

RNAi-CON1

RNAi-CON2

F

Relative caspase-3 activity

untreated UV treated
Figure 3
Figure 4

A

B

C

D

E

HIF-KD1-DsRed  HIF-KD1-xL

HIF-KD1-DsRed  HIF-KD1-xL

HIF-KD1-DsRed  HIF-KD1-xL

HIF-KD1-DsRed  HIF-KD1-xL

HIF-KD1-DsRed  HIF-KD1-xL

Relative caspase-3 activity

untreated  UV-treated
Figure 5

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