

Hypoxia-inducible factor-1 α enhances the malignant phenotype of multicellular spheroid HeLa cells *in vitro*

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Abstract. The purpose of this study was to clarify the direct effect of hypoxia-inducible factor-1 α (HIF-1 α) on tumor growth, apoptosis and migration *in vitro*. To achieve this aim, a comparison was made of the differences in growth rates, apoptotic indices and cell invasive ability in the human cervical cancer cell line HeLa and the HIF-1 α -blocked counterpart in a three-dimensional spheroid culture. A significant decrease in cell proliferation and invasion, and an increase in cell apoptosis were observed in HIF-1 α -blocked cells in the three-dimensional culture. The data indicated that a multicellular spheroid culture is an ideal model of hypoxia *in vitro* and that HIF-1 α is a significant regulator of adaptive processes that promote tumor cell malignant phenotypes, such as proliferation, anti-apoptosis and invasive ability.

Introduction

Tumor hypoxia is a significant factor in tumor physiology and treatment, as it appears to be closely associated with tumorigenesis, metastasis and chemoresistance (1). Hypoxia is a common characteristic of locally advanced solid tumors. Under this condition, reduced oxygen levels (hypoxia) lead to a set of cellular adaptations, including increased angiogenesis, erythropoiesis and a switch to glycolytic metabolism. Mounting evidence indicates that the effect of hypoxia on malignant progression is mediated by a series of hypoxia-induced proteomic and genomic changes that activate angiogenesis, anaerobic metabolism and other processes that enable tumor cells to survive or escape their oxygen-deficient environment. The critical regulatory gene that functions when

the oxygen level in tissues is low is the transcription factor hypoxia-inducible factor-1 (HIF-1) (2,3). Clinical studies have suggested that HIF-1 is a significant regulator of tumor cell adaptation to hypoxic stress and is crucial in cervical malignant progression and outcome (4,5). HIF-1 plays an essential role in the maintenance of oxygen homeostasis in metazoan organisms (3). The DNA binding complex of HIF-1 is a heterodimer comprising HIF-1 α and HIF-1 β subunits, both of which are basic helix-loop-helix transcription factors (6). HIF-1 α is constitutively expressed (7), but under normoxic conditions is hydroxylated at specific proline residues resulting in ubiquitination through the interaction with von Hippel-Lindau factor suppressor protein (pVHL) and proteosomal degradation (8,9). Under hypoxic conditions, proline hydroxylation is inhibited, preventing association with pVHL. Subsequently, HIF-1 α accumulates and associates with HIF-1 β to form a heterodimer that accumulates in the nucleus and activates a specific set of genes by binding to hypoxic response elements in the promoter region (10). The HIF-1 α protein complex mediates transcriptional responses to hypoxia by binding to hypoxia response elements on specific target genes (2), but the role of HIF-1 in tumor growth and development remains uncertain as a number of *in vivo* studies have drawn conflicting results. For example, certain studies have shown that the loss of HIF-1 function inhibits both angiogenesis and tumor growth (11-13), while other studies showed impaired growth ability, but no effect on angiogenesis (12,14-16). The main reason for this phenomenon appears to be that HIF is not expressed in monolayer culture cells under normal culture circumstances. Therefore, the appropriate *in vitro* model simulating the realistic situation *in vivo* is the most important factor in the study of HIF functional analysis.

In this study, the three-dimensional spheroid culture method was employed to study the possible role of HIF-1 α in the biological behavior of the cervical tumor cell line HeLa *in vitro*. A vector was constructed to express antisense HIF-1 α (anti-HIF-1 α -pEGFP) and transfect the latter into HeLa cells. Cell proliferation, apoptosis and migration were compared among the anti-HIF-1 α -pEGFP-transfected (HIF-1 α -blocked), pEGFP-transfected (mock, as a plasmid-transfection control) and untransfected cells. HIF-1 α was found to play a potentially pivotal role in the malignant phenotype of HeLa cells *in vitro*.

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Materials and methods

Cell culture. The human cervical carcinoma HeLa cell line was obtained from the American Type Culture Collection (ATCC, VA, Manassas, USA) and was cultured in RPMI growth medium supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. For the hypoxic culture, cells were maintained in an incubator with 1% O₂.

Vector construction. RNA was isolated from HeLa cells using TRIzol™ Reagent (Gibco BRL, USA) according to the manufacturer's instructions. RNA (2 µg) was used for cDNA synthesis by reverse transcription. The RNA samples were incubated at 70°C for 5 min with 0.5 µg oligo(deoxythymidine) primers in a final volume of 10 µl and then at 37°C for 60 min in a 25-µl reaction volume containing 125 mmol/l deoxynucleotide triphosphate, 200 units Muloney murine leukemia virus reverse transcriptase and Muloney murine leukemia virus RT buffer (Promega, USA). The cDNAs obtained were amplified by using the cloning primers: 5' CGG GAT CCG GTG ATT TGG ATA TTG AAG ATG AC 3' (upper) and 5' GAA GAT CTC ACT CAC AAC GTA ATT CAC ACA TA 3' (lower). The PCR profile was 95°C for 1 min, 94°C for 40 sec, 58°C for 40 sec and 72°C for 1 min for 30 cycles, followed by extension for 7 min at 72°C. The amplified products were purified using a PCR kit (New England Biotech, UK), ligated with pEGFP vector (Promega) by following the instruction manual. The recombinant plasmid was then screened by digestion and sequencing to confirm the blocked sequences of HIF-1, and was termed anti-HIF-1α-pEGFP.

Vector transfection and clone selection. HeLa cells were transfected with 3 µg pEGFP (as a blank control) or 3 µg anti-HIF-1α-pEGFP according to the protocol provided with the Lipofectamine 2000 transfection reagent (Life Technologies, Inc.). Briefly, 2x10⁵ cells were plated in 6-well plates and incubated with the appropriate plasmid DNA and Lipofectamine 2000 in serum-free medium for 5 h. Equal volumes of media containing 20% FBS were then added. After 24 h, the media were replaced with media containing 1 mg/ml G418. Surviving colonies were selected after 2 weeks and maintained in 300 µg/ml G418. Positive cell clones were selected and amplified. Changes in HIF-1α levels were confirmed by Western blotting in the hypoxic environment.

Spheroid culture. HeLa cells, the anti-HIF-1α-pEGFP transfected counterpart and the control blank vector-transfected cells were cultured to 95% confluence, seeded into agarose-coated 24-well plates at a density of 2,000 cells/well and cultured. Each well contained 200 µl of tissue culture medium, and the spheroids were fed every other day by carefully aspirating 100 µl of spent medium and replacing it with the same quantity of fresh medium.

Western blot analysis. Cells were lysed in a lysis buffer containing 50 mm Tris, pH 7.4, 150 mm NaCl, 0.5% NP-40, 50 mm NaF, 1 mm Na₃VO₄, 1 mm phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin and 25 mg/ml aprotinin. The lysates were cleared by centrifugation, and the supernatants were

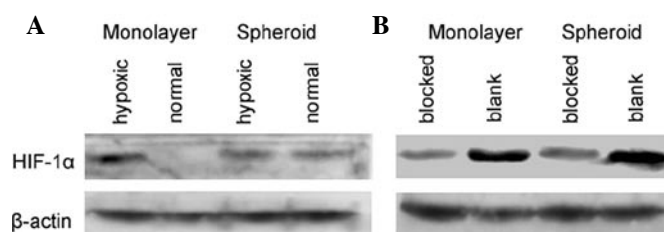


Figure 1. (A) HIF-1α protein was expressed in the hypoxic culture or normal spheroid culture but was not expressed under normal culture conditions. (B) Significantly decreased HIF-1α expression was noted in the anti-HIF-1α-pEGFP-transfected (blocked) HeLa cells in hypoxic conditions compared to the blank pEGFP vector-transfected cells in the monolayer culture. Similar results were also obtained in the multicellular spheroids.

collected. Equal amounts of lysate protein were used for the Western blot analyses with the indicated antibodies. Specific signals were visualized using the ECL chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA).

Analysis of cell proliferation and apoptosis by flow cytometry. After trypsinization for cell detachment, the cells were incubated in 50% FBS for 15 min to restore membrane integrity and centrifuged for 5 min at 1,200 rpm. Detached cells were stored via retention of the culture medium and recovered by centrifugation. Apoptotic cells were detected by assaying the Annexin V binding by flow cytometry (commercially available test, provided by Boehringer Mannheim). To exclude necrotic cells, we double-stained the cells with 5 µg/ml propidium iodide (PI) in PBS. Cells were fixed with 75% ethanol and digested with DNase-free RNase in PBS containing 5 µg/ml PI for DNA staining for 45 min at 37°C. PI and forward light scattering were detected using the flow cytometer FACSCalibur (Beckton-Dickinson) equipped with Cell Quest software. The data were analyzed using Cell Fit software. The experiment was repeated three times.

Spheroid invasion assays. Cell motility was assessed using the HABM-HEC model. Multicellular spheroids were plated at 100 spheroids/well in the upper chamber of the model. The outer chambers were filled with 0.5 ml of media containing 10% FBS. After 24 h, cells migrating to the undersurface of the filters were counted. The same five microscopic fields were used to count the number of cells passing to the undersurface of each filter.

Statistical analysis. All experiments were repeated at least three times. The Student's t-test was used to evaluate the differences between the experimental and control groups. $P < 0.05$ was considered to be statistically significant.

Results

Expression of HIF-1α protein in the monolayer-cultured HeLa cells and the multicellular spheroids. In the monolayer-cultured HeLa cells, no HIF-1α protein expression was detected under normal culture conditions. However, we observed HIF-1α expression under hypoxic conditions. Nevertheless, in the multicellular tumor spheroids, HIF-1α was expressed in hypoxic and normal cultures (Fig. 1A).

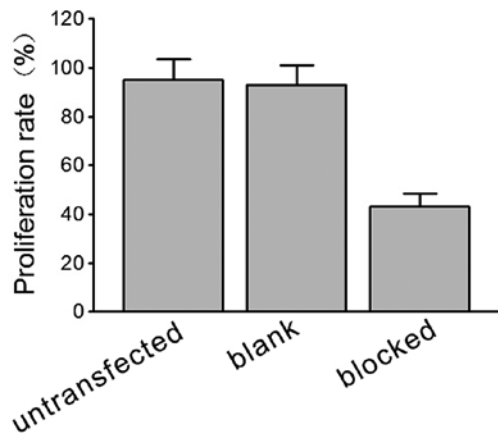


Figure 2. A marked decrease in proliferation was observed in the HeLa cell spheroids when compared to the blank pEGFP vector-transfected spheroid cells under normal culture conditions.

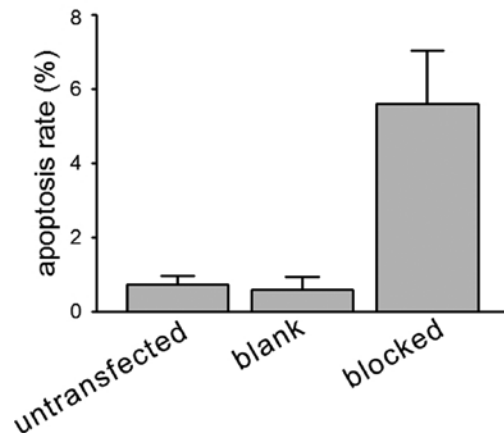


Figure 3. HIF-1 α -blocked HeLa cell spheroids had higher fold levels of apoptosis than those of the blank vector-transfected cell spheroids in the normal culture.

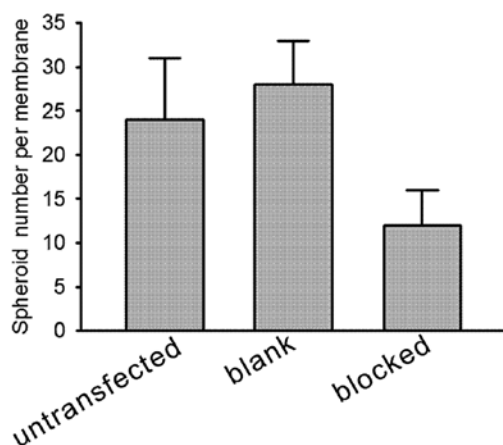


Figure 4. The rate of invasion of the HIF-1 α -blocked HeLa cells was much lower than that of the blank vector-transfected HeLa cells in the spheroids under normal conditions.

A significantly decreased HIF-1 α expression was noted in the anti-HIF-1 α -pEGFP-transfected (blocked) HeLa cells under hypoxic conditions, compared to the blank pEGFP vector-

transfected cells in the monolayer culture. Similar results were also obtained in the multicellular spheroids (Fig. 1B). These results confirm that HIF-1 α is expressed under hypoxic conditions and that the multicellular tumor spheroid was an ideal model of hypoxia *in vitro*.

Effect of HIF-1 α on multicellular spheroid growth and apoptosis. In the HIF-1 α -blocked HeLa cells, a marked decrease in proliferation was observed in the HeLa cell spheroids when compared to the blank pEGFP vector-transfected spheroid cells under normal culture conditions, as assessed by flow cytometry and the counting of cell numbers (Fig. 2).

Concomitantly, when the apoptotic indices were compared, HIF-1 α -blocked HeLa cell spheroids had higher fold levels of apoptosis than those of the blank vector-transfected cell spheroids (5.6 vs. 0.6%) in the normal culture (Fig. 3).

HIF-1 α protein promotes the invasive ability of HeLa cells. To evaluate the effect of HIF-1 α on cell invasive ability, an invasion assay was performed *in vitro* by testing the cells invading from the top well to the lower chamber. Our data showed that the invasion rate of the HIF-1 α -blocked HeLa cells was much lower than that of the blank vector-transfected HeLa cells in the spheroids under normal conditions (Fig. 4, $P < 0.01$). Findings of this study indicate that HIF-1 α protein promotes the invasive activity of tumor cells in a three-dimensional spheroid culture *in vitro*.

Discussion

Cells under hypoxic conditions express a series of genes that allow for survival and proliferation. HIF-1 α regulates the expression of more than 30 target genes (11), most of which play roles in tumor malignant behaviors, such as proliferation, invasion and metastasis (17,18). HIF-1 α expression is a common feature of solid human tumors and has been reported in many different tumor types (5,19-25). Moreover, the overexpression of HIF-1 α was found to be a poor prognostic indicator in a variety of tumors (4,26-28).

This study focused on whether HIF-1 α is involved in the cervical tumor malignant phenotype by affecting proliferation, apoptosis and tumor invasion of HeLa cells *in vitro*. Therefore, we established interference for the inhibition of HIF-1 α in HeLa cells. Since HIF-1 α rapidly undergoes ubiquitin-mediated degradation during normoxia, we detected HIF-1 α expression in monolayer cultured cells and in multicellular spheroids, respectively. HIF-1 α was not detected in HeLa cells in the monolayer culture in a normal culture condition. However, it displayed a strong increase at the protein level in multicellular spheroids under the same condition or in a monolayer under hypoxic conditions. The main cause of this phenomenon may be that in three-dimensional spheroid culture, oxygen diffusion was limited by the depth of the fluid medium and the smaller surface area to the volume compared to that in monolayer culture cells. Thus, the spheroid culture is an ideal model for the study of the mechanism of HIF-1 α *in vitro*.

We transfected the antisense HIF-1 α plasmid into the human cervical cancer cell line HeLa. Western blotting showed that the HIF-1 α expression was markedly down-regulated in the cloned antisense plasmid-transfected cells in the mono-

layer under a hypoxic condition or in multicellular spheroids in a normal culture condition.

We then compared the cell proliferation between HIF-1 α -blocked and blank plasmid-transfected cells. We found that spheroid HIF-1 α -blocked HeLa cells decreased proliferative ability when compared to the blank plasmid-transfected cells in the normal culture condition. The apoptotic rate of HIF-1 α -blocked cells was also significantly reduced in the spheroid cultured cells when compared to the monolayer cultured cells. Additionally, the HIF-1 α -blocked HeLa cell spheroids had higher fold levels of apoptosis than the normal HeLa cells (5.6% of cells in the HIF-1 α -blocked HeLa cell spheroids compared to 0.6% in the HeLa cells). Thus, in the spheroids, HIF-1 α has a dual role in the regulation of cell division and resistance to apoptosis. Studies have reported that hypoxia causes cell death partly by involving the pro-apoptotic HIF-regulated factor BNip3 (26,29,30). Nevertheless, in spheroids, overall HIF-1 has an anti-apoptotic effect as measured by the inhibition of caspase-3 activation in the proliferating compartment and by the final growth rate of the spheroid (31).

When the cell cycle was analyzed, enhanced transition from the G1 into the S phase was noted under hypoxic conditions. However, Wang *et al* (32) showed that the loss of HIF-1 α caused an increased progression into the S phase and abolished hypoxia-induced growth arrest. Goda *et al* found that HIF-1 α was required for cell cycle arrest during hypoxia and that BrdUrd labeling was increased in HIF-1 α null B cells in culture (33), which was also observed in HIF-1 α null chondrocytes *in vivo* (34). Taken together, the findings appear to be contradictory to our observation that the overall growth rate was slower in the anti-HIF-1 α HeLa spheroids. Other studies have shown that HIF-1 α -defective tumor cell lines grow more quickly than those with functional HIF-1 α in normoxia (12). However, we observed no difference in the growth rates of the HeLa and HIF-1 dysfunctional HeLa cell lines in the normoxic monolayer culture.

HIF-1 α protein has been found to be overexpressed in multiple types of human cancer and distant metastatic tissues (18). This overexpression of HIF-1 α may occur very early in carcinogenesis before histological evidence of angiogenesis or invasion (18). Regarding cell migration, the data presented in this study suggest a molecular mechanism by decreasing the protein level of HIF-1 α as an anti-metastatic strategy. However, the discrepancy between the extent by which anti-HIF-1 α decreases the HIF-1 α level and expression levels of its target genes must be considered. Transactivation of target genes by HIF-1 α is cell-type specific; thus, it should not be expected that the same battery of genes reported would be transactivated by HIF-1 α in other cell lines. Furthermore, the data presented in this study did not distinguish between direct and indirect regulation of the identified target genes by HIF-1 α . Nevertheless, our results indicate that antisense affects multiple steps in the complex process of invasion by inhibiting HIF-1 α .

This study therefore supports the hypothesis that HIF-1 α is a significant regulator of adaptive processes that promote tumor cell malignant phenotypes, such as proliferation, anti-apoptosis and invasive ability. The results of previous pre-clinical and clinical studies have established the theory that tumor hypoxia may promote malignant progression by a number of

mechanisms, including an increased expression of transcription factors and gene products involved in tumor propagation and the induction of genomic instability. Therefore, in developing treatment strategies for cancer patients, it is reasonable to consider approaches aimed at ameliorating tumor hypoxia in an effort to maximize the effects of cancer therapy.

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