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Treatment With siRNA and Antisense Oligonucleotides Targeted to HIF-1α Induced Apoptosis in Human Tongue Squamous Cell Carcinomas

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Treatment With siRNA and Antisense Oligonucleotides Targeted to HIF-1α Induced Apoptosis in Human Tongue Squamous Cell Carcinomas

Abstract

Overexpression of hypoxia inducible factor-1α (HIF-1α) in cancers has been correlated to a more aggressive tumor phenotype. We investigated the effect of HIF-1α knockout on the in vitro survival and death of human tongue squamous cell carcinomas (SCC-4 and SCC-9). Under normoxic condition, a basal level of HIF-1α protein was constitutively expressed in SCC-9 cells, albeit an undetectable level of HIF-1α messages. Exposure to hypoxia induced only a transient increase in mRNA transcript but a prolonged elevation of HIF-1α protein and its immediate downstream target gene product, VEGF. Under normoxic or hypoxic conditions, treatment of SCC-9 cells with AS-HIF-1α ODN suppressed both constitutive and hypoxia-induced HIF-1αa expression at both mRNA and protein levels; Knockout of HIF-αa gene expression via either AS-HIF-1α ODN or siRNA (siRNA HIF-1α treatment resulted in inhibition of cell proliferation and induced apoptosis in SCC-4 and SCC-9 cells. We also demonstrated that exposure of SCC-9 cells to hypoxia led to a time-dependent increase In the expression of bcl-2 and IAP-2, but not p53. The attenuated levels of bcl-2 and IAP-2, and the enhanced activity of caspase-3 after treatment with AS-HIF-1α ODN may contribute partly to the effects of HIF-1α blockade on SCC-9 cell death. Collectively, our data suggest that a constitutive or hypoxia-induced expression of HIF-1α In SCC-9 and SCC-4 cells is sufficient to confer target genes expression essential for tumor proliferation and survival. As a result, interfering with HIF-1α pathways by antisense or siRNA strategy may provide a therapeutic target for human tongue squamous cell carcinomas. © 2004 Wiley-Liss, Inc.

Keywords

Antisense, Apoptosis, Bcl-2, HIF-1α, IAP-2, siRNA, Apoptosis, Carcinoma, Squamous Cell, Cell Hypoxia, Cell Survival, Humans, Hypoxia-Inducible Factor 1, alpha Subunit, Oligonucleotides, Antisense, Phenotype, RNA, Messenger, RNA, Small Interfering, Tongue Neoplasms, Transcription Factors, Tumor Cells, Cultured, antisense oligonucleotide, caspase 3, gene product, hypoxia inducible factor 1alpha, iap 2 protein, protein, protein bcl 2, protein p53, small interfering RNA, unclassified drug, apoptosis, article, cancer growth, cell death, cell proliferation, cell survival, controlled study, enzyme activity, gene expression, gene overexpression, gene targeting, human, human cell, hypoxia, in vitro study, knockout gene, phenotype, priority journal, protein expression, RNA transcription, squamous cell carcinoma, tongue carcinoma

Disciplines

Dentistry | Oral and Maxillofacial Surgery | Oral Biology and Oral Pathology | Periodontics and Periodontology

Comments

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TREATMENT WITH siRNA AND ANTISENSE OLIGONUCLEOTIDES TARGETED TO HIF-1α INDUCED APOPTOSIS IN HUMAN TONGUE SQUAMOUS CELL CARCINOMAS

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Overexpression of hypoxia inducible factor-1α (HIF-1α) in cancers has been correlated to a more aggressive tumor phenotype. We investigated the effect of HIF-1α knockout on the *in vitro* survival and death of human tongue squamous cell carcinomas (SCC-4 and SCC-9). Under normoxic condition, a basal level of HIF-1α protein was constitutively expressed in SCC-9 cells, albeit an undetectable level of HIF-1α messages. Exposure to hypoxia induced only a transient increase in mRNA transcript but a prolonged elevation of HIF-1α protein and its immediate downstream target gene product, VEGF. Under normoxic or hypoxic conditions, treatment of SCC-9 cells with AS-HIF-1α ODN suppressed both constitutive and hypoxia-induced HIF-1α expression at both mRNA and protein levels. Knockout of HIF-1α gene expression via either AS-HIF-1α ODN or siRNA (siRNA$_{HIF-1\alpha}$) treatment resulted in inhibition of cell proliferation and induced apoptosis in SCC-4 and SCC-9 cells. We also demonstrated that exposure of SCC-9 cells to hypoxia led to a time-dependent increase in the expression of bcl-2 and IAP-2, but not p53. The attenuated levels of bcl-2 and IAP-2, and the enhanced activity of caspase-3 after treatment with AS-HIF-1α ODN may contribute partly to the effects of HIF-1α blockade on SCC-9 cell death. Collectively, our data suggest that a constitutive or hypoxia-induced expression of HIF-1α in SCC-9 and SCC-4 cells is sufficient to confer target genes expression essential for tumor proliferation and survival. As a result, interfering with HIF-1α pathways by antisense or siRNA strategy may provide a therapeutic target for human tongue squamous cell carcinomas.

Key words: *antisense; siRNA; apoptosis; HIF-1α; Bcl-2; IAP-2*

Hypoxia inducible factor 1 (HIF-1), a master transcriptional factor of oxygen-regulated genes, mediates a wide range of cellular and physiological adaptive responses to changes in oxygen tension.[1] HIF-1 belongs to the basic-helix-loop-helix-pas (bHLH/PAS) transcription factor family and is composed of 2 subunits, HIF-1α and HIF-1β (also known as ARNT, aryl hydrocarbon receptor nuclear translocator). Under normoxic condition, HIF-1α is modified at the proline residues (pro^{564} and pro^{402}) by prolyl hydroxylases,[2] interacts with the von Hippel-Lindau tumor suppressor protein (VHL), a recognition component of an E3 ubiquitin-protein ligase, and is targeted for proteasomal degradation.[3,4] Under hypoxic condition, however, HIF-1α protein is stabilized through the inactivation of an oxygen-dependent HIF-1α-prolyl hydroxylase,[3,4] and translocates to the nucleus where it dimerizes with HIF-1β unit. The HIF molecule binds to the consensus DNA sequence in the promoter of downstream target genes[1] whose products are essential in several key pathways enabling tumor cells to adapt to the hypoxic microenvironment.[5–9]

Intratumoral hypoxia, a characteristic feature of solid tumors, results from several factors including an inadequate neovascularization, irregular blood flow, and poorly compensated oxygen consumption by rapidly proliferating cancer cells. Adaptation to the hypoxic microenvironment leads to the selection of certain malignant tumor phenotypes, characterized by abnormal neovascularization,[10] invasion, metastasis and resistance to chemo- and radiotherapies.[11–14] Recent studies have correlated the over-expression of HIF-1α in common cancers with a more aggressive tumor phenotype and an advanced tumor grade,[15,16] implying HIF-1α as an independent marker of disease prognosis, and a potential predictor for mortality risk and treatment failure.[17–22] Besides hypoxia, certain oncogenes such as Ha-ras and v-Src[23,24] and tumor suppressor genes such as von Hippel-Lindau gene (pVHL),[25,26] phosphatase and tensin homologue (PTEN)[27,28] and p53[29,30] have also been indicated in the stabilization and activation of HIF-1α under normoxic conditions. Furthermore, certain growth factors or hormones such as EGF, PDGF, IGF-I, IGF-II, angiotensin II and thrombin have also been described to stabilize and activate HIF-1α under normoxic conditions through translational and posttranslational mechanisms.[31–33]

Besides its well-characterized role in the adaptation to the hypoxic stress, recent studies have shown that HIF-1α displays a pro-apoptotic or an anti-apoptotic effect in some cell types under certain experimental conditions. Under chronic or severe hypoxia, HIF-1α promotes apoptosis in some human tumor cells by inducing expression of cell death factors such as BNIP3 and NIX[34,35] or by stabilizing the tumor suppressor protein p53.[29] Paradoxically, several studies also reported that hypoxia or HIF-1α can obviously protect cells from apoptosis induced by different agents like serum deprivation,[36] interferon-γ,[37] tumor necrosis factor related apoptosis-inducing ligand (TRAIL)[38] and some chemotherapeutic agents.[39,40] Despite controversial findings, clinical studies have reported that high levels of HIF-1α in human squamous cell carcinomas seem correlated with tumor resistance to radiation and chemotherapy.[21,22] Likewise, HIF-1α-deficient transformed mouse embryonic fibroblasts and fibrosarcoma cells are more susceptible to chemotherapeutic agents and ionizing radiation than wild-type cells.[42] These findings imply that HIF-1α is a survival factor in tumor growth and can contribute to tumor treatment resistance by intervening with cellular apoptosis.[43]

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Despite several findings correlating HIF-1 α with tumor survival ability, the cellular mechanisms remain to be elucidated. We examined the effect of HIF-1α knockout via either antisense fragment, phosphorothioate oligodeoxynucleotide (AS-HIF-1α ODN) or small interfering RNA targeted to HIF-1α ($siRNA_{HIF-1\alpha}$), on the *in vitro* survival and death of human tongue squamous cell carcinomas (SCC-4 and SCC-9). Our findings suggest that depletion of HIF-1α activity results in significant cellular apoptosis via mechanisms involving the activation of caspase-3, and the downregulation of Bcl-2 and IAP-2 gene expressions.

MATERIAL AND METHODS

Tumor cell lines and culture

Two human tongue squamous cell carcinoma cell lines (SCC-4 and SCC-9) and a human hepatoma cell line (HepG2) were obtained from ATCC. SCC-4 and SCC-9 were cultured in DMEM/F-12 medium (Fisher Scientific, Tustin, CA) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and hydrocortisone (400 ng/ml). HepG2 cells were maintained in DMEM medium (Gibco BRL, Rockville, MD) supplemented with 10% FBS and antibiotics as described above. All cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Establishment of hypoxic culture condition

Cells were cultured to about 80% confluence and transferred to a hypoxic chamber with an auto purge airlock (Coy Laboratory Products Inc., Grass Lake, MI). Environmental hypoxic conditions (1%) were achieved in an airtight humidified chamber continuously flushed with a gas mixture containing 5% CO_2 and 95% N_2. Maintenance of the desired O_2 concentration was constantly monitored during incubation using a microprocessor-based oxygen controller (Coy Laboratory Products, Inc.).

HIF-1 α antisense oligonucleotide treatment of cancer cells

The HIF-1α antisense phosphorothioate oligodeoxynucleotide (AS-HIF-1α-ODN) and sense control (S-HIF-1α-ODN) were synthesized by GenoMechanix (Alachua, FL) according to published sequences (antisense sequence: 5′-GCC GGC GCC CTC CAT-3′; sense sequence: 5′-ATG GAG GGC GCC GGC-3′).[44] SCC-4, SCC-9 and HepG2 cells were plated onto 12-well or 6-well plates, grown to about 40–50% confluence, and exposed to either sense or antisense ODNs (final concentration, 200 nM) premixed with the Oligofectamine Reagent (Invitrogen, Grand Island, NY) in Opti-MEM-I (Invitrogen) for 4 hr. As mock transfection, cells were exposed to Oligofectamine alone in the absence of sense or antisense oligonucleotides.

siRNA treatment of cells

Small interfering RNA targeted to HIF-1α ($siRNA_{HIF-1\alpha}$), 5′-AGAGGUGGAUAUGUGUGGGdTdT-3′ and 5′-CCCACACAUAUCCACCUCUdTdT-3′, were synthesized and annealed (Dharmacon Research, Inc., Lafayette, CO) as described previously.[45] Cells were transfected with the oligonucleotide duplexes (200 nM) premixed with the Oligofectamine (Invitrogen) in Opti-MEM-I (Invitrogen) for 4 hr. As mock transfection, cells were exposed to Oligofectamine alone. A siRNA targeted to an irrelevant mRNA (Dharmacon Research) serves as nonspecific control.

RNA extraction and Northern blot analysis

Total RNA was isolated from cancer cells using TRIZOL (Gibco BRL). A total amount of RNA (15 μg) was separated on a 1.4% agarose gel containing formaldehyde and transferred onto nylon membranes (Amersham Pharmacia Biosciences, Piscataway, NJ). A cDNA probe complementary to human HIF-1α or VEGF was amplified using reverse transcription polymerase chain reaction (RT-PCR), subcloned into pGEM-T Easy Vector System (Promega, Madison, WI), labeled with α^{32}P-dCTP using the Rediprime II Random Prime DNA labeling System (Amersham Pharmacia Biosciences), and purified through Sephadex G-50 Quick Spin columns (TE) (Roche Diagnostics, Indianapolis, IN). After overnight hybridization membranes were processed and analyzed with a phosphor imaging scanner (ImageQuant, Molecular Dynamics, Sunnyvale, CA). Images were quantified using ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

RT-PCR analysis for HIF-1α mRNA Levels

RT-PCR analysis of HIF-1α and β-actin mRNA levels was carried out using the One-step RT-PCR Kit (QIAGEN) with primers specific to HIF-1α (forward primer 5′-TCACCACAGGACAGTACAGGATGC-3′ and reverse primer 5′-CCAGCAAAGTTAA AGCATCAGGTTCC-3′, 418 bp fragment) or specific to β-actin (forward primer: 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ and reverse primer: 5′-CCTAGAAGCATT TGCGGTGCACGATG-3′, 285 bp fragment).

Western blot analysis

Whole cell lysates were prepared as described by Arsham *et al.*[46] Briefly, treated and untreated cells were extracted with lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% Triton X-100, 10 mmol/L sodium fluoride, 20 mmol/L β-mercaptoethanol, 250 μmol/L sodium orthovanadate, 1 mmol/L PMSF and complete protease inhibitor cocktail (Sigma, St. Louis, MO), and incubated at 4°C for 30 min. The lysates were ultra-sonicated and centrifuged at 14,000*g* for 15 min. The supernatants were collected and stored at −70°C. Protein concentrations were determined by BCA methods. Protein (100 μg) was separated on 10% polyacrylamide-SDS gel and electroblotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia). After blocking with TBS/5% skim milk, the membrane was incubated overnight at 4°C with primary antibodies against HIF-1α (BD Transduction Lab, San Diego, CA), bcl-2, IAP-2 and p53 (Santa Cruz Biotechnology, Santa Cruz, CA), or the active caspase-3 (Chemicon, Temecula, CA), followed by second antibodies conjugated with peroxidase (1:5000) (Pierce, Rockford, IL) for 1 hr at room temperature. Signals were detected with ECL.

Apoptosis analysis

Assessment of cellular apoptotic morphologies. The morphologic changes characteristic of apoptotic cells after transfection with S-HIF-1α-ODN or AS-HIF-1α-ODN for 48 hr were evaluated using phase-contrast microscopy, and photographs were captured with a computer-imaging system.

Detection of apoptosis rate by ELISA. The rate of apoptosis in AS-HIF-1α-ODN or $siRNA_{HIF-1\alpha}$-treated cancer cells was assessed using the cell death detection $ELISA^{PLUS}$ System (Roche Diagnostics). The assay measures the amount of cytosolic histone-associated DNA fragments generated during the apoptosis. DNA fragments were measured according to the procedures as described in the manufacturer's protocol.

Flow cytometry. For analysis of apoptosis, cells transfected with sense or antisense HIF-1α ODNs were harvested and washed once with PBS at 500*g* for 5 min. Cell pellets were resuspended in Annexin-V-FLUOS staining solution (Roche Molecular Biochemicals) and incubated for 15 min at 15~25°C. Samples were analyzed on a FSCAN flow cytometer.

Cellular proliferation assay. SCC-9 cells were plated in 96-well plates at 10^4 cells per well. After transfected with $siRNA_{HIF-1\alpha}$, sense or antisense HIF-1α ODNs, cells were cultured under normal conditions. At different intervals, viable cells were determined using MTT Assay kit (Chemicon) according to the manufacturer's protocol. Each experiment was carried out in triplicate.

ELISA assay for VEGF production. VEGF production in the media was assayed with a commercially available ELISA kit (American Diagnostica Inc, Stamford, CT). Cells transfected with sense or antisense HIF-1α ODNs were cultured under normoxic or hypoxic conditions for 48 hr, and then media were collected for ELISA analysis according to the manufacturer's protocols. VEGF production was normalized to cell count (5×10^4).

Data analysis

Data are presented as the mean ± SEM for 3 separate experiments. A paired Student's test was employed for statistical analysis, with significant differences determined as $p < 0.05$.

RESULTS

HIF-1α protein is expressed constitutively in SCC-9 cells and is upregulated by hypoxia

Under normoxic condition, HIF-1α mRNA level was low to undetectable in SCC-9 cells (Fig. 1*a*, *lane a*); however, a stable basal level of HIF-1α protein was expressed throughout the course of the study (Fig. 1*b*). As cells were exposed to the hypoxic environment, a transient multifold increase in HIF-1α mRNA transcript was observed, with maximum induction at 3 hr (Fig. 1*a*, *lane c*). Hypoxia induced a rapid and sustained accumulation of HIF-1α protein in SCC-9 cells up to 8 hours (Fig. 1C) or longer (data not shown).

We next examined the effect of hypoxia-induced HIF-1α on its immediate downstream target gene, vascular endothelial growth factor (VEGF) in SCC-9. As expected, a similar pattern of induction of VEGF was observed (Fig. 1*a*, *lane c*). Moreover, ELISA results showed a robust increase in VEGF protein level in the conditioned media after exposure to hypoxia for up to 48 hr ($p < 0.01$) (Fig. 2*c*).

HIF-1α expression is suppressed by AS-HIF-1α-ODNs

To determine whether treatment with antisense ODNs targeted against HIF-1α (AS-HIF-1α-ODNs) suppresses HIF-1α mRNA and protein expression, SCC-9 cells were transfected with AS-HIF-1α-ODN for 24 hr, followed by RT-PCR and Western blot analyses. The results showed that HIF-1α mRNA level was completely attenuated by 200 nM AS-HIF-1α-ODNs under both normoxic and hypoxic conditions (Fig. 2*a*). Likewise, both basal and hypoxia-induced HIF-1α protein levels were nearly abolished by AS-HIF-1α-ODNs treatment (Fig. 2*b*). The effect of HIF-1α antisense treatment on the transactivation of downstream target gene was further confirmed by analysis of secreted VEGF in the conditioned media. AS-HIF-1α-ODN treatment led to a significant decrease in both basal and hypoxia-induced secreted VEGF as compared to sense HIF-1α-ODNs treatment or mock transfection (Oligofectamine treatment alone) ($p < 0.01$).

Knockout of HIF-1α inhibits SCC-9 cell proliferation

To explore the biological effects of HIF-1α abrogation in SCC-9 cells, we first study the effect of AS-HIF-1α-ODNs treatment on cellular proliferation using the MTT method. No growth inhibitory effect was observed in mock transfected cells or cells treated with sense ODNs (Fig. 3). The percentages of living cells labeled with bioreduced formazan were reduced to 78.6%, 61.6% and 38.4% after

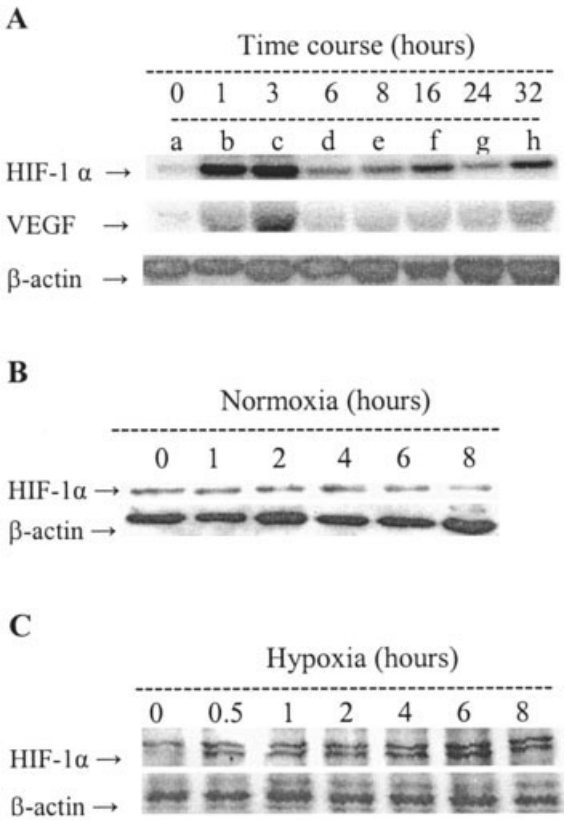

Figure 1 – Time course study of hypoxia-induced HIF-1α and VEGF expressions in SCC-9 cells. (*a*) Semi-confluent cultures of cancer cells were exposed to hypoxia (1% O_2) for different time intervals and total RNAs were analyzed for VEGF and HIF-1α transcripts using Northern blot. (*b*,*c*) SCC-9 cells were exposed to normoxia (*b*) or hypoxia (*c*) for different time intervals and whole cell lysates were analyzed for HIF-1α protein levels using Western blot. The data represent 3 independent experiments.

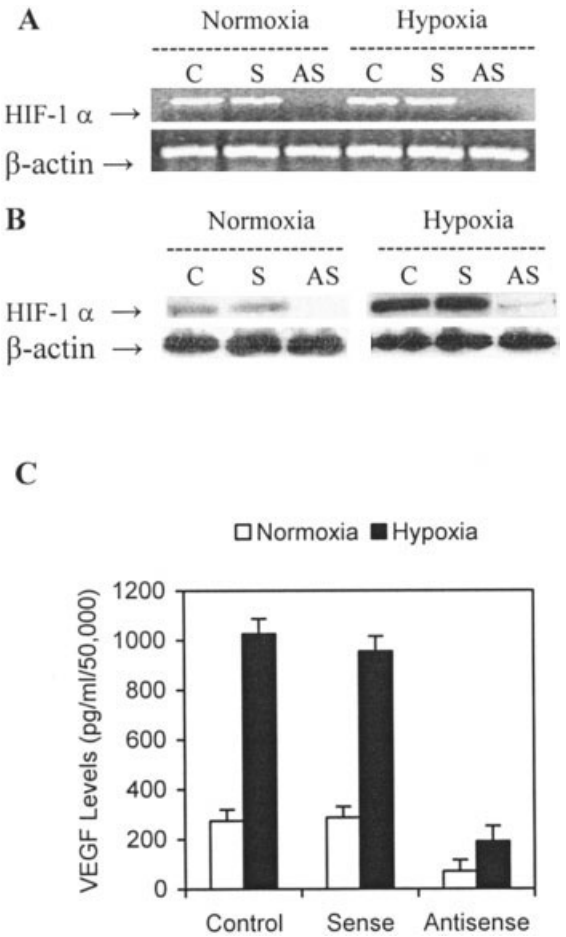

Figure 2 – Antisense-HIF-1α-ODN suppresses HIF-1α expression in SCC-9 cells. Semi-confluent SCC-9 cells were transfected with sense (S)- or antisense (AS)-HIF-1 α ODNs followed by exposure to normoxia or hypoxia for 24 hr. (*a*) Total RNAs were isolated and HIF-1 α or β-actin transcripts were analyzed by RT-PCR (C, mock transfected control, cells exposed to Oligofectamine reagent alone; S, sense; AS, antisense). (*b*) Whole cell lysates were assayed for HIF-1 α protein using Western blot. (*c*) VEGF levels in the conditioned media were measured by ELISA kit and normalized to cell counts (5×10^4). The results are representative of 3 independent experiments.

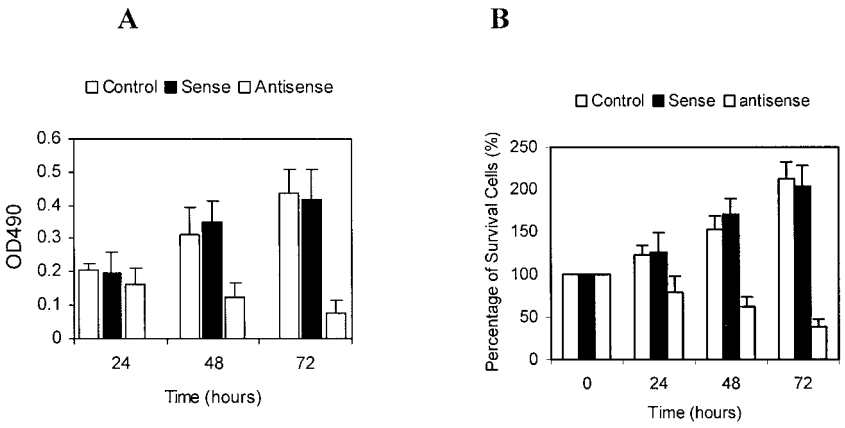

FIGURE 3 – Abrogation of HIF-1α inhibits proliferation of SCC-9 cells. (*a*) After transfection with HIF-1α sense (S) and antisense (AS) ODNs, cancer cells were cultured under normoxic conditions for the indicated time periods, followed by incubation with MTT tetrazolium compound, and absorbance at 490 nm was determined. (*b*) Results from (*a*) were described as percentage of living cells (survival %) as compared to the mock transfected control (Control, 100%). The data represent the mean ± SD of triplicate wells.

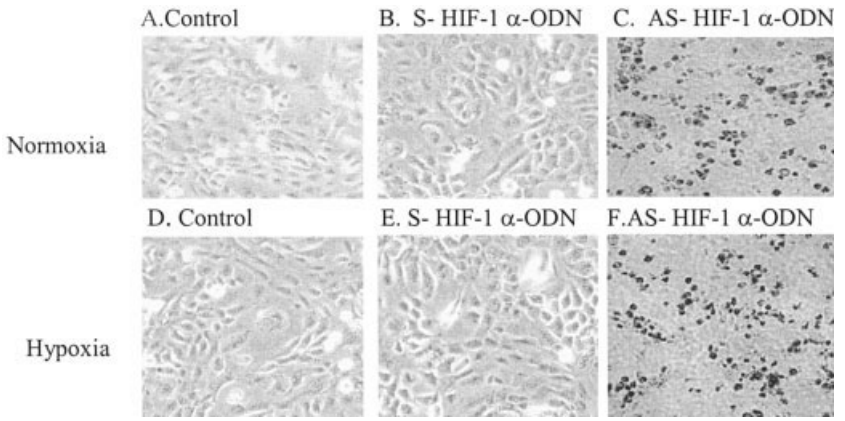

FIGURE 4 – Morphological studies of SCC-9 cells treated with antisense-HIF-1 α ODNs. After transfected with S- or AS-HIF-1α ODNs, cancer cells were cultured under normoxic or hypoxic conditions for 48 hr and examined under a phase-contrast microscope (20×). Cells exposed to Oligofectamine reagent alone served as mock transfected control.

treatment with AS-HIF-1α-ODNs for 24, 48 and 72 hr, respectively (Fig. 3*b*).

Apoptosis in SCC cells is induced by AS-HIF-1α-ODN treatment

To study the effect of HIF-1α knockout on cell apoptosis in tongue squamous cell carcinomas, we employed 3 separate approaches. First, we examined morphological changes using phase-contrast microscopy. Under both normoxic and hypoxic conditions, SCC-9 cells treated with AS-HIF-1α ODNs exhibited cellular phenotypes characteristic of apoptosis, including reduction in cell volume, nuclear chromatin condensation and membrane blebbing (Fig. 4*C*,*F*). No obvious morphological changes were observed in SCC-9 cells exposed to S-HIF-1α ODNs treatment or mock transfected cells (Fig. 4*a*,*b*,*d*,*e*, respectively).

To confirm the effect of AS-HIF-1α-ODN treatment on apoptosis in SCC-9 cells, DNA fragmentation, a characteristic feature of cellular apoptosis, was analyzed using a commercial DNA fragmentation ELISA kit. A time-dependent increase in DNA fragmentation with a peak of 6–8-fold increase at 48 hr was observed in SCC-9 cells treated with AS-HIF-1α-ODN (Fig. 5*a*). No changes in DNA fragments were observed in SCC-9 cells treated with S-HIF-1α-ODN or mock transfected cells over 48 hr. Similar findings were observed in another human tongue squamous cell carcinoma line, SCC-4, after treatment with the AS-HIF-1α-ODN (Fig. 5*b*).

We next explored whether hypoxia affects the apoptosis induced by AS-HIF-1α-ODN treatment. SCC-9 cells were treated with either S- or AS-HIF-1α ODNs and further cultured under normoxic or hypoxic conditions for additional 24 hr. Our results demonstrated a similar 3–4-fold increase in DNA fragmentation in SCC-9 cells treated with AS-HIF-1α-ODN under both normoxic and hypoxic conditions, as compared to mock transfection or cells treated with S-HIF-1α-ODN (Fig. 5*c*). There seem to be no significant differences in DNA fragmentation induced by AS-HIF-1α-ODN treatment between normoxic and hypoxic conditions ($p > 0.05$). These findings suggest that AS-HIF-1α-ODN-induced apoptosis in SCC-9 cells is independent of oxygen tension.

Last, we employed flow cytometric analysis to characterize the apoptotic profile of cells treated with AS-HIF-1α-ODN. Under normoxic conditions, no increase in the percentage of apoptotic cells was observed in SCC-9 cells treated with S-HIF-1α-ODNs (5.8 ± 1.4%) for 48 hr as compared to mock transfected control (6.3 ± 2.1%) ($p > 0.05$). However, treatment of SCC-9 with AS-HIF-1α-ODN for 48 hr led to a significant increase in the percentage of apoptotic cells (36.7 ± 8.5%) as compared to mock transfected control or S-HIF-1α-ODN treated cells ($p < 0.01$). Similar findings were demonstrated in SCC-4 cells treated with AS-HIF-1α-ODNs (Fig. 6). These results are consistent with findings from DNA fragmentation analysis (Fig. 5*a*,*b*).

HIF-1α depletion-induced apoptosis in SCC-9 cells was further confirmed by siRNA$_{HIF\text{-}1\alpha}$ treatment

To confirm the specificity of cellular apoptosis induced by HIF-1α knockout in SCC-9 cells, an alternative approach using siRNA$_{HIF\text{-}1\alpha}$ was employed. When transfected into cells, siRNA-$_{HIF\text{-}1\alpha}$ targets HIF-1α mRNA for degradation, thus blocking HIF-1α activity.[45] SCC-9 cells were treated with siRNA$_{HIF\text{-}1\alpha}$ for indicated time intervals followed by Western blot and ELISA analyses to assess HIF-1α protein expression and DNA fragmentation, respectively. Under both normoxic and hypoxic conditions, HIF-1α protein was significantly suppressed in SCC-9 cells treated with siRNA$_{HIF\text{-}1\alpha}$ (200 nM) in comparison to mock transfection controls or cells transfected with siRNA targeted to an unrelated mRNA (nonspecific control) (Fig. 7*a*). The depletion of HIF-1α level correlated with an increase in DNA fragmentation in SCC-9 cells after treatment with siRNA$_{HIF\text{-}1\alpha}$ for 24 and 48 hr as compared to mock transfection or nonspecific controls ($p < 0.01$) (Fig.

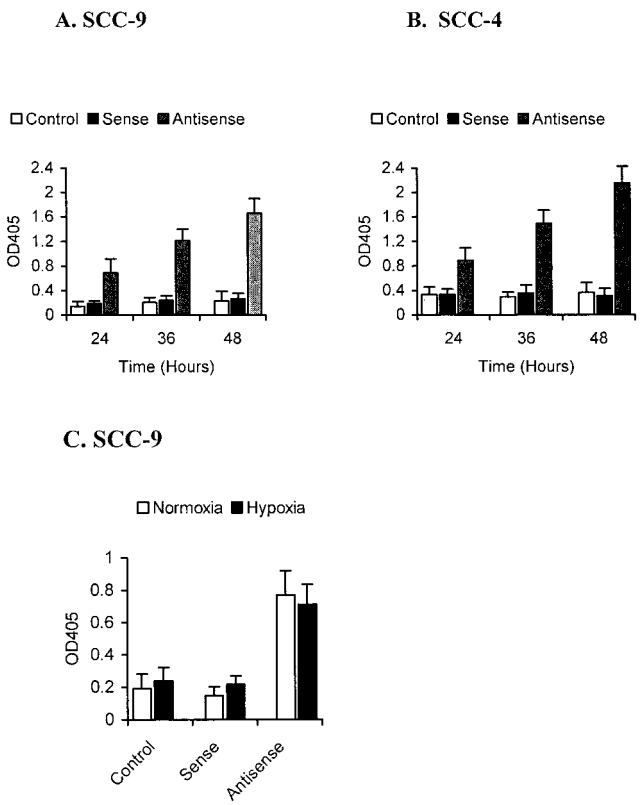

FIGURE 5 – Quantification of fragmented DNA by ELISA. After transfection with sense (S)- or AS-HIF-1α ODNs (AS), cells were cultured under normoxic conditions for different time intervals and DNA fragmentation was determined by ELISA as described in Material and Methods. (*a*) SCC-9 cell line; (*b*) SCC-4 cell line. (*c*) Hypoxic effects on DNA fragmentation in SCC-9 cells treated with AS-HIF-1α ODNs. Cells exposed to Oligofectamine reagent alone (mock transfection) served as control. Data are expressed as the mean ± SD and are representative of 3 independent experiments.

7*b*). Similar morphological changes characteristic of apoptosis were observed in SCC-9 cells exposed to siRNA$_{HIF\text{-}1\alpha}$ treatment (data not shown). These findings confirmed that the apoptosis induced by treatment with antisense oligonucleotides or siRNA was a specific result of HIF-1α loss-of-function.

To further establish the connection between apoptosis and HIF-1α expression, another cancer cell line, human hepatoma cell line (HepG2), that does not exhibit high levels of HIF-1α, was included. The basal level of HIF-1α protein in HepG2 cells was low to undetectable under normoxic condition (Fig. 8*a*), which is consistent with previous reports.[6,32] Treatment of cells with siRNA$_{HIF\text{-}1\alpha}$ suppressed hypoxia-induced HIF-1α protein accumulation (Fig. 8*a*). We examined the effects of siRNA$_{HIF\text{-}1\alpha}$ or antisense-HIF-1α-ODNs treatment on cellular proliferation or viability of HepG2 cells. Our results showed no decrease in the percentage of viable cells after treatment with siRNA$_{HIF\text{-}1\alpha}$ or AS-HIF-1α-ODNs for the indicated time intervals, as compared to mock transfections or cells treated with sense oligonucleotides or siRNA targeted to an irrelevant mRNA (Fig. 8*b*,*c*). These findings indicated that depletion of HIF-1α expression only induced apoptosis in cells harboring a constitutive level of HIF-1α under normoxic conditions.

Mechanisms underlying apoptosis induced by AS-HIF-1α-ODN treatment

To gain insights into the mechanisms underlying apoptosis induced by HIF-1α depletion in SCC-9 cells, a time course study was carried out to investigate the hypoxic induction of p53 gene, a well-known tumor suppressor. No change in p53 protein level was detected up to 24 hr of exposure to hypoxia (Fig. 9*a*) as determined by Western blot analysis. We explored whether hypoxia regulates the expression of another set of related anti-apoptotic molecules, Bcl-2 and IAP-2. SCC-9 cells were exposed to hypoxia for different time intervals and whole cell lysates were subjected to Western analysis. A time-dependent increased expression of both Bcl-2 and IAP-2 was consistently observed in SCC-9 cells exposed to hypoxia (Fig. 9*a*).

We then assessed whether HIF-1α depletion via antisense treatment has any effects on these apoptotic genes under both normoxic and hypoxic conditions. No changes in p53 protein expression were observed in cells treated with AS-HIF-1α-ODN under both normoxic and hypoxic conditions (Fig. 9*b*,*c*) as compared to mock transfections or sense controls. These results further confirm that p53 is not involved in the mechanisms of increased cell death in SCC-9 cells treated with AS-HIF-1α-ODN. Under similar experimental conditions, however, AS-HIF-1α ODN treatment significantly attenuated Bcl-2 and IAP-2 protein expression, whereas no change was observed in cells treated with S-HIF-1α-ODN or mock transfection controls (Fig. 9*b*,*c*).

Finally, we investigated whether activation of caspase-3 is involved in apoptotic pathways mediated by HIF-1α abrogation in SCC-9 cells. Results from Western blot analysis with an antibody specific against the active form of caspase-3 showed a multifold increase in the level of active caspase-3 in SCC-9 cells treated with AS-HIF-1α-ODN under normoxic conditions compared to S-HIF-1α-ODN treatment or mock transfection controls (Fig. 9*d*). Similar findings were observed under hypoxic condition (data not shown).

DISCUSSION

Recent studies have greatly extended the knowledge of hypoxia and hypoxia-inducible factor 1 (HIF-1) in tumor cell biology. We demonstrated that a low level of HIF-1α protein was constitutively expressed in human tongue squamous cell carcinoma line (SCC-9) under normoxic condition (Fig. 1*b*). This is in accordance with previous reports that HIF-1α protein is not only over-expressed in many types of cancer specimens including clear cell renal carcinomas,[47,48] brain tumors,[17] and others[15] but also constitutively expressed in *in vitro* cultured glioma cells,[17] pancreatic cancer cells,[41] and prostate carcinoma cells.[49] These findings support the contention that maintaining a constitutive level of HIF-1α is essential to adaptive cellular functions, such as cell proliferation and survival in the hostile microenvironment of tumor.[41,42]

Previous studies have demonstrated that HIF-1α protein is degraded rapidly in the presence of oxygen by the ubiquitin proteasome system. Under hypoxia this process is suppressed, allowing the stabilization and accumulation of this transcriptional factor with little effects on the expression of its messages.[33,50–52] In our present study, we showed that exposure of SCC-9 cells to hypoxia induced an early but transient increase in the expression of HIF-1α mRNA (Fig. 1*a*). However, a steady-state increase in HIF-1α protein level was maintained up to 8 hr after exposure to hypoxic condition (Fig. 1*c*). The hypoxia-induced transcriptional activation of HIF-1α in SCC-9 cells was further confirmed by an up-regulation of both VEGF mRNA and secreted protein in the culture media (Figs. 1*a*, 2*c*). These results were consistent with similar findings in other cancer cell lines reporting a transient induction of HIF-1α message and a prolonged stabilization of HIF-1α protein.[53,54]

Besides its role as a cellular survival factor, rapidly accumulating evidences suggest that HIF-1α possesses dual functions in mediating apoptosis of tumor cells, anti-apoptotic and pro-apoptotic, in response to various stimuli.[55] We adopted an effective antisense strategy that efficiently abrogated the constitutive as well as hypoxia-induced HIF-1α expression at both mRNA and protein levels in human tongue squamous cell carcinoma (SCC-9) (Fig. 2*a*,*b*); we have demonstrated for the first time that AS-HIF-1α-ODN treatment not only led to a time-dependent decrease in

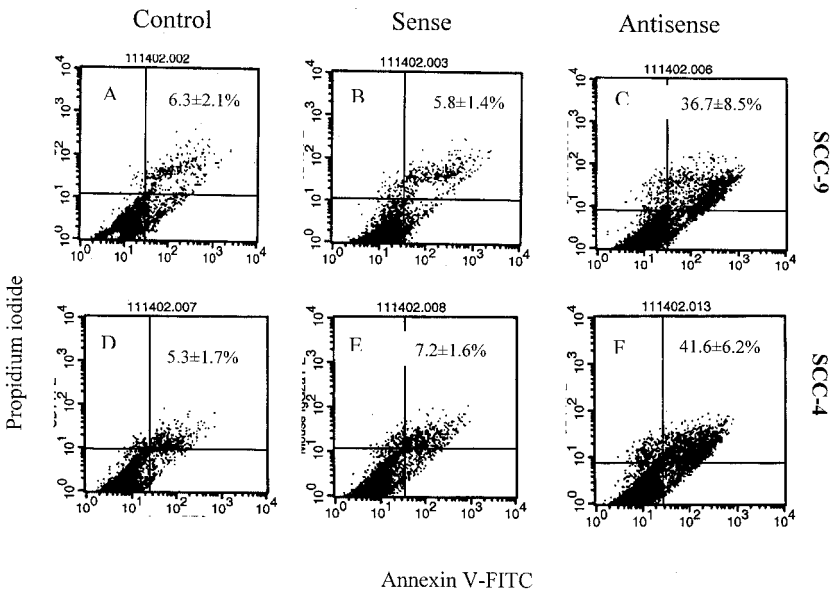

FIGURE 6 – Flow cytometry analysis of apoptosis in SCC-9 and SCC-4 cells. Cancer cells were transfected with S- or AS-HIF-1α ODNs and were maintained under normoxic conditions for 48 hr. Cells were collected and apoptotic cell death was analyzed by flow cytometry using annexin V-FITC/PI. Cells exposed to Oligofectamine reagent alone (mock transfection) served as control. Data are representative of 3 separate experiments.

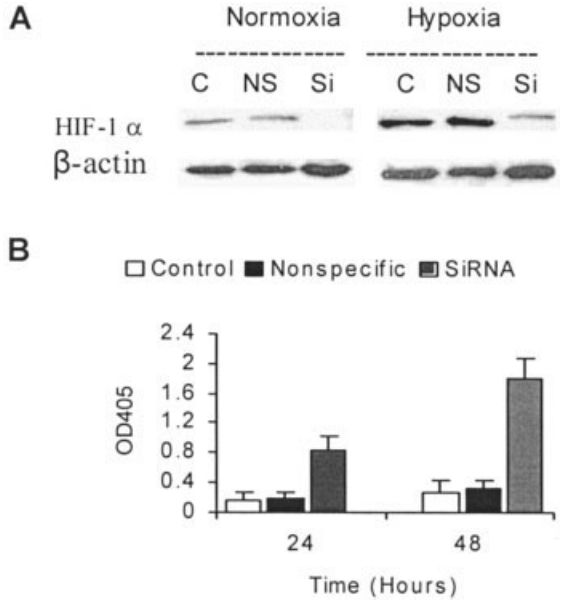

FIGURE 7 – Effects of siRNA treatment on HIF-1α protein expression and apoptosis in SCC-9 cells. (*a*) After transfection with siRNA-$_{HIF\text{-}1\alpha}$ (Si) or an siRNA targeted to an unrelated mRNA (nonspecific control, NS), cells were maintained under normoxia or hypoxia for 24 hr and whole cell lysates were isolated for Western blot analysis for HIF-1α protein expression. (*b*) ELISA assay on DNA fragmentation in SCC-9 cells after exposed to siRNA$_{HIF\text{-}1\alpha}$ or an siRNA targeted to an unrelated mRNA (nonspecific control, NS) for indicated time intervals. Cells exposed to Oligofectamine alone served as mock transfected control (*c*). Data are representative of 3 separate experiments.

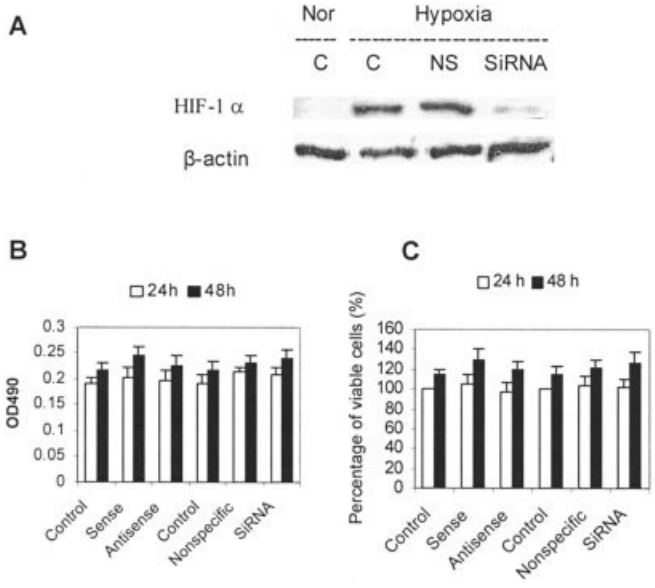

FIGURE 8 – Effects of siRNA treatment on HIF-1α protein expression and cellular viability in HepG2 cells. (*a*) After transfection with siRNA$_{HIF\text{-}1\alpha}$ (Si) or an siRNA targeted to an unrelated mRNA (nonspecific control, NS), cells were maintained under normoxia (Nor) or hypoxia for 24 hr and whole cell lysates were isolated for Western blot analysis for HIF-1α protein expression. (*b*) After transfection with sense (S)- or antisense (AS) ODNs, or transfection with siRNA$_{HIF\text{-}1\alpha}$ or an siRNA targeted to an unrelated mRNA (nonspecific control, NS) for indicated time intervals, cancer cells were cultured under normoxic conditions for the indicated time periods followed by incubation with MTT tetrazolium compound, and absorbance at 490 nm was determined. (*c*) Results from (*b*) were described as percentage of living cells (survival %) as compared to the mock transfection (100%, control). The data represent the mean ± SD of triplicate wells.

surviving cells (Fig. 3), but also triggered a similar increase in the percentage of apoptotic cells in SCC-9 and SCC-4 cells under both normoxic and hypoxic conditions (Figs. 4–6). Such biological effects of HIF-1α depletion on apoptotic induction was further confirmed by treatment of SCC-9 cells with an siRNA that specifically target HIF-1α mRNA for degradation (Fig. 7). On the contrary, treatment with AS-HIF-1α-ODNs or siRNA$_{HIF\text{-}1\alpha}$ had no obvious effects on cellular viability or apoptotic induction in HepG2 cells with an undetectable level of constitutive HIF-1α (Fig. 8). These results suggest that the apoptotic induction of human tongue squamous cell carcinomas treated with AS-HIF-1α-ODNs or siRNA$_{HIF\text{-}1\alpha}$ was a specific outcome of HIF-1α loss-of-function; furthermore, cellular apoptosis induced by the depletion of HIF-1α expression is cell-context dependent and is based primarily on the constitutive basal level of HIF-1α present in the cell.

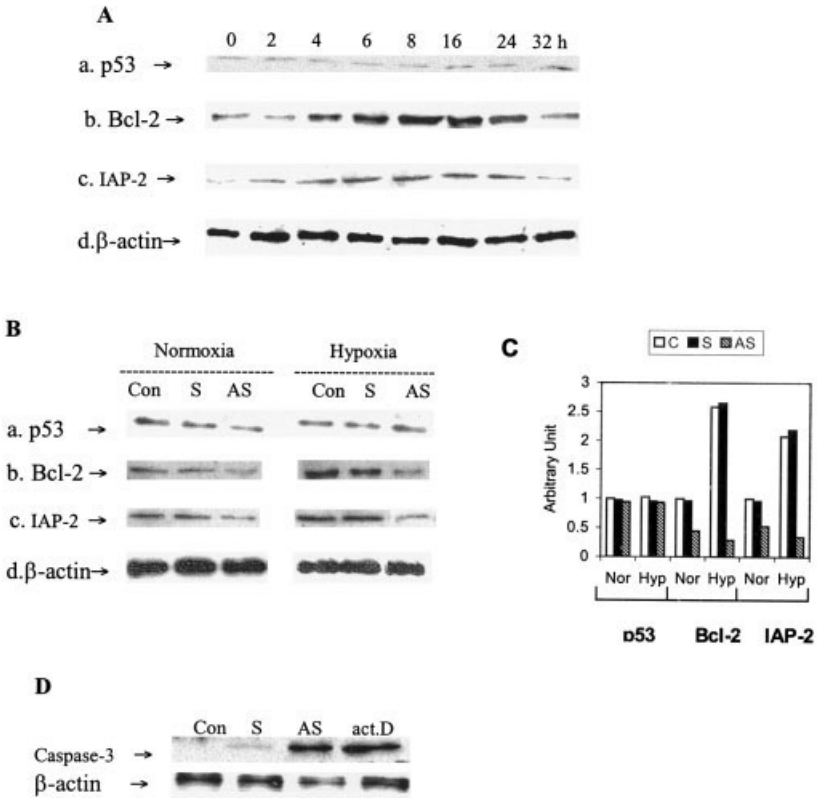

FIGURE 9 – Effects of hypoxia and AS-HIF-1α-ODN treatment on apoptosis-related genes expression and caspase-3 activity. (*a*) Semi-confluent SCC-9 cells were exposed to hypoxia for different time intervals and whole cell lysates were analyzed for p53, Bcl-2, and IAP-2 expression using Western blot. (*b*) SCC-9 cells were transfected with sense (S)- or AS-HIF-1α ODNs (AS) and cultured under normoxic or hypoxic conditions for 24 hr. Whole cell lysates were extracted for Western blot analyses. (*c*) Densitometric analysis of (*b*), with the normoxic control arbitrarily set as 1.0. (*d*) Western analysis of active caspase-3 expression in AS-HIF-1α-ODN treated SCC-9 cells under normoxic conditions using actinomycin D as positive control (act.D). Data are representative of 3 independent experiments.

Up to date, the exact mechanisms underlying the role of HIF-1α in the regulation of apoptosis remain poorly understood. Previous studies have shown that under severe or prolonged hypoxic condition, the stabilization of p53 protein is HIF-1α dependent,[29,56] which in turn leads to the induction of apoptosis.[29] Recently, Suzuki *et al*.[57] demonstrated that phosphorylation of HIF-1α is the key determinant in cell survival and death under hypoxia. The dephosphorylated form of HIF-1α promotes apoptosis by binding to and stabilizing p53. Phosphorylated HIF-1α promotes cell survival by forming an active complex with ARNT, and transactivates various target genes to allow cells to adapt to the stressed microenvironment.[57] We demonstrated that hypoxia had no effect on p53 protein levels in SCC-9 cells and depletion of HIF-1α expression did not affect p53 expression (Fig. 9), suggesting that in SCC-9 cells the apoptotic process induced by AS-HIF-1α-ODN treatment is p53-independent. This finding concurs with recent reports that increased apoptosis in HIF-1α deficient glioblastoma cells, mouse embryonic fibroblasts, and human fibrosarcoma cells did not involve p53 induction.[40,42]

Recently, both the Bcl-2 and IAP family members, as well as caspase-3 have been implicated in the regulation of hypoxia/HIF-1α mediated anti-apoptotic and cell survival processes.[38,40] Park *et al*.[38] reported that hypoxia induced upregulation of anti-apoptotic proteins such as Bcl-2, Bcl-X_L, and IAP family members, contributing to the hypoxia-mediated protective role in TRAIL-induced apoptosis in human lung carcinoma A549 cells.[38] In other cell systems, however, the hypoxia-induced up-regulation of IAP-2 expression has been shown to be independent of HIF-1.[58] Additionally, the enhanced apoptosis in human glioblastoma cells triggered by antisense knockdown of HIF-1α has been shown to be correlated with an increase in caspase-3 activity.[40] Consistent with above findings, our present study demonstrated that exposure of SCC-9 cells to hypoxia led to the up-regulation of both Bcl-2 and IAP-2 expression (Fig. 9*a*), and treatment with AS-HIF-1α-ODNs not only attenuated Bcl-2 and IAP-2 expression but also greatly enhanced the active caspase-3 levels under both normoxic and hypoxic conditions (Fig. 9*b–d*). These results suggest that Bcl-2, IAP-2 and caspase-3 may possibly be involved in the regulation of apoptosis induced by treatment with AS-HIF-1α-ODNs in human tongue squamous cell carcinomas.

Accumulating evidences have indicated that HIF-1α links hypoxia, angiogenesis and malignant phenotypes of lethal cancers, thus serving a promising target for future cancer molecular therapy.[59] Sun *et al.*[50] reported that knockdown of HIF-1α by using antisense HIF-1α retroviral vector reduces tumor vessel density and enhances cancer immunotherapy. Kung *et al*.[61] demonstrated that the specific blockade of the interaction of HIF-1α with its binding protein transcriptional co-activators, p300 and CREB, leads to the attenuation of hypoxia-inducible gene expression and diminution of tumor growth. Most recently, Krishnamachary *et al*.[45] successfully adopted a small interfering RNA directed against HIF-1α ($siRNA_{HIF\text{-}1\alpha}$) and inhibited hypoxia or HIF-1α overexpression-stimulated matrigel invasion by HCT116 human colon carcinoma cells. These results have provided further supporting evidences that interfering with HIF-1α pathways may serve as promising targets for suppressing tumor resistance and achieving more efficient clinical outcomes in head and neck cancer therapies.

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