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Hypoxia-inducible factor-1α mediates oral squamous cell carcinoma invasion via upregulation of α5 integrin and fibronectin

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ABSTRACT

With progressive and rapid growth of malignant tumors, cancer cells in an ischemic condition are expected to develop an increased potential for local invasive growth. To address this hypothesis, we first examined the effect of hypoxia on the invasiveness of oral squamous cell carcinoma (OSCC) cells using the Matrigel invasion assay. We then investigated the effect of hypoxia on the protein and mRNA expression of α5 integrin and fibronectin, which are major factors involved in tumor cell invasion. We showed that (i) hypoxia increased the invasiveness of OSCC cells, (ii) α5 integrin and fibronectin protein and mRNA expression levels were increased in OSCC cells under hypoxic conditions, (iii) hypoxia stimulated autocrine secretion of fibronectin in OSCC cells, (iv) administration of $siRNA_{HIF\text{-}1\alpha}$ caused a significant decrease in α5 integrin and fibronectin protein, confirming that HIF-1α plays a role in their induction, and (v) $siRNA_{HIF\text{-}1\alpha}$ abrogated hypoxia-induced cell invasion. Collectively, these data suggest that hypoxia promotes OSCC cell invasion that is elicited by HIF-1α-dependent α5 integrin and fibronectin induction.

Introduction

The inner regions of cancerous tumors become hypoxic as they grow and rapidly expand. Hypoxia is a state where oxygen tension drops below normal limits and is a common feature of malignant tumors [1]. A key regulatory protein involved in the cellular adaptive response to hypoxia is hypoxia-inducible factor-1 (HIF-1), which is a heterodimer consisting of HIF-1α and HIF-1β [2]. HIF-1β is constitutively expressed, but HIF-1α has a short half life under normoxic conditions and is rapidly degraded. However, under hypoxic conditions, HIF-1α is stabilized and induces the transcription of a number of downstream target genes involved in physiologic and pathologic processes [3,4]. Previous studies have shown that hypoxia causes HIF-1α accumulation in several human malignancies, including cancers of the head and neck, lung, and uterine cervix [5–7].

Invasion and metastasis are the major characteristics of malignant tumors, and the primary cause of cancer death. For cancer cells to become invasive they must reorganize their molecular profiles at the cell surface. This disrupts contact with the basement membrane and allows recognition of previously unencountered molecules in the extracellular matrix (ECM) of the stroma. Cancer cells then attach to surrounding ECM proteins by interacting with their receptors. Integrins are the most important adhesion receptors that mediate cell attachment to ECM [8]. A correlation between integrin receptors and invasiveness has been observed in a variety of cancers [9–11]. For example, integrin αVβ3 is expressed in invasive melanoma but is absent in benign nevi or normal melanocytes [9].

Hypoxia-induced HIF-1α accumulation may play an important role in cancer cell invasion by affecting the expression of integrin receptors. Data have shown that intratumoral hypoxia increases the potential for cancer cells to invade [12,13]. This indicates that the hypoxic tumor microenvironment may act as a physiological stimulus to activate the expression of genes controlling invasion. However, the exact role of HIF-1α in cellular invasion remains controversial, and its mechanism of action has not also been clearly defined. We have examined the invasiveness of OSCC cells under hypoxic conditions and the role of HIF-1α in molecular and functional changes related to invasiveness of OSCC cells.

Materials and methods

Cell culture and treatment. YD10B OSCC cells were gifted from the Department of Oral Pathology, College of Dentistry, Yonsei University (Seoul, Korea). They were maintained in Dulbecco's

Abbreviations: HIF-1α, hypoxia-inducible factor-1α; siRNA, small interfering RNA; OSCC, oral squamous cell carcinoma.

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modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (GIBCO-BRL, Rockville, MD). Cells were cultured at 37 °C in a humidified 5% CO_2–95% air incubator. For hypoxic treatment, cells were incubated in a multi-gas incubator (MCO-5M, Sanyo, Japan) that was equilibrated at 1% O_2, 5% CO_2, and balanced N_2.

Western blot analysis. Cells were harvested, pelleted by centrifugation, washed with ice-cold phosphate-buffered saline (PBS), and lysed on ice for 20 min with RIPA buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, leupeptin (5 mg/ml), and aprotinin (5 mg/ml)]. The cell lysates were centrifuged and supernatants were collected. The cell lysates were separated on 10% SDS–polyacrylamide gels and transferred electrophoretically onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA) using a wet transfer kit. The membranes were blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline (TBS, pH 7.6) containing 0.1% Tween (TBST). Membranes were then incubated overnight at 4 °C with antibodies against HIF-1α, α5 integrin (BD Biosciences, Lexington, KY), fibronectin (Cell Signaling, Beverly, MA), or actin (Santa Cruz Biotechnology, Santa Cruz, CA). Following three washes in TBST, the blots were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The antibody–antigen complexes were visualized with 2 ml of SuperSignal West-femto reagent (Pierce, Rockford, IL).

In vitro invasion assay. Cell invasiveness was determined using the Matrigel invasion system. Transwell polycarbonate filters (8-μm pore size, Costar, Cambridge, MA) were coated with 80 μl of Matrigel (BD Biosciences, Bedford, MA) at 1:3 dilutions in serum-free medium. They were allowed to gel at 37 °C for 1 h and then placed in 24-well tissue culture plates. OSCC cells (4×10^4) were seeded into the Transwells in 200 μl of medium containing 1% FBS. Complete medium (600 μl) containing 10% FBS was added to the lower chamber, and the plate was incubated at 37 °C in a 5% CO_2/95% air incubator (20% O_2) for 24 h. For hypoxic treatment, Transwell-containing plates were placed in a 1% O_2, 5% CO_2 and 94% N_2 chamber and incubated at 37 °C for 24 h. The ECM gel and the cells on the upper surface of the filter were removed with a cotton swab, and the filters were detached from the Transwell using a scalpel. The filters were stained with hematoxylin and eosin, mounted on glass slides, and the cells were counted.

Reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was reverse transcribed using the OneStep RT-PCR Kit (Qiagen), and cDNA was amplified in a final volume of 50 μl. The primer sequences were as follows: α5 integrin forward primer 5′-CCCA GACTTCTTTGGCTCTG-3′, α5 integrin reverse primer 5′-GCAAGATC TGAGCCTTGTCC-3′, fibronectin forward primer 5′-CGAACAACATGA GAGCACACC-3′, and fibronectin reverse primer 5′-TCACTGTGA CAGCAGGAGCATC-3′, actin forward primer 5′-TTAGCTGTGCTCGCG CTACTCTCTC-3′, and actin reverse primer 5′-TCGGATTGATGAAA CCCAGACACA-3′. After the initial denaturation at 95 °C for 15 min, PCR amplification was performed by denaturation for 1 min at 94 °C, annealing for 30 s at 58 °C (α5 integrin), 63 °C (fibronectin), or 60 °C (actin), primer extension for 1 min at 72 °C, and final extension for 10 min at 72 °C. Complementary DNAs for α5 integrin, fibronectin and actin were amplified for 28 cycles.

Preparation of conditioned medium. Cells were seeded into 6-cm tissue culture plates and maintained in DMEM containing 10% FBS for 16 h. The plates were then placed in a normoxic or hypoxic chamber for an additional 48 h. The medium was collected and used for trichloroacetic acid precipitation (TCA), an enzyme-linked immunosorbent assay (ELISA) assay, or invasion assays.

Trichloroacetic acid (TCA) precipitation. Proteins in the conditioned medium were precipitated with 10% TCA (final concentration) and centrifuged at 10,000*g* for 10 min. Precipitated pellets were solubilized in 200 μl of 0.2 N NaOH and analyzed for fibronectin by Western blotting.

siRNA-mediated protein knockdown. OSCC cells were seeded in antibiotic-free DMEM containing 10% FBS at a density of 4×10^4 cells per well in 24-well plates. The next day, cells were transfected with 80 nM of siRNA directed against HIF-1α or a non-targeting siRNA (ON-TARGET plus Non-targeting siRNA) using DharmaFECT 4 (Dharmacon Inc., Chicago, IL). Following 6 h incubation, the medium was changed, and the cells were incubated for an additional 24 h. The plates were then placed in the 1% hypoxic chamber and incubated for an additional 24 h. The cells were then harvested for use in further experiments.

Enzyme-linked immunosorbent assay (ELISA). Fibronectin in the conditioned medium was assayed using a human fibronectin EIA kit (Takara, Japan) according to the manufacturer's protocols.

Results

Hypoxia induces HIF-1α accumulation and enhances the invasiveness of OSCC cells

When OSCC cells were cultured under hypoxic conditions, HIF-1α protein levels were markedly increased at 3 and 6 h (Fig. 1A). To determine whether hypoxia affects OSCC cell invasion, cells were incubated in ECM gel-coated Transwell chambers maintained in

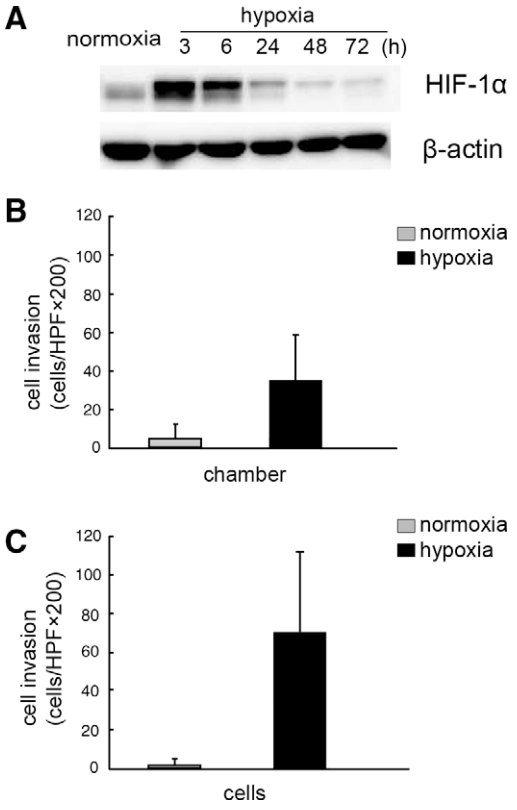

Fig. 1. Hypoxia increases YD10B oral squamous cell carcinoma (OSCC) cell invasion. (A) Cells were cultured in 20% O_2 (normoxia) or 1% O_2 (hypoxia). Cell extracts were subjected to SDS–PAGE and immunoblot analysis to detect HIF-1α accumulation. (B) Cells were incubated in extracellular matrix (ECM) gel-coated Transwell chambers maintained in 20% O_2 (normoxia) or 1% O_2 (hypoxia). After 24 h, the numbers of invaded cells were counted. (C) Cells were grown under normoxia or hypoxia chambers for 48 h. Then cells were seeded in ECM gel-coated Tranwell chambers, and invasion assays were performed under normoxic conditions.

20% O_2 (normoxia) or 1% O_2 (hypoxia). As shown in Fig. 1B, OSCC cell invasion was significantly facilitated under hypoxic as compared to normoxic conditions. We next wanted to determine whether hypoxia-induced invasiveness was a consequence of cellular changes in OSCC cells or not. For this, an invasion assay was performed under normoxic conditions, using cells that had been previously grown under hypoxic conditions for 48 h. OSCC cells pre-exposed to hypoxic conditions were more invasive than cells that were simply grown in a normoxic condition (Fig. 1C).

Hypoxia increases α5 integrin and fibronectin expression in OSCC cells

We showed that hypoxia increased HIF-1α expression levels and OSCC cell invasion. We thus hypothesized that HIF-1α accumulation promotes the expression of genes that control tumor cell invasion. To define a molecular basis for the observed effects of hypoxia and HIF-1α accumulation on tumor cell invasion, we sought to identify HIF-1α target genes that encode proteins with established roles in the process of invasion. We found that hypoxia significantly induced α5 integrin expression in a time-dependent manner (Fig. 2A). Since α5 integrin is the receptor for fibronectin, we next examined fibronectin protein expression under normoxic or hypoxic conditions. Similar to α5 integrin, the expression of fibronectin was also significantly induced by hypoxia, but not normoxia, in OSCC cells (Fig. 2B). To determine whether hypoxia-induced α5 integrin and fibronectin expression was regulated at the level of transcription, the mRNA levels of α5 integrin and fibronectin were examined using semi-quantitative RT-PCR. α5 Integrin and fibronectin mRNA induction occurred following 6 h of hypoxia exposure, and maximal levels were observed after 24 h (Fig. 2C). These findings may indicate that hypoxia-induced OSCC invasion is mediated by an increased production of fibronectin and its α5 integrin receptor.

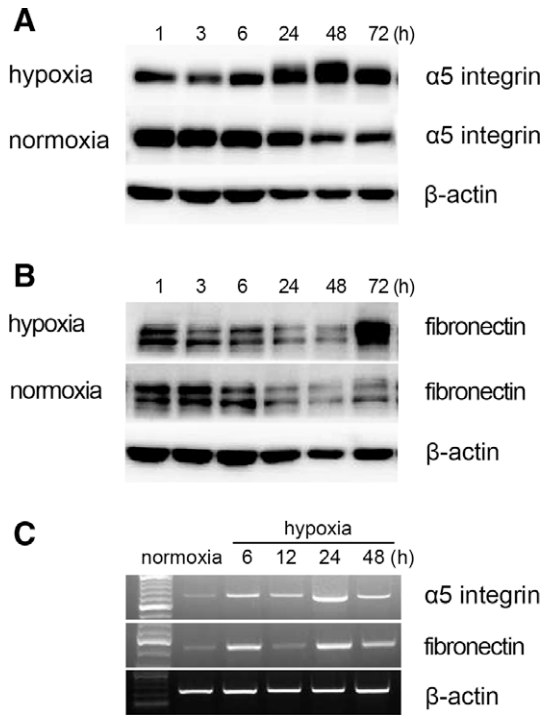

Fig. 2. Hypoxia induces the expression of α5 integrin and fibronectin in OSCC cells. Cells were incubated in normoxic or hypoxic chambers for the times indicated. The cells were harvested for Western blot (A, B) or reverse transcription-polymerase chain reaction (RT-PCR) analysis (C).

Hypoxic OSCC cells stimulate autocrine secretion of fibronectin

We next wanted to verify that invasive OSCC cells under hypoxia synthesize new ECM, in particular fibronectin, to enhance cell migration. For this, conditioned media from cells was exposed to hypoxia for 48 h, collected, and the proteins in the media were precipitated. Using Western blot analysis, we showed that conditioned media from hypoxic cells contained higher levels of fibronectin protein as compared to conditioned media from normoxic cells (Fig. 3A).

To establish the role of secreted molecules in OSCC cell invasion, hypoxia-conditioned media was placed in the lower chamber of the Transwell chamber instead of adding new fresh media. Although the experiment was performed under normoxia, hypoxia-conditioned medium increased OSCC cell invasiveness as compared to normoxia-conditioned medium. This suggests that protein(s), such as fibronectin, in hypoxia-conditioned medium may function as chemotactic factors that mediate tumor cell invasion (Fig. 3B).

Inhibition of HIF-1α decreased α5 integrin and fibronectin expression and reduced OSCC cell invasion

A siRNA knockdown approach was used to verify the role of HIF-1α in the increased expression of α5 integrin and fibronectin, and subsequent elevated invasion in hypoxic condition. HIF-1α knockdown was confirmed by a reduction in mRNA and protein levels of HIF-1α (Fig. 4A and B). HIF-1α degradation prevented the upregulation of α5 integrin and fibronectin protein expression in response to hypoxia. Transfection of siRNA targeted towards an irrelevant mRNA ($siRNA_{cont}$) had no significant effect on the expression of HIF-1α, α5 integrin and fibronectin. In addition, HIF-1α knockdown significantly decreased the amount of fibronectin released into the culture medium of cells exposed to hypoxia (Fig. 4C).

We next examined cell invasion using the HIF-1α-knockdowned cells. Under hypoxic conditions, the HIF-1α-silenced OSCC cells lost the hypoxia-induced ability to invade Matrigel. Transfection of $siRNA_{cont}$ had no inhibitory effect on cellular invasion, implying that the reduced invasiveness of $siRNA_{HIF\text{-}1\alpha}$-transfected cells was a result of HIF-1α loss-of-function. To explore the interaction between α5 integrin and fibronectin in the invasion

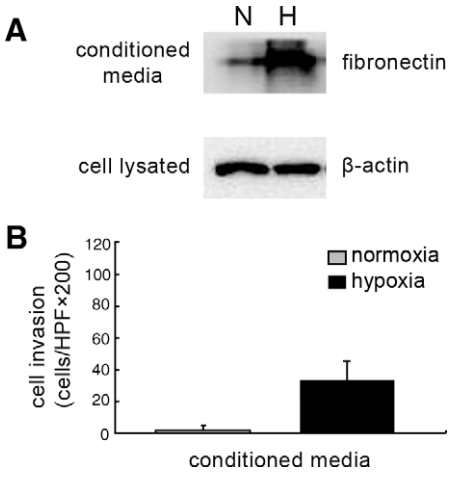

Fig. 3. Exposure to hypoxic conditions increases fibronectin secretion into culture medium and promotes cellular invasion. Conditioned media under normoxic or hypoxic conditions was collected at 48 h. (A) Proteins in the culture media were precipitated by 10% trichloroacetic acid and analyzed by Western blotting. (B) Conditioned media was used in invasion assays performed in normoxic conditions.

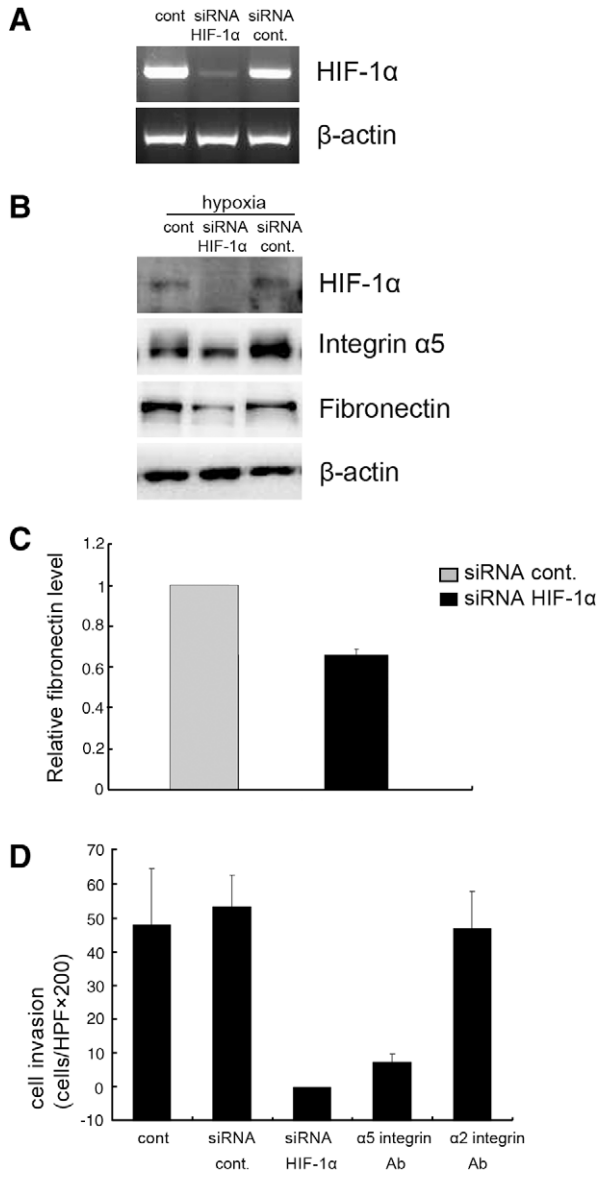

Fig. 4. The effect of HIF-1α siRNA on α5 integrin and fibronectin expression and OSCC cell invasion. Cells were transfected with non-targeting siRNA ($siRNA_{cont}$) or siRNA directed against HIF-1α ($siRNA_{HIF\text{-}1\alpha}$) using DharmaFECT reagent for 24 h. (A) After additional 24 h, HIF-1α knockdown was confirmed by RT-PCR. (B) Transfected cells were incubated in a 37 °C, 1% O_2 incubator for 24 h. Cell lysates were subjected to immunoblot analysis using antibodies against HIF-1α, α5 integrin, and fibronectin. (C) Transfected cells were incubated in a 37 °C, 1% O_2 incubator for 24 h. Fibronectin in conditioned media was analyzed using an enzyme-linked immunosorbent assay. (D) Mock-transfected or siRNA-transfected cells were seeded onto Matrigel-coated filters of Transwells and incubated for 24 h in 1% O_2 with or without 20 μg/ml blocking antibodies. Cells on the underside of the filters were counted.

process, we used a neutralizing antibody against α5 integrin (20 μg/ml; BD Biosciences), which blocks its association with fibronectin. OSCC cell invasion, which was potently increased under hypoxic conditions, was significantly decreased by the anti-α5 integrin antibody whereas anti-α2 antibody (20 μg/ml; BD Biosciences) had no effect on the inhibition of OSCC cell invasion under hypoxic conditions (Fig. 4D). These findings suggest that, under hypoxic conditions, increased OSCC cell invasion is significantly dependent on the interaction between HIF-1 α-induced α5 integrin and fibronectin.

Discussion

Clinical studies have revealed that HIF-1α is overexpressed in the invasive portions of human brain and colon tumor biopsies. This suggests that intratumoral hypoxia-induced HIF-1α accumulation may promote cancer cell invasion [6,14]. This model is supported by studies demonstrating that cancer cells transiently subjected to hypoxia show increased rates of invasion through the basement membrane [13,15]. In addition, a recent report has shown that overexpression of HIF-1α increases colon carcinoma cell invasion in vitro [16]. Consistent with these results, our data showed that hypoxia-exposed HIF-1α-expressing OSCC cells are more invasive as compared to cells in normoxic conditions. Furthermore our study suggests that hypoxia promotes cancer cell invasion by inducing the expression of mesenchymal cell markers such as fibronectin. Recently, several reports have suggested that epithelial–mesenchymal transition (EMT), which is characterized by a decreased expression of epithelial markers (E-cadherin, cytokeratin, etc.) and/or an increased expression of mesenchymal markers (vimentin, fibronectin, etc.), significantly favors cancer cell invasion [15]. Our study has not determined whether hypoxia promotes EMT in OSCC cells, but it is clear that hypoxia-dependent changes allow OSCC cells to acquire features of mesenchymal-like cells and invasiveness.

Cancer cells degrade the ECM and invade the surrounding stroma by producing proteases. The degraded ECM is replaced by fibronectin and other ECM proteins. During this process, cancer cells must form transient cell-ECM attachments at their invasive front. Thus, alterations of adhesive property in cancer cells is an important factor in tumor invasion, and changes in integrin expression frequently occur in tumor cells that transform into invasive and metastatic phenotypes. Semenza et al. suggested that HIF-1α regulates genes that encode such invasion-related factors [17]. The present study not only suggests that the stimulatory effects of hypoxia and HIF-1α accumulation modulate α5 integrin and fibronectin expression, but that the enhancement of new cell-ECM attachments by fibronectin-rich ECM deposition confers a selective advantage for tumor cell invasion. Collectively, these are important driving forces in hypoxia-induced cells invasion. Cannito et al. show that conditioned culture media under hypoxia induces a significant increase in the invasiveness of various cancer cell lines [15]. This increased invasiveness was dependent on autocrine factors released by the cancer cells. Consistent with their data, we show that conditioned culture media from OSCC cells under hypoxic conditions significantly increased invasion of cells in normoxic status. This implies that OSCC cells under hypoxic conditions also release autocrine factors, and the released autocrine factors can help invasion of adjacent normoxic cells in vivo. We show that hypoxia potentiated the release of fibronectin from OSCC cells, suggesting that released fibronectin by hypoxic stimuli plays a major role in invasion of not only hypoxic cells but also normoxic tumor cells by accelerating new cell-fibronectin attachment.

Numerous studies have shown that hypoxia and/or HIF-1α induces transcription and protein expression of α5 integrin in a variety of human cancer cell lines in vitro [18–21]. The relationship between α5 integrin and hypoxia-induced cancer cell invasiveness was not investigated in those studies. Most studies have investigated the role of urokinase-type plasminogen activator receptor (uPAR) in hypoxia-induced invasiveness. These reports demonstrated that uPAR is a hypoxia-inducible gene important for tumor invasion under hypoxic conditions [13]. A few studies have shown that hypoxia-induced upregulation of α5 integrin promotes invasive cell behavior [22–24]. However, these data are specific to trophoblast invasion during the implantation process. We show that siRNA against HIF-1α blocked induction of α5 integrin and

that neutralizing the function of α5 integrin inhibited hypoxia-induced invasion of OSCC cells. This provides a direct link between hypoxia, HIF-1α-regulatory α5 integrin gene expression and OSCC cell invasion. Our findings suggest that hypoxia-induced invasion is accomplished by an upregulation of α5 integrin. In addition, the ligand (fibronectin)/α5 integrin interaction would be further enhanced by HIF-1α-dependent induction of fibronectin. Thus, HIF-1α-dependent increased expression of these proteins might synergistically enhance cell-ECM interaction in response to hypoxia, thereby critically involving in tumor invasion induced by hypoxia.

In summary, we have shown that hypoxia increases the protein expression of α5 integrin and fibronectin. This occurs at the level of transcription in a HIF-1α-dependent manner. Furthermore, increased interaction between α5 integrin and fibronectin confers a selective advantage for OSCC cell invasion. Therefore, targeting hypoxia or HIF-1α may reduce OSCC cell invasion and metastasis.

Acknowledgments

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