SPANDIDOS PUBLICATIONS

Zebularine-induced reduction in VEGF secretion by HIF-1α degradation in oral squamous cell carcinoma

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Abstract. Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis in oral squamous cell carcinoma (OSCC). In this study, we used the novel DNA methyltransferase inhibitor zebularine (Zeb) to investigate epigenetic influences on the secretion of VEGF-A in the OSCC cell line HSC-3. Under normoxic conditions, we found that Zeb inhibited secretion in a dose-dependent manner by reducing the activity of hypoxia-inducible factor-1α (HIF-1α). Treatment of the cells with the proteasome inhibitor MG132 protected the HIF-1α protein from Zeb-mediated epigenetic regulation. In addition, our study revealed that neither the PI3K/Akt nor the p53 signaling pathway is required for Zeb-induced HIF-1α degradation. In short, Zeb influenced the stability of the HIF-1α protein and the activity of its targets, such as VEGF, in HSC-3 cells in normoxic conditions. This study has laid the foundation for a novel anti-cancer approach, which may find applications in molecular staging.

Introduction

Oral squamous cell carcinoma (OSCC) is the most aggressive malignant neoplasm of the head and neck region and remains morbid and often fatal. The most important prognostic indicator in cases of OSCC is metastasis to the cervical lymph nodes or distant organs (1). It is widely recognized that the development of OSCC, as of other cancers, involves a multistep process characterized by the progressive accumulation of genomic aberrations, including deletions and mutations in the genomic sequence, or by epigenetic mechanisms (2). In particular, epigenetic abnormalities in OSCC affect a number of genes involved in crucial cellular pathways, such as the cell cycle, apoptosis, angiogenesis and cancer cell invasion and metastasis (3). Recent work has demonstrated that epigenetic transcriptional silencing by promoter methylation of tumor suppressor genes in OSCC is a relatively common and important feature of oral carcinogenesis (4). Epigenetic changes in cancer cells are the consequence of a combination of alterations in chromatin structure, including the hypermethylation of CpG islands and the modification of histones (5). Epigenetic modifications, such as the hypomethylation of cellular DNA by DNA methyltransferase (DNMT) inhibitors or the hyperacetylation of DNA-associated histone proteins by histone deacetylase (HDAC) inhibitors, are novel therapeutic strategies, particularly for OSCC, which exhibits an inherent resistance to chemotherapeutic agents (6). While several well-tolerated HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), have already become available, the application *in vivo* of DNMT inhibitors, such as 5-aza-cytidine (5-aza-CR) and 5-aza-2'-deoxycytidine (5-aza-CdR), has been limited due to low stability in neutral solutions and high toxicity (7). Recently, zebularine (Zeb), a newer cytidine analog, was established as a novel and orally available inhibitor of DNMT with a preferential response in cancer cells. It has been shown to be more stable and less toxic than other DNMT inhibitors, leading to the re-expression of epigenetically silenced genes (8,9). For this reason, Zeb was evaluated in this study as an epigenetic anti-cancer agent against OSCC.

Established chemotherapy regimens for OSCC include a combination of 5-fluorouracil with platinum-containing drugs such as cisplatin and carboplatin. However, treatment of OSCC with these drugs does not always substantially induce a positive response. Despite recent advances in the management of cancer, most patients with advanced OSCC develop local or regional recurrence and metastatic disease leading to a poor survival rate (1). Angiogenesis is required at almost every step of tumor progression and metastasis. Thus, the inhibition of angiogenesis is considered to be one of the most promising strategies in the development of novel anti-neoplastic therapies. Angiogenesis is defined as the *de novo* formation of blood vessels from a pre-existing vasculature by sprouting and maturation. These processes are tightly controlled by pro-angiogenic and anti-angiogenic factors and their receptors, which regulate one or more of the key events involved (10). The appearance of new blood vessels in malignant tumors is known as the angiogenic switch (11). Because genetic aberrations of tumor suppressor genes and oncogenes influence angiogenesis, the angiogenic switch occurs when tumor cells acquire genetic

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changes that make them switch to the angiogenic phenotype. As a result, tumor cells produce pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, and shift to a positive balance in favor of angiogenesis.

The VEGF family consists of at least seven members (VEGF-A, -B, -C and -D, viral VEGF, snake venom VEGF and placental growth factor), Of these, VEGF-A is known to play an important role in tumor angiogenesis (12). Tumor cells are able to secrete VEGF in an autocrine or paracrine fashion to stimulate the proliferation and attraction of endothelial cells (13). This secretion is generally regulated by local oxygen concentration. VEGF stimulates endothelial cells to secrete proteases and plasminogen activators, resulting in the degradation of the vessel basement membrane, in turn allowing cells to invade the surrounding matrix. After subsequent migration and proliferation, cells finally differentiate to form a new vessel.

Elevated expression of VEGF has been observed in many human cancers, including colorectal, breast, non-small cell lung and ovarian cancers (14). In particular, all VEGF members were expressed at different levels in OSCC, suggesting a possible relationship between VEGF levels and the development of cancer angiogenesis (15). Anti-angiogenic factors have attracted special attention as potential new anti-cancer agents possessing different modes of action than the drugs currently used. It has been demonstrated that the tumor suppressor gene p53 negatively regulates VEGF expression, although its mutation is common in OSCC. In addition, the $p16^{INK4a}$ gene, whose promoter is targeted by the predominant methyltransferase gene DNMT1, is a negative regulator of VEGF gene expression (16). In contrast, VEGF secretion is positively affected in cancer cells by hypoxia-inducible factor-1α (HIF-1α), a key transcriptional regulator regulated by the PI3K (phosphatidylinotidol 3-kinase)/Akt (protein kinase B) pathway (17).

Taken together, both activated oncogenes and inactivated tumor suppressor genes contribute to the up-regulation of VEGF expression in cancer cells, where epigenetic abnormalities contribute in several ways to oncogenesis and may activate oncogenes or silence tumor suppressor genes. If the silencing of certain genes by DNA hypermethylation is involved in VEGF activation, thus playing a role in angiogenesis, then the demethylation of anti-angiogenic genes by epigenetic inhibitors should result in the down-regulation of VEGF transcription and angiogenic potential.

In contrast to the increasing knowledge of epigenetic aberrations in cancer cells, almost nothing is known of the role of DNA methylation and histone modifications in the regulation of gene expression for angiogenesis. Because the regulation of VEGF expression in OSCC cells is pivotal to angiogenic properties, we investigated whether epigenetic mechanisms are involved in the regulation of the secretion of VEGF-A in the OSCC cell line HSC-3. For the epigenetic approach, we used pharmacological means to restore DNA methylation, and found that the secretion of VEGF and the activation of its transcriptional regulator HIF-1α in HSC-3 cells is inhibited by a demethylating agent (Zeb). Our results show that VEGF is epigenetically regulated in an indirect manner through modifications to the promoter of upstream genes.

Materials and methods

Reagents. Zeb was obtained from Sigma (St. Louis, MO). SAHA was obtained from Alexis Biochemicals (San Diego, CA). The proteasome inhibitor MG132 and PI3K inhibitor LY294002 were purchased from Calbiochem (La Jolla, CA). All other chemicals used were commercially available.

Cell culture. HSC-3 cells (human OSCC), HSC-4 cells (human OSCC) and A549 cells (human non-small cell lung adenocarcinoma) were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Japan). These carcinoma cell lines were cultured in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10 or 1% (v/v) heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). All cell lines were cultured at 37˚C in a humidified atmosphere of 5% CO_2.

Cell viability assays. Cell viability was evaluated by the trypan blue exclusion assay. The cytotoxic effects of Zeb were also determined using a cell counting kit (Dojindo Lab, Kumamoto, Japan). Cells were seeded in 96-well plates ($1x10^4$ cells/well) for 24 h and treated with Zeb for 48 h. Cell viability was determined using the MTT (WST8) assay. Absorbance (450 nm) was directly proportional to the number of living cells in culture.

Analysis of DNMT activity. To quantify the DNMT activity of cells, we used a DNA methyltransferase activity assay kit (Epigentek Inc., Brooklyn, NY). Briefly, cells were incubated for 48 h with or without Zeb (220 μM; IC_{50}), then nuclear protein was extracted using a nuclear extraction kit (Active motif, Carlsbad, CA). Nuclear extracts from untreated and Zeb-treated cells were incubated in wells coated with a cytosine-rich DNA substrate. DNMT transfers a methyl group from S-adenosylmethionine to the 5' position of cytosine bases to methylate the DNA substrate. The methylated DNA can be recognized with anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzymatic activity, can be colorimetrically quantified through an ELISA reaction.

Global DNA methylation. To quantify the global DNA methylation of cells, a DNA methylation quantification kit (Epigentek Inc.) was used. In this assay, DNA was immobilized on a strip with high affinity for the DNA. The methylated fraction of DNA was recognized by 5-methylcytosine antibody and quantified through an ELISA reaction. Results are expressed as the absolute percentage of 5-methylcytosine.

Measurement of VEGF-A and VEGF-C secretion. ELISA kits for VEGF-A165 and -C (R&D System, Minneapolis, MN) were used. Cells ($2x10^4$ cells/well) were seeded in 12-well flat-bottomed culture plates and incubated in medium with 10% FBS for 24 h, then treated with Zeb (120 μM) for 48 h. The cells were washed and incubated in medium with 1% FBS for 2-4 days. The amounts of VEGF secreted were determined by ELISA according to the manufacturer's

ns. In brief, cultured supernatants were incubated ered protein base for 2 h at room temperature in a 96-well plate coated with a mouse monoclonal antibody against VEGF-A or -C. After three washes, anti-VEGF-A or -C polyclonal antibody conjugated to horseradish peroxidase (HRP) was added and incubated for 2 h at room temperature. Absorbance was measured at 450 nm in a microplate reader. For standardization, serial dilutions of recombinant human VEGF-A or -C were assayed. Results were normalized to cell counts at the end of treatment ($1x10^5$).

Measurement of Akt phosphorylation. The amount of phosphorylated Akt relative to the total amount of Akt in HSC-3 cells was measured using an ELISA kit (Active Motif). Cells were seeded in 96-well plates ($2x10^4$ cells/well). After stimulation, the cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature and washed three times with PBS containing 0.1% Triton X-100. Endogenous peroxidase was quenched with 1% H_2O_2 and 0.1% azide, blocked with antibody-blocking buffer for 1 h and incubated overnight with various dilutions of primary antibody at 4˚C. After being washed, cells were incubated with secondary antibody for 1 h at room temperature and washed again. Cells were incubated with developing solution for 2-20 min at room temperature in the dark, and then stop solution was added. Absorbance was measured at OD_{450}.

HIF-1α assay. To analyze HIF-1α/hypoxia response element (HRE) interaction, an ELISA-based assay was performed according to the manufacturer's instructions (Active Motif). Nuclear extracts (5 μg) were incubated in 96-well plates precoated with the HRE consensus oligonucleotides. HIF-1α antibody was added to the reactions and an HRP-conjugated secondary antibody was used to quantify the HRE-binding activity of the nuclear extracts. Results are expressed as the absorbance at 450 nm.

Statistical analysis. Data are presented as the means ± SD. Multiple comparisons were performed by Scheffe's test. P-values <0.05 were regarded as significant.

Results

Secretion of VEGF-A under serum-starved conditions. To assess whether serum or serum-derived growth factors affect the secretion of VEGF, HSC-3 cells (OSCC, mutant p53) were incubated for 4 days in medium supplemented with 1% FBS (serum-starved conditions). The cultured supernatants were then collected from the wells and the VEGF in the medium was measured using an ELISA for human VEGF-A. The baseline VEGF level was relatively high even under non-starved conditions (10% FBS). Under serum-starved conditions, there was a significant increase in the amount of VEGF secreted into culture supernatants (Fig. 1A). We next assessed whether VEGF was secreted under serum-starved conditions in a time-dependent manner. The cultured medium from HSC-3 cells was incubated for different periods and was assayed for secreted VEGF. VEGF levels increased progressively, with production detectable as early as day 1 and reaching maximum secretion at day 4 (Fig. 1B). It was concluded that serum starvation promoted the secretion of VEGF in the human OSCC cell line (HSC-3).

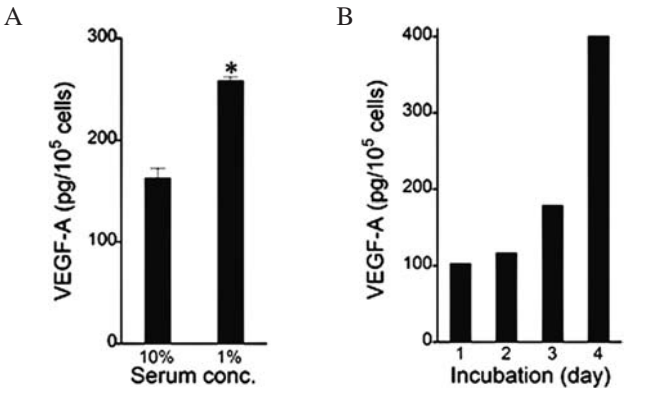

Figure 1. VEGF-A secretion in serum-starved conditions. HSC-3 cells ($2x10^4$ cells/well) were cultured in 10% serum medium for 24 h. (A) After removal of the medium, cells were cultured in 1% (starved) or 10% (complete) serum medium for 4 days. (B) Cells were cultured in 1% serum medium for 1-4 days. The cultured medium was collected and VEGF-A was measured by ELISA. Cells were counted at the end of culture and results were normalized to cell counts ($1x10^5$). *P<0.05 compared to 10% serum medium.

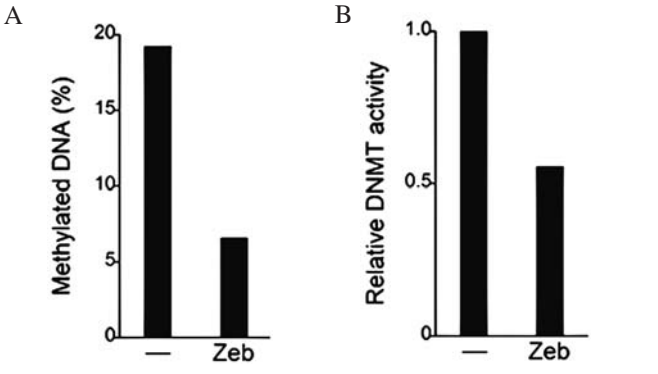

Figure 2. Effects of Zeb on DNA methylation and DNMT activity. HSC-3 cells ($1x10^6$ cells/dish) were cultured with Zeb (220 μM) for 48 h. (A) DNA was purified from untreated and Zeb-treated cells, and the global DNA methylation of cells was quantified. Results are expressed as an absolute percentage. (B) In addition, nuclear protein was extracted and DNMT activity measured. The effect of Zeb on DNMT activity is shown as a ratio relative to the untreated control. Results are representative of three independent experiments.

Effects of Zeb on DNA methylation and DNMT activity. To confirm a possible basis for the gene induction effects of Zeb, we examined the reduction in global DNA methylation levels and DNMT activity elicited by Zeb treatment. In a previous study, we had examined the anti-proliferative effects of Zeb to determine cellular inhibitory concentrations (IC) against HSC-3 cells after 48 h of treatment (9). The IC values were as follows: IC_{20}, 120 μM and IC_{50}, 220 μM. Zeb treatment (IC_{50}) was able to consistently reduce the methylation levels of DNA in HSC-3 cells (Fig. 2A). In a similar fashion, DNMT enzymatic activity under the same conditions was 44% lower in Zeb-treated cells (Fig. 2B).

Effects of Zeb on VEGF-A and -C secretion in carcinoma cell lines. To study the effects of an epigenetic inhibitor (Zeb) on the secretion of VEGF, we first determined the dose-response effects of Zeb on the serum-starved secretion of VEGF-A in

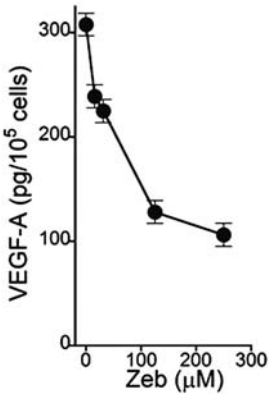

Figure 3. Dose-dependent reduction of VEGF-A secretion by Zeb. HSC-3 cells were cultured for 24 h then treated with Zeb (0-250 μM) for 48 h. After the medium was removed, cells were further cultured in serum-starved medium with or without the same doses of Zeb for 4 days. The cultured medium was collected and VEGF-A was measured by ELISA. Results represent the mean ± SD of values per 1x10^5 cells from three independent experiments.

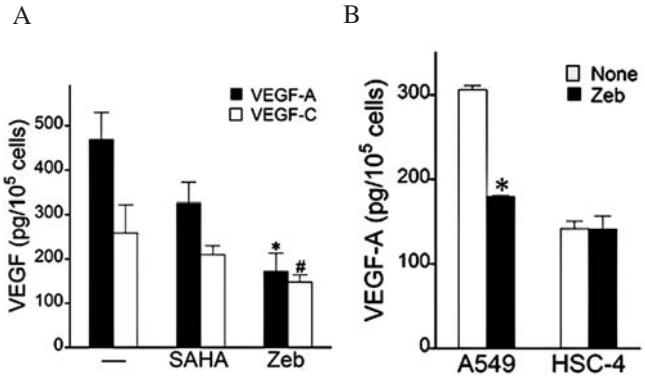

Figure 4. Effects of Zeb and SAHA on the secretion of VEGF-A and -C. (A) HSC-3 cells were cultured with or without Zeb (120 μM) for 48 h. After the medium was removed, cells were treated with Zeb or SAHA (1.5 μM) in serum-starved medium for 4 days. (B) After pre-culture, Zeb was added to A549 (200 μM) or HSC-4 cell culture (15 μM). Cells were cultured in serum-starved medium for 4 days. The cultured medium was collected and VEGF-A and -C were measured by ELISA. Results represent the mean ± SD of values per 1x10^5 cells from three independent experiments. $^{*,\#}$P<0.05 compared to each medium alone.

HSC-3 cells. At increasing concentrations, Zeb significantly inhibited the secretion of VEGF-A in a dose-dependent manner (Fig. 3). The secretion was reduced by 60% at IC_{20} and 65% at IC_{50}.

We also examined the levels of VEGF-C in the cultured medium of HSC-3 cells after treatment with Zeb or SAHA. Zeb significantly reduced the amount of VEGF-C as well as VEGF-A secreted. For both VEGF-A and -C secretion, treatment with the HDAC inhibitor SAHA (IC_{20}, 1.5 μM) resulted in less extensive reduction than did treatment with Zeb (Fig. 4A). We then examined the effects of Zeb on VEGF-A levels in the cultured medium of other carcinoma cell lines. As shown in Fig. 4B, the level of VEGF-A in A549 cells (wild p53) was closer to that of the OSCC cell line HSC-3. However, the OSCC cell line HSC-4 (mutant p53) secreted less VEGF. Secretion-reducing effects of Zeb were observed in A549 cells. We conclude that these epigenetic agents strongly inhibit the secretion of VEGF in carcinoma cell lines containing OSCC, regardless of p53 status.

Role of Akt kinase in VEGF-A reduction elicited by Zeb. We focused on the mechanisms responsible for the Zeb-induced reduction in the secretion of VEGF-A in HSC-3 cells, initially by checking whether a reduced response of the Akt pathway could account for this effect. A significant suppression of Akt phosphorylation was observed after 4 h of treatment with the PI3K-specific inhibitor LY294002 (30 μM), while no effect was exhibited on the total protein levels of Akt (Fig. 5A). When the Akt signaling pathway was inhibited by LY294002 in HSC-3 cells, a reduction in the level of secretion was detected in a dose-dependent manner (Fig. 5B).

To study the effect of Zeb on the activation of Akt, cells were treated with Zeb and levels of phospho-Akt protein were measured. Zeb (IC_{20}) had only a weak effect on the phosphorylation of Akt, while an apparent effect on the secretion of VEGF was detected under the conditions (Fig. 5A). Taken together, our findings suggest that, in HSC-3 cells, a Zeb-induced reduction in the secretion of VEGF-A does not occur via the PI3K-Akt pathway, although the pathway does regulate basal secretion.

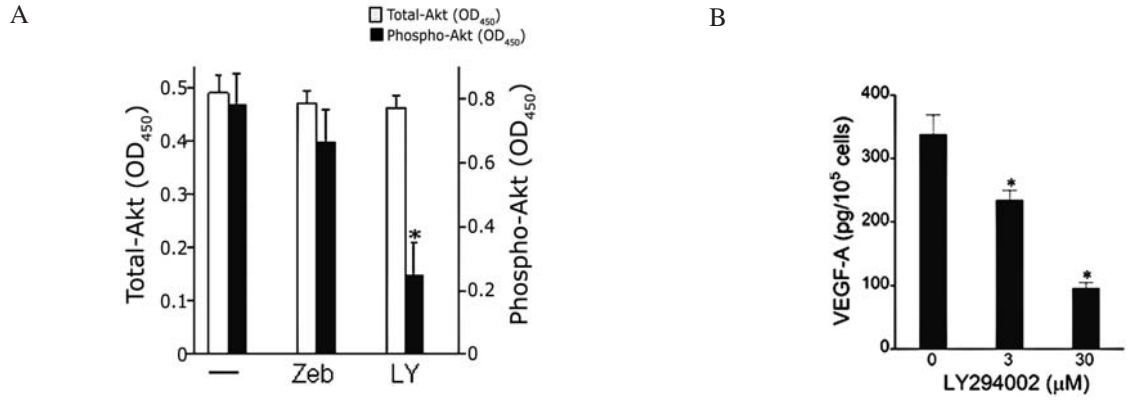

Figure 5. Role of Akt kinase in VEGF-A reduction elicited by Zeb. (A) Measurement of total and phosphorylated Akt. HSC-3 cells were cultured with or without Zeb (120 μM) for 48 h. After the medium was removed, cells were cultured in serum-starved medium with Zeb or LY294002 (30 μM) for 4 h and fixed. Total and phoshpo-Akt were assayed in triplicate. Results represent the mean ± SD of values of OD_{450}. (B) Effect of a PI3K inhibitor on VEGF-A secretion. Cells were cultured in serum-starved medium with or without LY294002 (0-30 μM) for 4 days and VEGF-A was measured by ELISA. Results represent the mean ± SD of values per 1x10^5 cells from three independent experiments. *P<0.05 compared to medium alone.

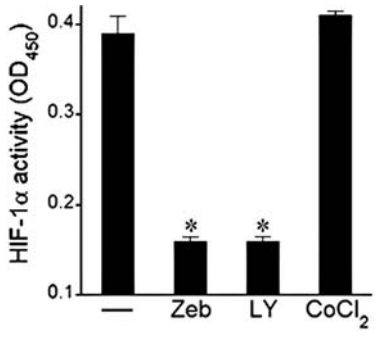

Figure 6. Role of HIF-1α in VEGF-A reduction elicited by Zeb. HSC-3 cells were cultured with or without Zeb (220 μM) for 48 h. After the medium was removed, cells were cultured in serum-starved medium with Zeb or LY294002 (30 μM) for 4 h, and nuclear proteins were extracted. Each sample was added to 96-well plates coated with oligonucleotide containing an HRE site, and assayed. Nuclear protein from COS-7 cells treated with $CoCl_2$ was assayed as a positive control. Results represent the mean ± SD of OD_{450} values from three independent experiments. *P<0.01 compared to medium alone.

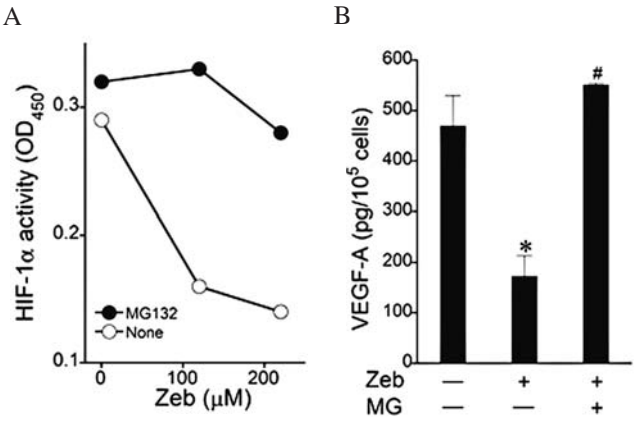

Figure 7. Promotion of HIF-1α degradation by Zeb. (A) Effect of MG132 on HIF-1α activity. HSC-3 cells were treated with Zeb (0-220 μM) in the absence or presence of MG132 (1 μM) for 48 h. After the medium was removed, cells were cultured in serum-starved medium with Zeb and/or MG132 for 4 h. Nuclear proteins were added to 96-well plates coated with oligonucleotide containing an HRE site, and assayed. Results are representative of three independent experiments. (B) Effect of MG132 on VEGF-A secretion. Cells were cultured in serum-starved medium with or without Zeb and/or MG132 for 4 days, and VEGF-A was measured by ELISA. Results represent the mean ± SD of values per $1x10^5$ cells from three independent experiments. *P<0.05 compared to medium alone; #P<0.05 compared to Zeb alone.

Role of HIF-1α in VEGF-A reduction elicited by Zeb. In normoxic conditions, HIF-1α is continuously expressed and simultaneously degraded through the ubiquitin-proteasome pathway, and the binding of HIF-1α to the promoter region of VEGFs stimulates expression (18). To study the inhibitory effect of Zeb on HIF-1α activity in normoxic conditions, cellular protein was extracted from nuclei and analyzed by ELISA. As shown in Fig. 6, the nuclear localization of HIF-1α was significantly decreased in Zeb-treated cells, whereas control cells showed relatively high levels of HIF-1α activity. COS-7 cells treated with the hypoxia-mimetic $CoCl_2$ showed similar levels of secretion to control cells. The activity of HIF-1α was substantially reduced in the presence of PI3K inhibitor. These results suggest that activation of the PI3K/Akt pathway is necessary for HIF-1α protein to accumulate, whereas Zeb decreases levels of HIF-1α protein by a different mechanism than that of PI3K inhibitor.

Promotion of HIF-1α degradation by Zeb. It is possible that the Zeb-mediated inhibition of HIF-1α is due to the promotion of HIF-1α degradation. To test this possibility, the effect of Zeb on the degradation pathway of HIF-1α was investigated in the presence or absence of the proteasome inhibitor MG132. As shown in Fig. 7A, the level of HIF-1α activity was significantly decreased by Zeb in a dose-dependent manner, significantly increased by combined treatment with Zeb and MG132, and was only slightly increased by treatment with MG132 alone. The Zeb-induced reduction of VEGF secretion returned to the control level in the presence of MG132 (Fig. 7B). Taken together, these findings indicate that Zeb treatment induces a reduction in the levels of both VEGF and HIF-1α, and that Zeb inhibits VEGF expression through proteasome-based ubiquitination of the HIF-1α pathway in OSCC cells.

Discussion

The malignant potential of OSCC is closely associated with local expansion and lymph node metastasis (1). Extended local invasion involves important surrounding structures, which makes it difficult to complete tumor resection and reduces cancer curability (19). Angiogenesis is a major event in the progression of OSCC, during which several angiogenic factors are recruited (10). One factor known to be involved in tumor-associated angiogenesis is VEGF, reported to be highly expressed in 43% of OSCCs (20). The tumor microenvironment is best characterized as a fluctuation of hypoxic and nutrient-deprived conditions, which leads to epigenetic and genetic adaptations of clones and increased invasiveness and metastasis. Recently, it was demonstrated that the demethylation of VEGF-C by 5-aza-CdR can activate the expression of VEGF-C mRNA in gastric cancer cell lines (21). In contrast, 5-aza-CdR inhibited the production of VEGF protein by human lung cancer cell lines, especially in hypermethylated cells (22). Because 5-aza-CdR is a generalized non-specific cytotoxic agent, in addition to being a demethylating agent, differences between these two will be important to the understanding of the mechanisms underlying the epigenetic changes caused by demethylating agents. In this study, we used the low toxic agent Zeb and observed that treating HSC-3 cells with it reduced the secretion of VEGF-A (and VEGF-C) through the augmentation of the proteasome-dependent degradation of HIF-1α. This might also play a pivotal role in the prevention of angiogenesis.

VEGF expression in tumor tissues is induced under hypoxic conditions. This induction is a multistage process in which HIF-1α plays an important role (17). Generally, oxygen tension in normal tissues has a mean of approximately 7% oxygen; in tumors, the mean oxygen tension is approximately 1.5%. HIF-1α is a transcription factor that activates the transcription of many genes, including VEGF genes (18). HIF-1α dimerizes with HIF-1ß and translocates into the nucleus, where the functionally active HIF-1α/HIF-1ß complex activates the transcription of VEGF genes. HIF-1α can transactivate the hypoxia response element (HRE)-containing promoter of

VEGF genes. In addition, HIF-1α expression can be induced through the activation of PI3K/Akt or MAPK signaling, which regulate tumor angiogenesis via HIF-1α/VEGF (23). O_2-dependent degradation of HIF-1α by the 26S proteasome is regulated by the hydroxylation of specific proline residues that are recognized by the von Hippel-Lindau (VHL) tumor suppressor protein (24). VHL regulates in normoxia the ubiquitin-mediated proteolysis of HIF-1α, and its inactivation results in increased cellular HIF-1α expression. Furthermore, the stability of the HIF-1α protein is also regulated through an O_2-independent pathway involving the interaction of HSP90 and receptor for activated C kinase 1 (RACK1) with the PAS domain (25). HSP90, as a molecular chaperone, modulates the conformation of the HIF-1α/Arnt heterodimer, making it suitable for interaction with HRE and preventing it from undergoing proteasome-dependent degradation (26). HSP90 and RACK1 compete to bind to the PAS-A subdomain of HIF-1α. The binding of RACK1 leads to the ubiquitination and degradation of HIF-1α. Consequently, agents that block HSP90 function and/or the PI3K-Akt and MAPK signaling pathways may inhibit tumor angiogenesis by decreasing HIF-1α and VEGF expression. For example, the farnesyltransferase inhibitor SCH66336 inhibits the secretion of VEGF by OSCC cells by inhibiting the interaction between HIF-1α and HSP90, resulting in the proteasomal degradation of HIF-1α (27). It has been shown that inhibition of PI3K by LY294002 abrogated the expression of HIF-1α and VEGF in prostate cancer cell lines (28). However, our results show that treatment with Zeb inhibits basal VEGF secretion without down-regulating phospho-Akt in HSC-3 cells (Fig. 5A). Therefore, Zeb appears to affect the level of HIF-1α through mechanisms other than the inactivation of PI3K-Akt.

In this study, we demonstrated the functional relevance of HIF-1α in the down-regulation of VEGF expression in Zeb-treated OSCC cells. In tumors, transcriptional silencing is often associated with the aberrant methylation of the promoter region of genes. The VHL tumor suppressor gene is disrupted through the mutation or methylation of the promoter in most sporadic clear cell renal carcinomas. The resulting loss of VHL protein function is thought to stabilize HIF-1α, leading to the induction of VEGF expression. Re-expression of VHL by 5-aza-CdR can modulate the expression of VEGF, indicating that the promoter remains otherwise intact (29). Zeb treatment globally changes the methylation of the genome and, therefore, it is likely that in addition to affecting VHL it affects the expression of many other genes. One specific CpG island in the $p16^{INK4a}$ gene was found methylated in 20% of oral cytological samples of OSCC (30). Demethylating agents, such as 5-aza-CdR, can decrease methylation levels and have been shown to re-establish $p16^{INK4a}$ expression, consistent with the inhibition of $p16^{INK4a}$ expression by methylated promoter sequences in OSCC cell lines (31). The continuous application of Zeb to T24 human bladder carcinoma cells induced and maintained $p16^{INK4a}$ gene expression and sustained demethylation of the 5' region, preventing re-methylation (32). Another study has also indicated that demethylation of the $p16^{INK4a}$ gene results in down-regulation of VEGF expression in lung cancer cell lines (22). The tumor suppressor p53 has been identified as an important factor in VEGF expression under both normoxic and hypoxic conditions (33). Thereafter, it was demonstrated that VEGF expression may also be regulated by $p16^{INK4a}$ via a p53-independent pathway (34). In our study, a p53-dependent pathway could not explain the inhibition of VEGF secretion, because HSC-3 cells have a mutated, non-functional p53. The exact mechanisms by which the expression of the $p16^{INK4a}$ tumor suppressor gene causes the secretion of VEGF to decrease remain unclear. However, it is likely that G_1 arrest, due to $p16^{INK4a}$ expression, suppresses the properties of various factors related to VEGF secretion. The retinoblastoma protein (pRb) is required for cell cycle arrest by $p16^{INK4a}$. It has been demonstrated that enhanced expression of pRb2/p130 down-regulates VEGF expression *in vitro* as well as in a nude-mouse xenograft model (35). These findings suggest that $p16^{INK4a}$ may play a regulatory role in the secretion of VEGF via the p16-pRb pathway. Further studies are required to explore the mechanism linking the function of $p16^{INK4a}$ with the secretion of VEGF.

Research has demonstrated that HDAC1 is hypoxia-inducible, and that a HDAC inhibitor also serves as a potent anti-angiogenic agent against cancer cells over-expressing HDACs (36). In addition, HDAC inhibitors have been shown to down-regulate hypoxia-response genes (VEGF) and hypoxia-induced angiogenesis by the suppression of HIF-1α activity (36,37). In this study, the HDAC inhibitor SAHA reduced the secretion of VEGF-A, and may therefore inhibit angiogenesis in OSCC (Fig. 4A). Their most likely mechanism of action involves their effects on the HSP70/HSP90 axis, where they prevent the proper folding and maturation of the HIF-1α protein and thus promote its degradation by the proteasome in an ubiquitin-independent process (38).

In conclusion, we have shown that epigenetics can play a role in the regulation of the angiogenic factor VEGF. Our findings reveal novel anti-angiogenic and anti-cancer activities of Zeb in OSCC. Treatment with Zeb augmented proteasome-mediated degradation of the HIF-1α protein, resulting in decreased secretion of VEGF. Epigenetic restoring might render cancer cells non-aggressive, and agents acting as DNMT or HDAC inhibitors might be combined with established chemotherapy for cancer. This work has laid the foundation for a novel anti-cancer approach, whereby a DNMT inhibitor can be used to modulate the angiogenic properties in OSCC. Zeb has several features that make it suitable for clinical trials.

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