Resistance of SMMC-7721 hepatoma cells to etoposide in hypoxia is reversed by VEGF inhibitor

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Abstract. Hypoxia is associated with resistance to chemotherapy in a number of human cancer types; particularly in hepatocellular carcinoma (HCC), which is a highly vascularized tumor. To develop a potential combination therapy strategy that is capable of overcoming the hypoxia-induced insensitivity to chemotherapy, the HCC cell SMMC-7721 was employed to investigate the hypoxia-induced chemoresistance to etoposide. Increased levels of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) were observed when SMMC-7721 cells were exposed to hypoxia, and exposure of tumor cells to hypoxia impaired etoposide-induced DNA damage, as indicated by the failure of upregulation of γHA2X. Etoposide-induced apoptosis and cell cycle arrest of SMMC-7721 was also impaired in hypoxia. However, co-treatment with anti-VEGF significantly restored etoposide-induced cell apoptosis and cell cycle arrest, as indicated by the elimination of B-cell lymphoma 2 (Bcl-2), procaspase 3, cyclin B1 and Cdc2. Furthermore, anti-VEGF eliminated phosphorylation of AKT, ERK and IκB-α resulting from hypoxia, suggesting the involvement of VEGF in the activation of the survival pathways. In conclusion, the present study suggests a significant role of VEGF in the chemoresistance of etoposide in hypoxia. A rational chemotherapy should be developed based on a combination of etoposide and anti-VEGF.

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Abbreviations: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; HCC, hepatocellular carcinoma; $rhVEGF_{165}$, recombinant human vascular endothelial growth factor (165); NF-κB, nuclear factor κB.

Key words: hypoxia, etoposide, resistance, HIF-1α, VEGF

Introduction

The term hypoxia describes a reduction in the normal level of tissue oxygen tension, which may be acute or chronic. Hypoxia is the most notable and prevailing characteristic of the microenvironment of solid tumors (1). Consequently, adaptations to hypoxia are necessary for the survival of tumor cells in such microenvironments. Hypoxia-inducible factor-1 (HIF-1) is a transcriptional complex that is activated in response to hypoxia and other growth factors, and has a significant role in tumor progression, invasion and metastasis (2,3). A pool of studies has provided evidence indicating that the overexpression of HIF-1α subunit is associated with a poor prognostic outcome as well as resistance to chemotherapy and radiation (4-7). It is also well established that HIF-1α regulates multiple proangiogenic factors, including vascular endothelial growth factor (VEGF). Hypoxia-induced VEGF may activate the MAPK-related VEGF receptor signaling pathway to elicit anti-apoptotic effects in a self-activating manner and induce angiogenesis primarily through its interaction with two tyrosine kinase receptors expressed in vascular endothelial cells, VEGFR1 (Flt1) and VEGFR2 (Flk1/KDR) (8). Furthermore, there is emerging evidence demonstrating that VEGF may also form an autocrine loop with HIF-1α, contributing to tumor cell survival as well as drug resistance during hypoxia in neuroblastoma, osteosarcoma, rhabdomyosarcoma and breast cancer cells (9-11). The anticancer effect of the pharmacological VEGF antagonism beyond interference with angiogenesis is therefore a significant area of cancer research.

Hypoxia and DNA damage occur simultaneously when therapies that cause DNA damage are applied to tumors bearing hypoxic regions. Cells may be eliminated following

DNA damage through various forms of programmed death, including apoptosis, autophagy, mitotic catastrophe and necrosis. Among these, a common paradigm is that chemotherapeutic agents stimulate cells to undergo apoptosis, implying that impairment of apoptotic pathways may be involved in the development of chemoresistance (12).

Hepatocellular carcinoma (HCC) is one of the most common types of cancer and a leading cause of cancer-related mortality worldwide. As a highly vascularized tumor with poor prognosis, elevated levels of VEGF have been identified and a greater expression of VEGF has been associated with shorter survival in HCC patients (13-15). Therefore, inhibition of VEGF is an attractive target in HCC treatment (16). Significant clinical and biologic research into bevacizumab, a humanized monoclonal antibody that binds VEGF prior to its attachment to its natural receptors, has been undertaken with regard to HCC, since systemic therapy with cytotoxic agents has demonstrated a notable benefit (17-19).

Given the potential roles of chemotherapeutic agents and VEGF antagonism in cancer therapeutics, one rational strategy would be to combine anti-VEGF treatment with DNA damage agents such as etoposide. We used the SMMC-7721 hepatoma cell line in the present study, which is an identified chemoresistant phenotype when subjected to hypoxia, with elevated serum VEGF levels contributing to tumor growth and metastasis. This study investigates the role of this combination strategy and provides evidence that pre-exposure of SMMC-7721 cells to hypoxia weakens etoposide-induced cytotoxicity. Notably, the anti-VEGF strategy facilitated DNA damage ability of etoposide followed by cell cycle delay and apoptosis induction, which served to overcome hypoxia-driven etoposide resistance.

Materials and methods

Reagents and antibodies. Etoposide was purchased from Sigma-Aldrich (St. Louis, MO, USA); a stock solution of 100 mM was prepared with dimethyl sulfoxide and stored at -20˚C. The stock solution was further diluted with the appropriate medium immediately prior to use. The antibodies to HIF-1α and Flt1 (VEGFR1) were purchased from Calbiochem (La Jolla, CA, USA) and R&D systems (Minneapolis, MN, USA), respectively. The antibodies to VEGF, p53, Bcl-2, Bax, procaspase 3, cyclin A, cyclin B1, cdc2, AKT, ERK, α-tubulin and β-actin, as well as HRP-labeled secondary anti-goat, anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The antibodies to γH2AX, p-AKT (Ser473), p-ERK, p-IκB-α (Ser32) and GAPDH were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Recombinant human $VEGF_{165}$ ($rhVEGF_{165}$) and a neutralizing antibody against VEGF (anti-mouse monoclonal antibody) were purchased from R&D Systems.

Cell culture and establishment of hypoxia culture conditions. Human HCC SMMC-7721 cells (Shanghai Institute of Biological Sciences, Shanghai, China) were cultured in RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum containing 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). The cells were kept in a humidified atmosphere of 5% CO_2 and 20% O_2 at 37˚C. Environmental hypoxic conditions (0.6% O_2) were achieved in an airtight humidified chamber continuously flushed with a mixed gas where N_2 was used to compensate for the reduced O_2 level.

Cytotoxicity assay. Cell proliferation was determined by a standard 3-[4,5-dimethylthia-zol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates, and hypoxic cells were allowed to attach for one day before exposure to etoposide at concentrations specified or vehicle control alone. Plates were assayed 48 and 72 h after the initiation of the designated treatment. All experiments were repeated three times.

Flow cytometric determination of cell cycle. To determine the phase distribution of the DNA content, propidium iodide (PI) staining was performed. Following the designated treatment of actively proliferating cultures, cells were collected and washed twice with phosphate-buffered saline (PBS) buffer and fixed in 70% ice-cold ethanol overnight. The cell pellet was re-suspended in PBS plus 0.5 mg/ml RNaseA at 37˚C for 30 min prior to staining with 5 mg/ml PI (Sigma-Aldrich) at room temperature in the dark for another 30 min. The analysis was performed with a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

Flow cytometric determination of apoptosis. Cells were collected and washed twice with ice-cold PBS buffer following the designated treatment of actively proliferating cultures. Subsequently, cells were stained with annexin V-fluorescein and PI according to the descriptions in the commercial apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). Analysis was performed with a FACScan flow cytometer (BD Biosciences).

Protein expression. Proteins were extracted in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris, 2 mM EGTA, 2 mM EDTA, 25 mm NaF, 25 mm glycerophosphate, 0.2% Triton X-100, 0.3% NONIDET P-40 and 0.1 mM PMSF). Total protein concentrations of whole cell lysates were determined using the Bio-Rad reagent (Bio-Rad, Hercules, CA, USA) bicinchoninic acid method. Equal amounts of 40 μg total protein were loaded per lane. Proteins were fractionated on 8-12% Tris-Glycine pre-cast gels (Novex, San Diego, CA, USA), transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA, USA), and probed with primary antibodies and then HRP-labeled secondary antibodies. Proteins were visualized using ECL western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

Results

Hypoxia impairs etoposide-induced cytotoxicity. To examine the effect of hypoxia, SMMC-7721 cells were exposed to normoxia or hypoxia (0.6% O_2), respectively, in the presence of serum to avoid glucose deprivation or cell cycle inhibition. It was noted that etoposide exerted significant concentration- and time-dependent cytotoxicity in a normoxic atmosphere, as shown in Fig. 1A. Conversely, an impaired anti-proliferative

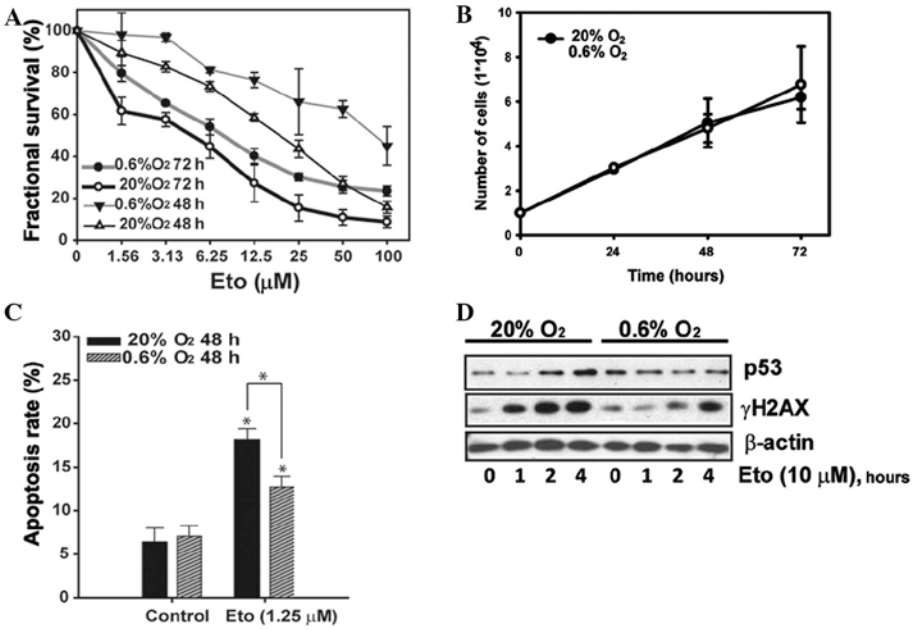

Figure 1. Hypoxia-induced etoposide resistance in SMMC-7721 cells. (A) Hypoxia impairs etoposide-induced anti-proliferative ability in SMMC-7721 cells. Cells were exposed to various concentrations of etoposide for 48 or 72 h under normoxia or hypoxia followed by MTT assay. (B) Growth curves of SMMC-7721 cells under normoxia and hypoxia. Cells were seeded in 24-well plates in duplicate wells and were trypsinized and manually counted every day. The data were normalized by setting the value at day 0. (C) SMMC-7721 cells were administered etoposide for 48 h, then collected for apoptosis analyses by flow cytometry. (D) Hypoxia impairs etoposide-induced DNA damage ability in SMMC-7721 cells. Cells were treated with etoposide (10 μM) for 0-4 h before they were collected for p53 and γH2AX detection. Data are presented as the means ± standard deviation (n=3). The statistical significance of cell cycle and apoptosis results was analyzed using a two-sided Student's t-test; *P<0.01. Eto, etoposide.

effect was observed under the hypoxic environment. To exclude the possibility that the diminished cytotoxicity of etoposide was due to a general anti-proliferative effect of hypoxic conditions that render more slowly cycling cells, cell growth curves were analyzed under various oxygen conditions, as shown in Fig. 1B, to demonstrate that hypoxia alone did not lower cell proliferation within 72 h. However, the etoposide-induced apoptosis was significantly reduced (Fig. 1C).

Etoposide is known to stabilize the cleavable complex formed between topoisomerase II and DNA, and subsequently form strand breakage. The topoisomerase II inhibitor causes replication stress and generates the dominant damage-induced phosphorylation of the minor histone H2A (γH2AX), which has become one of the most widely used measures of DNA damage and is observed within a short-term exposure to etoposide (20). We treated SMMC-7721 cells for 0-4 h with a relatively high concentration (10 μM) and then measured the γH2AX level. The western blotting results shown in Fig. 1D reveal that a significant increase of γH2AX was observed after 1 h of etoposide treatment, which reached a platform at 4 h. Since p53 was reported to be involved in apoptosis in response to DNA damage, p53 accumulation was also analyzed (21). A significant accumulation of p53 was detected following a 2 h challenge of etoposide. However, far less significant expression of γH2AX and p53 was observed in hypoxia (Fig. 1D).

Anti-VEGF intervention improves etoposide-induced apoptosis in hypoxia. By culturing SMMC-7721 cells under hypoxic conditions, increased levels of HIF-1α and VEGF were observed (Fig. 2A). To investigate the effect of VEGF in hypoxia-induced chemoresistance to etoposide, we exposed SMMC-7721 cells to etoposide with or without anti-VEGF under hypoxia and further detected the apoptosis. As expected, VEGF interference increased the apoptotic fraction (Fig. 2B).

To demonstrate how this combination strategy might affect etoposide function in DNA damage under hypoxia, SMMC-7721 cells were treated with or without anti-VEGF upon etoposide administration (10 μM). Combined treatment of anti-VEGF and etoposide significantly upregulated γH2AX and p53 levels, to a level similar to that observed with a single treatment of etoposide in normoxia (Fig. 2C).

Mitochondria play a pivotal role in the regulation of apoptosis by regulating the balance between anti-apoptotic family members including Bcl-2 and pro-apoptotic family members including Bax. To determine whether the downstream activators of apoptosis were induced, we analyzed the expression of Bcl-2, Bax and procaspase 3. A significant increase in Bax as well as significant decreases in Bcl-2 and procaspase 3 were observed upon etoposide treatment in normoxic conditions (Fig. 2D). However, the expression of these proteins was not significantly affected by etoposide alone in hypoxia, while the anti-VEGF combination helped to further downregulate Bcl-2 and procaspase 3, which was consistent with apoptosis data (Fig. 2B). Taken together, the anti-VEGF combination strategy may affect mitochondrial-related apoptosis and improve etoposide-induced SMMC-7721 apoptosis under hypoxia.

Anti-VEGF facilitates etoposide-induced cell cycle arrest under hypoxia. Hypoxia is also reported to induce cell cycle arrest in certain tumors, leading to chemo- and/or radio-resistance (22). SMMC-7721 cells were treated with various concentrations of etoposide for 48 h before analyses of cell

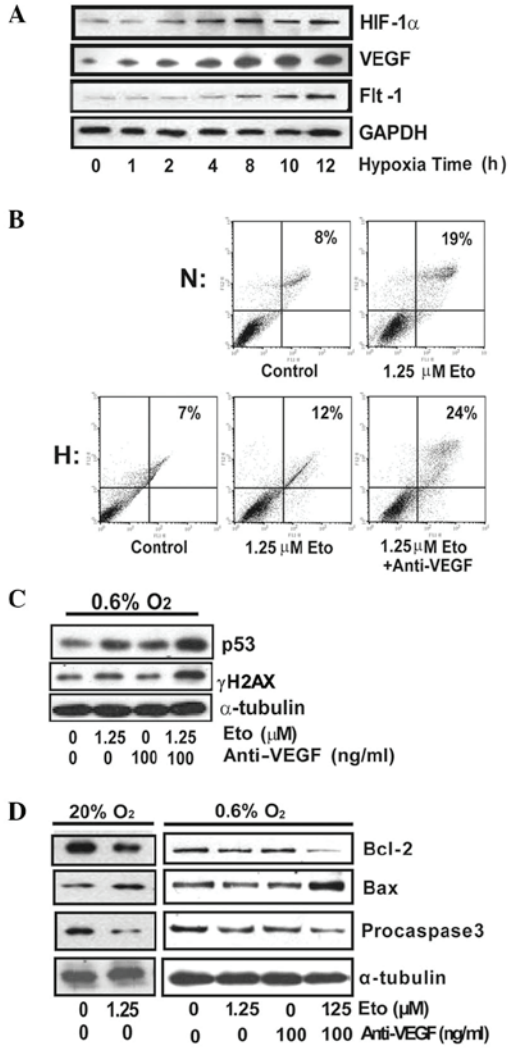

Figure 2. Anti-vascular endothelial growth factor (VEGF) intervention improves etoposide-induced apoptosis in hypoxia. (A) Cells were exposed to hypoxia for 0-12 h and the lysates were immunoblotted against hypoxia-inducible factor-1α (HIF-1α), VEGF and Flt1 antibody. (B) Cells were treated with etoposide alone in normoxia or treated with both anti-VEGF antibody and etoposide in hypoxia, and apoptosis was analyzed after 48 h exposure. (C) Cells were incubated with anti-VEGF for 24 h followed by an additional exposure to etoposide (1.25 μM) for 4 h under hypoxic conditions. Cells were then harvested, extracted and subjected to immunoblotting for γH2AX and p53 expression. (D) Cells were incubated with anti-VEGF for 24 h and then treated with etoposide for 24 h before they were collected for Bcl-2, Bax and procaspase 3 analysis. Eto, etoposide.

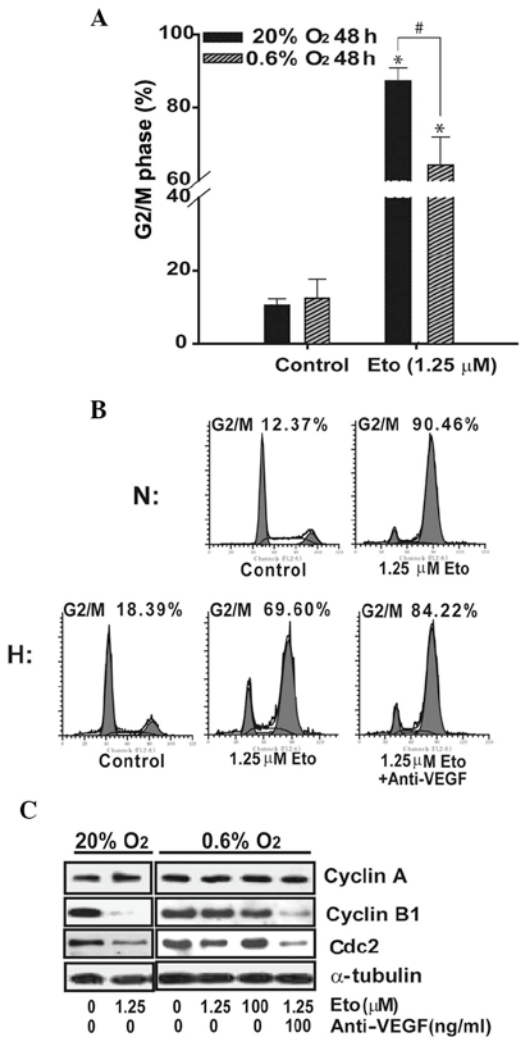

Figure 3. Anti-vascular endothelial growth factor (VEGF) facilitates etoposide-induced cell cycle arrest under hypoxia. (A) SMMC-7721 cells were treated with etoposide for 48 h then underwent cell cycle analysis by flow cytometry. (B) SMMC-7721 cells pre-incubated with anti-VEGF for 24 h followed by etoposide exposure for 48 h were collected for cell cycle analyses by flow cytometry. N, normoxia; H, hypoxia. Results from a representative experiment are presented. (C) G2/M phase-related proteins cyclin B1, cdc2, p-cdc2 (Thr161) and α-tubulin were assessed by western blotting as described in Fig. 2B. *P<0.01; $^{\#}$P<0.05. Eto, etoposide.

cycle phase distribution were performed. Hypoxia alone did not appear to induce cell cycle arrest since there were no notable differences between the hypoxic and normoxic cells. Etoposide exhibited a concentration-dependent effect in G2/M arrest (data not shown here). As shown in Fig. 3A, 48-h treatment of single etoposide (1.25 μM) triggered the arrest of 87.3±3.5% cells (control, 10.5±1.7%) in the G2/M phase under normoxic conditions. In contrast, a rate of only 64.1±7.7% (control, 12.4±5.3%) was observed under hypoxia. However, an increased proportion of G2/M phase arrested cells was observed when cells were treated with both anti-VEGF and etoposide, suggesting that VEGF reduced etoposide in cell cycle arrest (Fig. 3B).

Next, we assessed the effects of etoposide alone or combined with anti-VEGF on cell cycle regulating proteins. In normoxia, challenge with etoposide alone resulted in a significant decrease in cyclin B1 and Cdc2 (Fig. 3C). However, in hypoxia, etoposide only caused a slight decrease in cyclin B1 and Cdc2. Significant downregulation of cyclin B1 and Cdc2 was observed when the cells were co-treated with etoposide and anti-VEGF, which was similar to that observed under normoxia.

Anti-VEGF combination strategy is correlated with the AKT, ERK and nuclear factor-κB (NF-κB) pathway. Current studies suggest that the AKT and ERK pathways are the most relevant survival pathways in tumor cells (23). We thus assessed whether or not AKT and ERK play a role in hypoxia-induced insensitivity to etoposide in SMMC-7721 cells. As shown in Fig. 4, hypoxia triggered phosphorylation of AKT and ERK. Administration of etoposide alone resulted in a slight decrease

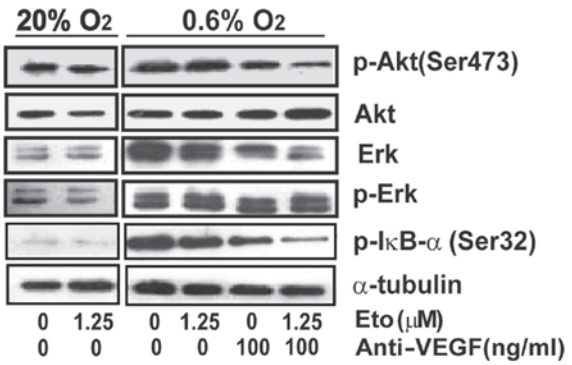

Figure 4. Nuclear factor-κB (NF-κB) but not AKT or ERK is involved in overcoming hypoxia resistance by anti-vascular endothelial growth factor (VEGF) combination strategy. Cells were treated as in Fig. 3; cell lysates were immunoblotted for AKT, p-AKT, ERK, p-ERK and p-IκB-α (Ser32) using corresponding antibodies. Eto, etoposide.

in the phosphorylation of AKT and ERK. However, anti-VEGF alone or the combination treatment completely eliminated the phosphorylation of these proteins, suggesting that the AKT and ERK pathways are involved in anti-VEGF-restored sensitivity to etoposide.

The transcription factor NF-κB has been implicated in resistance to chemotherapeutic treatment. Recent evidence has demonstrated significant cross-talk and the inter-independence of NF-κB and HIF-1α signaling (24,25). NF-κB is a transcriptional activator of HIF-1α and, conversely, HIF-1α accumulation is shown to promote NF-κB. Since phosphorylation of IκB-α at Ser32 is essential for the release of active NF-κB, phosphorylation at this site is a significant marker of NF-κB activation (26,27). Here, we demonstrate that phosphorylation of IκB-α at Ser32 was induced by hypoxia which was eliminated by VEGF inhibition, suggesting the involvement of NF-κB.

Discussion

Hypoxia-induced chemoresistance is an aspect of tumor biology that has received increasing attention over the last decades, with the characterization of HIFs being of particular significance (29,30). Genetic approaches and small-molecule inhibitors targeting HIF-1 have proven effective in decreasing hypoxia-induced resistance to chemotherapeutics in tumors (31,32). Previous studies suggest that VEGF/Flt1 may also form an autocrine loop with HIF-1α, which contributes to cell survival and drug resistance during hypoxia (9-11). Although KDR is generally considered to be the major mediator of the mitogenic and angiogenic effects of VEGF (33), emerging studies demonstrate that Flt1 is present and functional in a diverse group of tumors. In addition, Flt1 is the only receptor associated with VEGF that contains a binding site for HIF-1α, which further supports the recruitment of the VEGF/Flt1 system in HIF-1α-driven tumor progression. Bevacizumab has been shown to improve treatment outcomes in selected patients with advanced colorectal, breast and non-small cell lung cancer. However, it is also being evaluated among patients with earlier-stage cancer, and among patients with other types of cancer. The results of a phase II clinical trial (18,19) revealed that the combination of two targeted therapies, bevacizumab and erlotinib, exhibited anticancer activity in patients with advanced HCC and is therefore worthy of further study.

The DNA damaging reagent etoposide has been demonstrated to induce apoptosis in a variety of tumor cell lines harboring either wild-type or mutant p53. Although the signaling pathways mediating etoposide-induced apoptosis are unclear, one pathway may involve p53 since DNA damage induced by etoposide has been shown to activate p53. It is also known that p53 protein is a potent negative regulator of HIF-1α and VEGF in hypoxia. p53 represses VEGF during hypoxia by binding the transcription factor SP1 and inhibiting its ability to bind the VEGF promoter (34). HIF-1α activity may also be inhibited by p53, which directly binds HIF-1α and targets protein for degradation and thereby downregulated VEGF transcription (35,36). In addition, the ability of p53 to inhibit the HIF system is mediated by its physical interaction with HIF-1α and does not even require its transcriptional activity. Using γH2AX as an indicator of DNA damage, we observed that hypoxia weakened etoposide-induced DNA damage. Notably, the anti-VEGF combination strategy enhanced γH2AX and p53 expression. Subsequently, cell cycle and apoptosis data suggested that the anti-VEGF combination with etoposide could also restore etoposide function to a level comparable with that observed in normoxia.

Previous studies have shown indirect links between HIF-1α and NF-κB transcription pathways (37). The inactive NF-κB is primarily localized in the cytoplasm, and its activation involves its release from IκB-α and translocation to the nucleus. This phosphorylation induces the degradation of IκB-α by the ubiquitin-proteasome system and the release of NF-κB for nuclear translocation. Preventing NF-κB activation exerts a tumor-suppressive effect by promoting apoptosis of transformed cells which would otherwise have given rise to cancer. In addition, NF-κB is a transcriptional activator of HIF-1α, and basal NF-κB activity is required for HIF-1α accumulation in normoxia and during hypoxia. Previous studies have demonstrated significant cross-talk and the inter-independence of NF-κB and HIF-1α signaling, indicating that NF-κB is a transcriptional activator of HIF-1α and, conversely, HIF-1α accumulation is shown to promote NF-κB (24,25). Furthermore, *in vitro* and *in vivo* studies have already shown that targeted inhibition of NF-κB sensitizes tumor cells to chemotherapy and radiation (38).

In this study, we combined etoposide, a commonly used chemotherapeutic agent, with monoclonal VEGF antibody to evaluate its role in hypoxia-induced resistance in hepatoma SMMC-7721 cells. The results revealed that hypoxia impaired etoposide function in DNA damage and resultant cell death and resulted in drug resistance. In addition, interference of VEGF inhibited hypoxia induction of HIF-1α. The anti-VEGF combination treatment enhanced etoposide efficacy by the attenuation of DNA damage followed by limited cell cycle delay and/or apoptosis and reversed drug resistance in SMMC-7721 cells.

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