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Hypoxia promotes etoposide (VP-16) resistance in neuroblastoma CHP126 cells

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Hypoxia is widespread in solid tumors as a consequence of poorly structured tumor-derived neovasculature, which is recognized to play a role in the resistance of cancer cells to chemotherapy. Etoposide (VP-16), a drug commonly used in chemotherapy, leads to enhanced accumulation of cell populations in G2/M phase and increases levels of apoptosis as a topoisomerase II inhibitor. We evaluated the effects of hypoxia on the response of the neuroblastoma cell line CHP126 to VP-16, in order to delineate the mechanisms responsible for the hypoxia-induced chemoresistance of this clinically conventional anti-cancer agent, with an insight to determining potential indications in neuroblastoma therapy. In this study, physiological hypoxia was shown to attenuate G2/M arrest and apoptosis induced in CHP126 cells by VP-16. It suppressed drug-related Cdk1 activity with a less elevation of regulator proteins such as cyclin B1, Cdk7 and reduced caspase activation and PARP cleavage compared to the efficiency observed in normoxic condition, which were significantly relative with hypoxia-driven inhibition of p53 and p-ERK1/2 activation. These results clearly demonstrated that hypoxia had a protective effect against VP-16-induced cytotoxicity, which is likely to provide a further therapeutic knowledge in neuroblastomas.

1. Introduction

Hypoxic microenvironments are frequently found in solid tumors as a result of morphologically and functionally inappropriate vascularization, irregular blood flow, anemia and high oxygen consumption of rapidly proliferating malignant cells. It is thereby associated with malignant progression, lower sensitivity to chemotherapy and radio therapy, increased metastatic potential and poor prognosis (Unruh et al. 2003; Le et al. 2004). Hypoxia may directly induce tumor resistance via deprivation of molecular oxygen needed for some drugs to induce DNA damage. Indirectly, hypoxia may lead to treatment resistance by modulating gene expressions resulting in resistance to cell death. Thus tumor oxygenation is now recognized as an important determinant for the outcome of radiotherapy and other anti-cancer therapies in a number of tumor types (Bussink et al. 2003; Harrison and Blackwell 2004).

Neuroblastoma is the most common extracranial solid tumor of childhood derived from the sympathetic nervous system and accounts for 8% to 10% of all cancers in children. Even with intensive multiagent induction chemotherapy, high-dose therapy with autologous stem cell rescue, radio therapy, surgery, and maintenance chemotherapy with 13-cis-retinoic acid, the survival for this group of patients is poor (Matthay et al. 1999). Because there is a correlation in neuroblastoma between low stage of differentiation and poor clinical outcome, hypoxic adaptation could contribute to increased malignancy of this tumor (Karlsson et al. 2005).

Etoposide (VP-16), a topoisomerase II (topo II) inhibitor that is used in the treatment of neoplastic disease, results in cell cycle arrest at the G2/M DNA damage checkpoint at non-lethal

concentrations. It is also a potent inducer of apoptosis at higher concentrations (Kaufmann 1998; Jahnke et al. 2007). Although it is well established that hypoxia renders tumor cells resistant to chemotherapy, less is known about the importance of hypoxia in pediatric tumors as well as the elements currently available as for the mechanisms underlying hypoxia relative protection. Therefore, we investigated in this study the effect of hypoxia on undergoing cell cycle arrest and/or apoptosis induced by VP-16 in human neuroblastoma CHP126 cells.

2. Investigations and results

2.1. Hypoxia impairs the effect of VP-16 on CHP126 cells proliferation

CHP126 cells were treated with various concentrations of VP-16 for 24 h, 48 h or 72 h respectively under both normoxic and hypoxic conditions, then the antiproliferative effect of VP-16 was determined. As shown in Fig. 1A, VP-16 exerted noticeable concentration- and time- dependent cytotoxicity in normoxic CHP126 cells, whereas viable treated cell numbers in hypoxia remained almost unchanged compared to those without drug addition. The IC₅₀ values of drug incubation for 48 and 72 h were $0.78 \pm 0.09 \mu\text{M}$ and $0.24 \pm 0.02 \mu\text{M}$ in 20% O₂ atmosphere, while they were both above 50 μM in 3% O₂ condition. These results confirmed that hypoxia consequently lowered the efficiency of VP-16. Besides, CHP126 cells were treated with 0.25 to 1 μM of VP-16 required for the G2/M arrest and/or the apoptosis induction in the subsequent experiments.

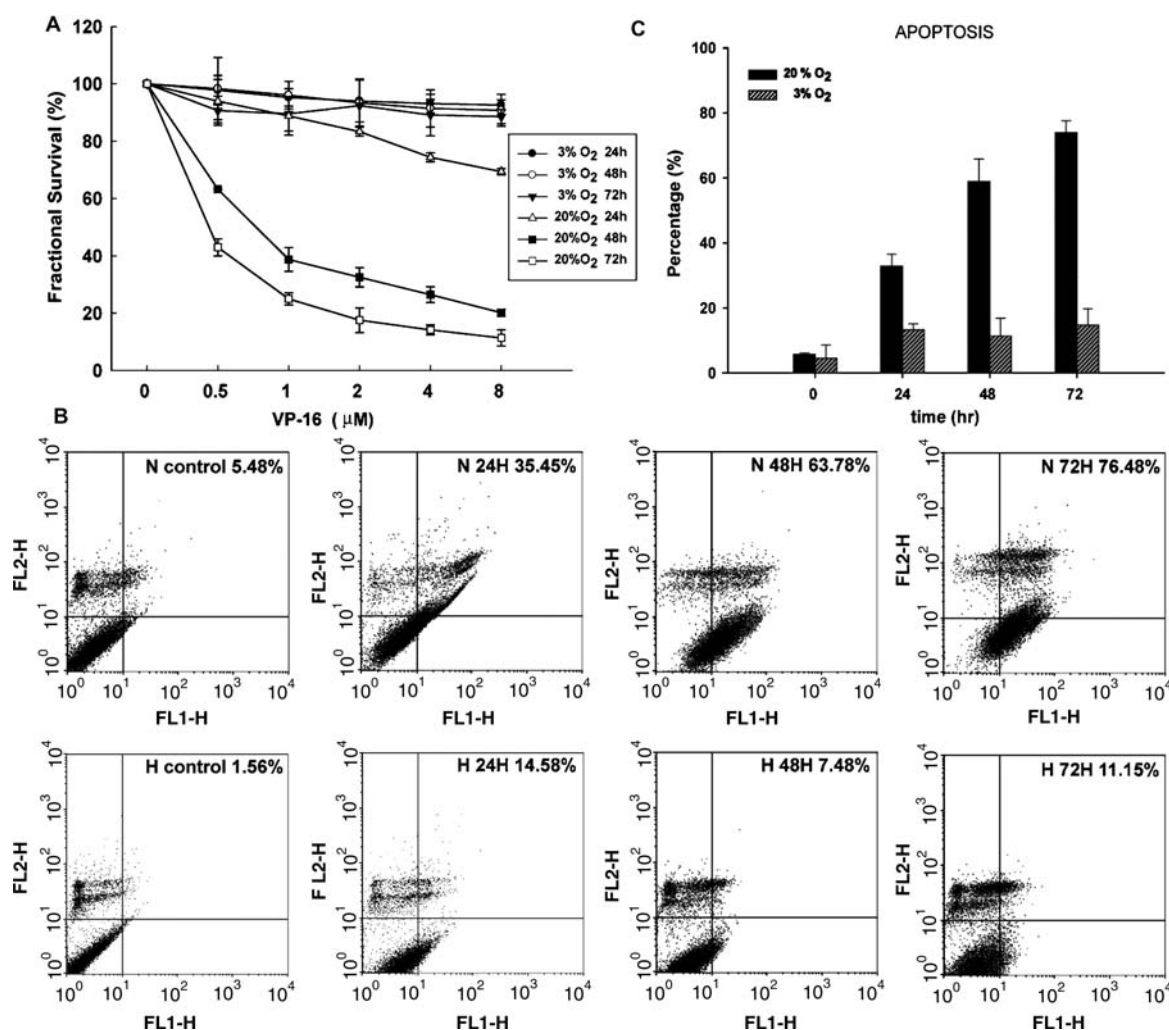


Fig. 1: Different cytotoxicity of VP-16 on human neuroblastoma CHP126 cells in normoxia and hypoxia. A, Cells were exposed with various concentrations of VP-16 for 24, 48, 72 h in hypoxia and in normoxia. Results are representative of three independent experiments. B/C, Hypoxia impairs VP-16 induced time-dependent apoptosis. CHP126 cells were incubated under normoxic (N) or hypoxic (H) conditions with or without etoposide (0.50 μM) for 24, 48 or 72 h. Detection of apoptosis by flow cytometry was then performed using the Annexin V/PI apoptosis detection kit

2.2. Hypoxia weakens VP-16-induced G2/M arrest in CHP126 cells

To determine the cell cycle effects of the drug, CHP126 cells were treated with 0.5 to 2 μM VP-16 for 24 h before they were collected for cellular DNA content analysis using flow cytometry. The percentage of subG1 phase cells decreased dose-dependently after treatment, and concurrently a relatively large fraction of the cells accumulated in the G2/M phase, indicating that CHP126 cells progressed through G1 and S phases, when exposed to non-lethal concentrations of VP-16, and blocked at G2/M phase (data not shown).

As illustrated in Fig. 2A/B, VP-16 treatment resulted in a concentration-dependent accumulation of normoxic CHP126 cells in the G2/M phase, and the percentages were 15.14%, 31.17% and 40.27% respectively at the concentration of 0, 0.25 and 0.50 μM. On the contrary, hypoxia conferred protection against VP-16 as a considerably decreased number of cells arrested in G2/M phase were observed, with the percentages 11.84%, 17.05% and 17.19%.

2.3. Hypoxia confers protection against VP-16-induced apoptosis

VP-16 is also a potent inducer of apoptosis at higher concentrations. In our study, Annexin V/PI staining and flow

cytometric analysis were used to determine the effects of VP-16 on apoptosis. As shown in Fig. 3A, VP-16 induced an evident dose-dependent cell death, from 6.98% (control) to 42.04% (1 μM, 24 h), in CHP126 cells grown under normoxia. The apoptotic rates for 0, 24, 48 and 72 h were 5.48%, 35.45%, 63.78%, and 76.48%, respectively when treated with 0.50 μM, in a time-dependent manner (seen in Fig. 1B/C). However, there was an approximate value of 20% in hypoxic VP-16-treated (24 h) CHP126 cells, regardless of concentrations of 0.25, 0.50 or 1 μM. Similarly, cells alive did not reduce significantly even when drug exposure time was prolonged. These results revealed that VP-16 exhibited clearly reduced efficacy under hypoxic conditions in CHP126 cells.

2.4. Hypoxia modulates changes of protein expressions related to cell cycle and apoptosis

Cyclin-dependent protein kinases have been suggested to play a key role in the activity of chemotherapeutic agents (shown in Fig. 2C). A notable concentration-dependent elevation of cyclin B1, which is suggested to be essential for mitosis, was observed in normoxia when exposed to 0.25 or 0.50 μM VP-16. In contrast, only modest up-regulation took place in hypoxic condition. Our results also showed that treatment with VP-16 resulted in the augmentation of p-Cdk1 (Thr-161, active form) in normoxia but a basically unchanged level in hypoxia. Cyclin-dependent kinase

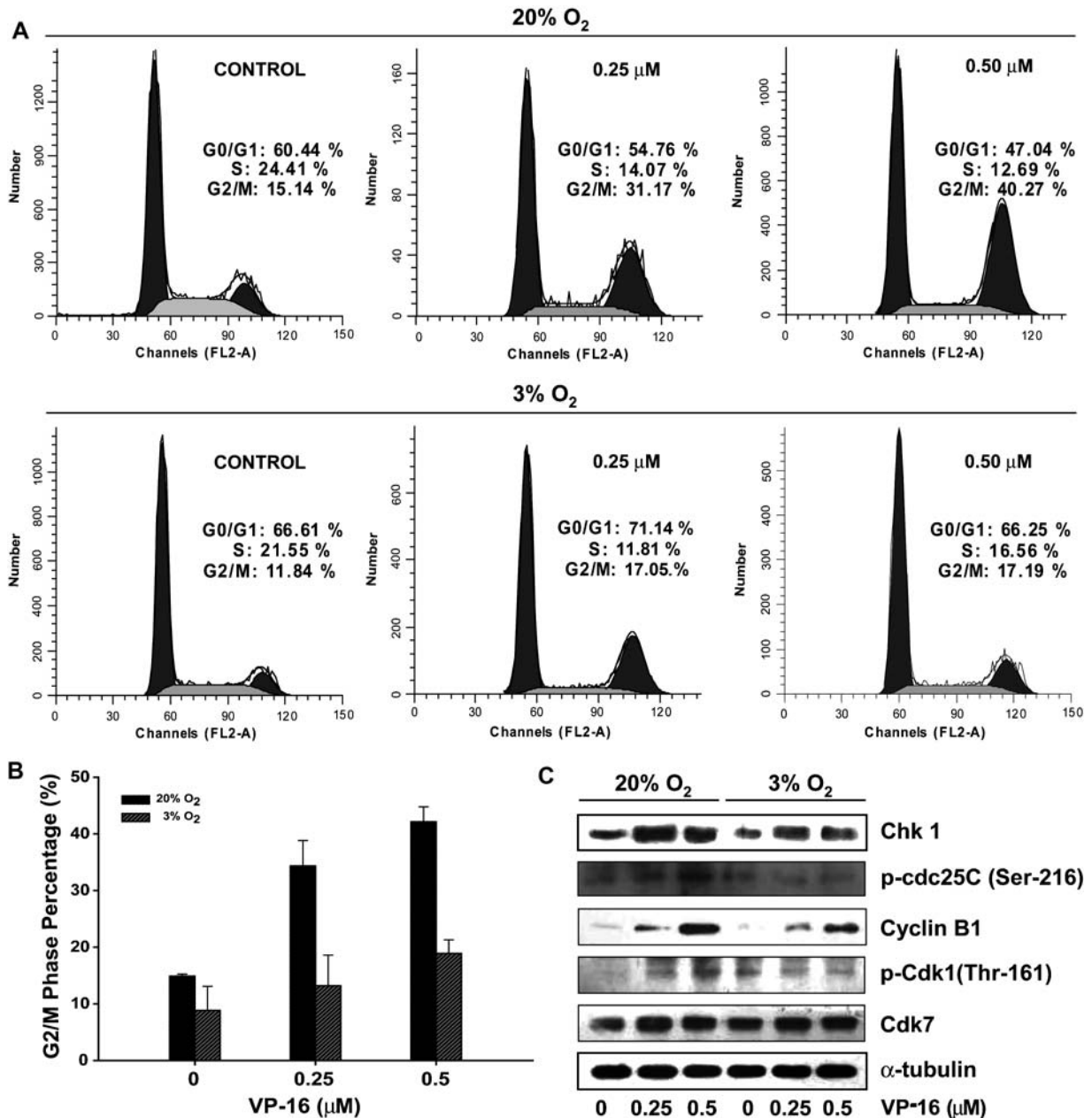


Fig. 2: Hypoxia impaired the ability of VP-16 in inducing G2/M arrest. A/B, Cell cycle profiles of CHP126 cells grown in the absence or presence (0.25 or 0.50 μ M) of VP-16 were examined by flow cytometry. Cells were incubated under normoxic or hypoxic conditions with or without VP-16 for 24 h. C, p-Cdk1(Thr-161), Cyclin B1, CDK7, Chk1 and p-cdc25C (Ser216) was determined by western blotting, using the corresponding specific antibodies respectively. α -tubulin was used to assess the total amount of proteins loaded on the gel

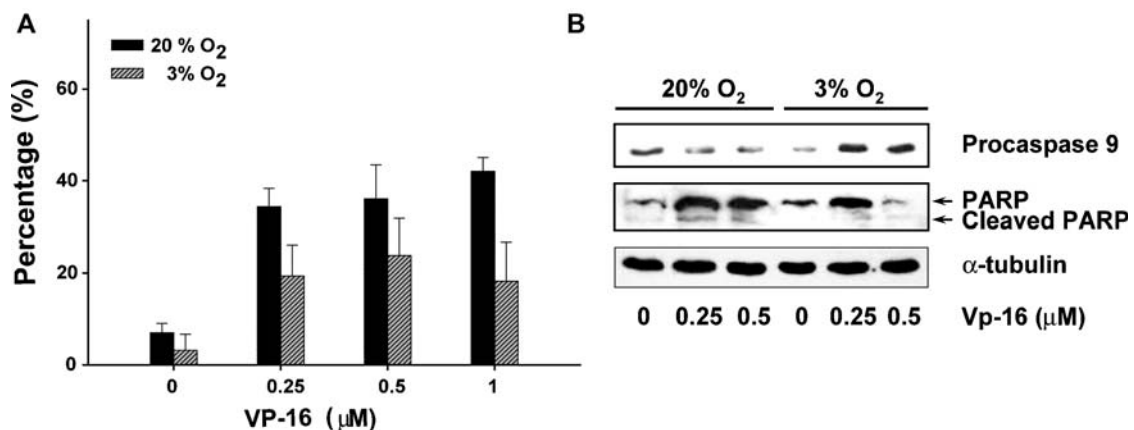


Fig. 3: Hypoxia impaired the ability of VP-16 in inducing apoptosis. A, CHP126 cells were incubated under normoxic or hypoxic conditions with (0.25, 0.50 or 1 μ M) or without VP-16 for 24 hours. Detection of apoptosis by flow cytometry was performed using the Annexin V/PI apoptosis detection kit. B, procaspase 9, PARP and its cleaved 85 kDa fragment were detected in total cell extracts by western blotting, using a specific anti-procaspase 9, anti-PARP respectively. α -tubulin was used as loading protein

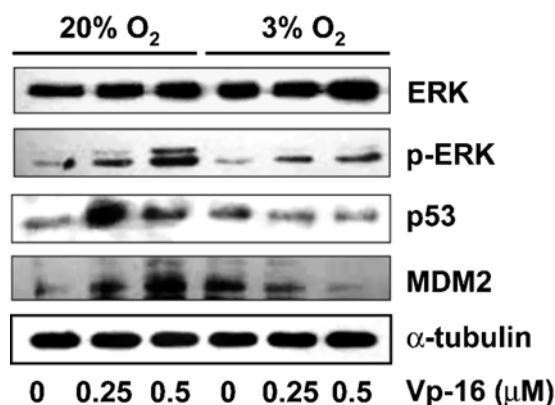


Fig. 4: Hypoxia conferred protection against VP-16 cytotoxicity via ERK and p53. CHP126 cells were exposed to the same treatment as mentioned in the previous figures. Then ERK activation, p53 and MDM2 were detected in total cell extracts by western blotting. α -tubulin was used to assess the total amount of proteins loaded on the gel

7 (Cdk7), which is critical for cell cycle and transcriptional programs (Larochelle et al. 2007; Wang et al. 2007; Yu et al. 2007) and plays an essential role for Cdk1 activation through the phosphorylation on threonine 161, resembled that of p-Cdk1. Moreover, we found an overproduction of total Chk1 (Matsuura et al. 2008) whereas much weaker upregulation was detected in hypoxic counterpart cells. To our expect, the level of p-Cdc25C (Ser 216, inactive form), an important substrate for Chk1, was elevated after VP-16 treatment under normoxic conditions, and this increase in p-cdc25C (Ser 216) coincided with the appearance of Chk1 and was also correlated with the course of G2/M arrest. However, p-Cdc25C changed slightly in hypoxia.

Cysteine proteases (caspases) play an important role in apoptosis induced by DNA damage through the proteolysis of specific targets. A proapoptotic signal activates initiator caspase that activates effector caspases and caspase 9 is induced in response to cytotoxic agents as previously reported (Thornberry and Lazebnik 1998). Also, the appearance of the 85 kDa proteolytic fragment of poly (ADP-ribose) polymerase (PARP) is indicative of caspase activation and apoptosis induction (Duriez and Shah 1997; Boldt et al. 2002), which is readily apparent in response to treatment with clinically relevant doses of VP-16 (Budman et al. 1994; Lowis et al. 1998). Western blot analysis demonstrated that CHP126 cells with VP-16 exposure for 24 h in normoxia did undergo apoptosis by the decrease of procaspase 9 expression and the appearance of PARP cleavage, which consisted with the results by flow cytometric determination (shown in Fig. 3). Accordingly, hypoxia attenuated VP-16-induced apoptosis: an elevated procaspase 9 as well as a marked decrease in PARP cleavage was observed, both of which played an anti-apoptotic role in VP-16 exposure as surrogate markers.

2.5. Hypoxia confers protection via p53 and ERK related pathways

The p53 tumor suppressor protein is activated by DNA damage and other cellular stress, the induction of cell cycle arrest, DNA repair and apoptosis (Ohnishi 2005; Nam et al. 2006; Lin and Wang 2008). In addition, MDM2 is often taken as a reference of p53 alteration, which is activated by p53 and then in turn inhibits p53-mediated transcription, shuttles p53 out of the nucleus, and targets p53 for ubiquitin/proteasome-mediated proteolysis as autoregulatory feedback loop to tightly regulate p53 levels (Lahav et al. 2004; Oren et al. 2002; Moll and Petrenko 2003). As expected, exposing CHP126 cells to either 0.25 or 0.50 μ M VP-16 for 24 h caused an obvious increase in

total p53 as well as MDM2 expression in normoxia. Of note is the repression of the VP-16-induced activation of p53 under hypoxia, besides of the inhibition of its transcriptional targets MDM2 (shown in Fig. 4). Moreover, ERK activation contributes to either cell cycle arrest or apoptosis in response to low or high intensity DNA insults, respectively. In this regard, we sought to explore consequences of such activation in response to DNA damage. After drug exposure, there was a much more significant concentration-dependent enhancement of p-ERK1/2 expression in normoxia as contrast to which we observed in hypoxia (shown in Fig. 4). Taken together, VP-16 modulated cell arrest and/or apoptosis at least in part through a p53- and ERK-dependent mechanism and hypoxia conferred protection partly via the failure of VP-16-induced ERK induction beside the stabilization of p53.

3. Discussion

Regions of tissue hypoxia often arise in aggressive solid tumors, which may impair the treatment efficiency thus frequently causing chemoresistance (Karlsson et al. 2005). As is widely known, chemotherapeutic drugs induce DNA damage and cell cycle arrest, which can, in case of prolongation, induce cell death through different mechanisms. In this end, we studied the effect of hypoxia on VP-16 related cytotoxicity as above-mentioned in neuroblastoma CHP126 cells, which would provide a novel insight into our knowledge on hypoxia-induced chemotherapy resistance and further suggest probable strategy to overcome this resistance.

VP-16 treatment resulted in a cell cycle delay with the cells remaining in G2/M throughout the interval of our experiments. It has been well established that Cdk1/cyclin B complexes are involved in regulation of the G2/M phase and the M phase transition. The activation of Cdk1/cyclin B is also positively regulated by Cdc25, which is well-known to be negatively regulated by phosphorylation on the serine-216 (Ser216) during interphase or in response to DNA damage, and therefore induces G2/M arrest. The Cdk1/cyclin B complex is inactivated by both degradation of cyclin B (Glutzer et al. 1991) and dephosphorylation of Thr-161 (Lorca et al. 1992) during mitosis. In our study, Cdk1 remained phosphorylated at Thr-161 during VP-16-induced G2/M delay, with a simultaneous elevation of Cyclin B1. Chk1 as well as the substrate p-Cdc25C (Ser 216) exhibited elevated protein levels after VP-16 treatment. On the contrary, all the proteins mentioned above changed insignificantly under hypoxia. Both Western Blot results and flow cytometric analysis indicated that VP-16 resulted in G2/M phase arrest of CHP126 cells and that hypoxia promoted resistance to it relative cell cycle arrest by modulating gene expressions.

In the wide range of mammalian cell lines where these effects have been characterized, cell cycle arrest is followed by, ultimately, cell death. VP-16 addition in normoxia induced caspase activation and PARP cleavage indicated the occurrence of apoptosis, while hypoxic environment encouraged drug resistance to cell death. Intriguingly, cyclin B1 and Cdk1 are also known to be regulators of a variety of apoptotic stimuli (Porter et al. 2000; Liu et al. 2007; Higginbottom et al. 2007), and the possibility of a functional link between cell cycle regulatory pathways and apoptosis may give a promising lead. Evidence that Cdk1 activity is required for apoptosis is provided by the observation that the overexpression of a dominant negative Cdk1 mutant blocks Fas/APO1-induced apoptosis in Jurkat cells (Yao et al. 1996) and that cells with a Cdk1 mutant are unable to undergo apoptosis in response to a variety of stimuli when cultured at the restrictive temperature (Shi et al. 1994) and premature activation of cyclin-dependent kinases occurs very early in the apoptotic

process. Others also reported that the cyclin B1 protein may act as a regulator of apoptotic fate following DNA damage, whose accumulation is also known to be a critical component in inducing p53-independent apoptosis (Chiu et al. 2005) and even high expression of cyclin B1 predicts a favorable outcome in patients with follicular lymphoma (Björck et al. 2005). Although the importance of cyclin B1 and Cdk1 activation in the process of apoptosis remains to be explored, it is conceivable that the insufficient accumulation of these proteins due to hypoxia may contribute more or less to VP-16 resistance in CHP126 cells.

DNA damage stimuli, including VP-16, activate mitogen-activated protein kinase (ERK1/2) in primary. It is repeatedly reported that the activation of the ERK pathway has been linked to cell proliferation and survival. However, growing evidences demonstrate that ERK can have a dual effect on proliferation and the activation of ERK can also halt the cell cycle by inducing the expression of cell cycle inhibitor protein such as p21 and p27 (Marshall 1999) or interfere with apoptosis on several levels. ERK activation is required for cell cycle arrest upon DNA damage, and facilitates DNA damage-induced apoptosis. Furthermore, p53 is also implicated in the maintenance of the G2 DNA damage checkpoint apart from its involvement in the G1 control (Taylor and Stark 2001), although the effects of p53 on the G2- to M-phase transition are less well characterized. When overexpressed in the absence of DNA damage, p53 may cause the accumulation of cells in the G2/M phase and thereby inactivate a protective cell cycle checkpoint. In the case of p53-induced apoptosis, the effects were associated with a transcriptionally dependent manner through induction of genes such as bax, or by post-transcriptional mechanisms (Tweddle et al. 2003). In the present study, VP-16 induced stabilization of p53 in neuroblastoma CHP126 cells as previously reported (Krieg et al. 2006; Sermeus et al. 2008), as well as produced ERK activation, indicating that VP-16 modulates the expression of numerous genes at least in part through a p53- and ERK-related mechanism; hypoxic condition, however, conferred protection via inactivation of p53 and ERK against VP-16 treatment. It has also been reported (Tang et al. 2002) that DNA damage-induced ERK activation is independent of p53 which seemed rather appealing as acquisition of p53 mutations has been a controversial area in neuroblastoma research.

Although other mechanisms like the overexpression of P-glycoprotein and MRP1 were previously related to drug resistance (Lee et al. 2006; de Cremoux et al. 2007; Wartenberg et al. 2003), a broader knowledge of drug resistance mechanisms are required before the survival rates can significantly improve as the multifactorial nature of drug resistance. In summary, our findings suggest that hypoxia clearly impaired efficiency of VP-16 treatment in CHP126 cells and thus contributed to resistance via regulating cell responses to both cell cycle and apoptosis. Moreover, we explored to demonstrate the activation of p53 and ERK pathways were involved in the mechanism of this resistance, aiding in more understanding of hypoxia-relevant chemoresistance.

4. Experimental

4.1. Reagents and antibodies

VP-16 was purchased from Sigma (St. Louis, MO), a stock solution of 10 mM was prepared with dimethyl sulfoxide (DMSO) and stored at -20°C . The stock solution was further diluted with the appropriate medium immediately before use. The primary antibodies to procaspase-9, PARP, ERK1/2, p-ERK1/2, p53, MDM2, Chk1, cyclin B1, p-Cdk1, p-Cdc25C, α -tubulin and HRP-labeled secondary anti-goat, anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). ECL, a western blot detection reagent, was purchased from Amersham Biosciences (Piscataway, NJ).

4.2. Cell culture and establishment of hypoxia culture condition

Human CHP126 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), containing 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Sigma, St. Louis, MO). The cells were kept in a humidified atmosphere of 5% CO_2 and 20% O_2 at 37°C . Environmental hypoxic conditions (3% O_2) were achieved in an airtight humidified chamber continuously flushed with a gas mixture containing 5% CO_2 and 95% N_2 .

4.3. Cytotoxicity assay

Cell proliferation was determined by a standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. CHP126 cells were seeded in 96-well plates (5000 cells/well), and were cultured in normoxia and hypoxia respectively. The hypoxic cells were allowed to attach 1 day before the exposure to VP-16 at concentrations specified or vehicle control alone. Plates were assayed at 24, 48 and 72 h after the initiation of drug addition. All experiments were repeated three times.

4.4. Flow cytometry

For determining phase distribution of DNA content, propidium iodide (PI) staining was employed. Briefly, cells collected were washed twice and fixed in 70% ice-cold ethanol overnight. After washed twice with ice-cold PBS, cell pellet was resuspended in PBS buffer plus 0.5 mg/ml RNaseA at 37°C for 30 min prior to 5 mg/ml PI (Sigma, St. Louis, MO) at room temperature in the dark for another 30 min. Analysis was performed with FACSscan flow cytometer (BD Biosciences, San Jose, CA). Annexin V-fluorescein and PI staining was used according to the descriptions in the commercial apoptosis detection kit (BD Pharmingen, San Diego, CA) followed by flow cytometry for determination of apoptosis.

4.5. Western analysis

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, 0.1 mM PMSF). Total protein concentrations of whole cell lysates were determined using the Bio-Rad reagent (Bio-Rad) BCA method (PIERCE, Rockford, IL). 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), transferred to Immobilon-P Transfer Membrane (Millipore Corporation, Billerica, Massachusetts), and probed with primary antibodies and then HRP-labeled secondary antibodies. Proteins were visualized using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

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