Hypoxia Induces Paclitaxel-Resistance through ROS Production

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Abstract – Oxygen supply into inside solid tumor is often diminished, which is called hypoxia. Many gene transcriptions were activated by hypoxia-inducible factor (HIF)-1 α . Here, we investigated the effect of hypoxia on paclitaxel-resistance induction in HeLa cervical tumor cells. When HeLa cells were incubated under hypoxia condition, HIF-1 α level was increased. In contrast, paclitaxel-mediated tumor cell death was reduced by the incubation under hypoxia condition. Paclitaxel-mediated tumor cell death was also inhibited by treatment with DMOG, chemical HIF-1 α stabilizer, in a dose-dependent manner. A significant increase in intracellular ROS level was detected by the incubation under hypoxia condition. A basal level of cell density was increased in response to 10 nM H₂O₂. HIF-1 α level was increased by treatment with various concentration of H₂O₂. The increased level of HIF-1 α by hypoxia was reduced by the treatment with N-acetylcysteine (NAC), a well-known ROS scavenger. Paclitaxel-mediated tumor cell death was increased by treatment with NAC. Taken together, these findings demonstrate that hypoxia could play a role in paclitaxel-resistance induction through ROS-mediated HIF-1 α stabilization. These results suggest that hypoxia-induced ROS could, in part, control tumor cell death through an increase in HIF-1 α level.

Keywords: HeLa cell, Hypoxia, HIF-1 α , ROS, Paclitaxel

INTRODUCTION

Hypoxia in cells and tissues contributes to the gene expression that participates in angiogenesis, vasorelaxation, metabolism, and cell proliferation/survival and resistance to anti-cancer drugs (Ke and Costa, 2006; Dong and Shin, 2008). Oxygen supply to the tumor cells is often diminished because tumor cells in solid tumor grow faster than the endothelial cells that build up the blood vessels (Folkman, 1990). Hypoxia in solid tumors is one of major causes to resistance against most anticancer drugs and, importantly, appears to accelerate malignant progression and increase metastasis (Hockel and Vaupel, 2001; Chan *et al.*, 2007; Selvendiran *et al.*, 2009). Transcription factor, hypoxia-inducible factor alpha (HIF)-1 plays an essential role in the cellular response to changes in oxygen tension (Papandreou *et al.*, 2006).

HIF-1 is composed of two subunits, HIF-1 α and HIF-1 β (Wang *et al.*, 1995). While HIF-1 β subunit is constitutively

expressed, HIF-1 α subunit is inducible to hypoxia. Therefore, HIF-1 activity depends on the availability of the HIF-1 α subunit and provides a molecular basis for the adaptations of cancer cells to hypoxia (Semenza, 2000). HIF-1 α is rapidly degraded in normoxia (Salceda and Caro, 1997) but the degradation of HIF-1 α is inhibited in hypoxia (Huang *et al.*, 1998). Many hypoxia-inducible genes are controlled by HIF-1 α (Wang *et al.*, 1995).

Reactive oxygen species (ROS) can be produced by serum-deprivation, LPS-stimulation and peroxiredoxin II-deficiency (Moon, 2008; Kim and Moon, 2009). ROS cause tissue injury in one hand and promote tissue repair in another hand (Maulik and Das, 2002). Hypoxia-induced mitochondrial ROS have been shown to be necessary for the stabilization of HIF-1 in hypoxic cells (Brunelle *et al.*, 2005; Guzy *et al.*, 2005; Kaelin, 2005; Mansfield *et al.*, 2005). Hypoxia activates gene transcription via a mitochondria-dependent signaling process that involves increased level of ROS (Chandel *et al.*, 1998). HIF-1 α expression can be also induced through H₂O₂ production (Gao *et al.*, 2004).

Paclitaxel is likely to play a major role as cancer chemotherapy in human malignancies. Paclitaxel blocks cells in the G2/M phase of the cell cycle and such cells are unable

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to form a normal mitotic apparatus. Microtubule cytoskeleton is reorganized by paclitaxel treatment and then it turns into extensive parallel arrays or stable bundles of microtubules in cells growing in tissue culture (Rowinsky *et al.*, 1992; Martin, 1993; Horwitz, 1994). Paclitaxel-resistance is induced by ROS production and HIF-1 α stabilization during cancer treatment (Kim *et al.*, 2008). However, little is known about whether hypoxia-induced ROS could lead to the drug-resistance to paclitaxel.

Here, we investigated the effect of hypoxia on paclitaxel-resistance induction in HeLa cervical tumor cells. Our data showed that hypoxia could play a role in tumor cell resistance to anticancer agents like paclitaxel through ROS-mediated HIF-1 α stabilization. It suggests that hypoxia-induced ROS could, in part, control tumor cell death to anti-cancer agents through HIF-1 α stabilization.

MATERIALS AND METHODS

Reagents

Antibodies which are reactive with HIF-1 α came from

BD Biosciences (San Jose, CA). 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) was from Molecular Probes (Eugene, OR). Dimethyloxallyl glycine (DMOG) was from Cayman Chemical (Ann Arbor, MI). Except where indicated, all other materials are obtained from the Sigma chemical company (St. Louis, MO).

Cell culture

HeLa cells were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) cell bank (Taejeon, Korea). Cells were maintained and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Kansas City, MO), 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin.

MTT assay

We quantified paclitaxel-treated cell density using colorimetric assay described for measuring intracellular succinate dehydrogenase content with MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Denizot

Fig. 1. Paclitaxel-mediated tumor cell death was decreased under hypoxia condition. (A) Cells were incubated in hypoxia condition for 0.5, 1, 1.5, 3, and 6 h. Cell lysates were prepared and HIF-1 α was detected by western blot analysis. (B) Cells were incubated with 2 μ M paclitaxel for an appropriate time under normoxia or hypoxia condition. Then, cells were fixed and stained with propidium iodide (50 μ g/ml) with RNase (25 μ g/ml). Hypodiploid cells were analyzed with flow cytometry. * p < 0.05, ** p < 0.01, hypodiploid cell population was significantly different from control in normoxia or hypoxia condition. & p < 0.05, && p < 0.01, hypodiploid cell population in hypoxia condition was significantly different from those in normoxia at each time point. (C) Cells were incubated with various concentrations of paclitaxel for 48 h in normoxia or hypoxia condition. Cell density was measured by MTT assay. Data represent mean $\pm$ SED. * p < 0.05, ** p < 0.01, cell density in paclitaxel-treated group was significantly different from control. # p < 0.05, ## p < 0.01, cell density in normoxia condition was significantly different from control in hypoxia condition at each concentration of paclitaxel.

and Lang, 1986). Confluent cells were cultured with various concentrations of paclitaxel for 48 hour. Cells were then incubated with 50 $\mu\text{g}/\text{ml}$ of MTT at 37°C for 2 hour. Formazan formed were dissolved in dimethylsulfoxide (DMSO). Optical density (OD) was read at 595 nm.

Western blot analysis

Cells were lysed in ice-cold lysis buffer, containing 0.5% Nonidet P-40 (vol./vol.) in 20 mM Tris-HCl, at a pH of 8.3; 150 mM NaCl; protease inhibitors (2 $\mu\text{g}/\text{ml}$ aprotinin, pepstatin, and chymostatin; 1 $\mu\text{g}/\text{ml}$ leupeptin and pepstatin; 1 mM phenylmethyl sulfonyl fluoride (PMSF); and 1 mM Na_4VO_3 . Lysates were incubated for 30 minutes on ice prior to centrifugation at 14,000 rpm for 5 minute at 4°C . Proteins in the supernatant were denatured by boiling for 5 minute in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose membranes. Following this transfer, equal loading of protein was verified by Ponceau staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.5% Tween 20), then incubated with the indicated antibodies. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence (ECL) (Pierce, Rockford, IL).

Flow cytometry analysis

Level of reactive oxygen species (ROS) was determined by incubating HeLa cells with 10 μM DCF-DA for 30 minutes at 37°C . The cells were detached by the incubation with 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA) for an additional 5 minute at 37°C . Fluorescence intensity of 10,000 cells was analyzed by CELLQuest™ software in FACScalibur™ (Becton Dickinson, San Jose, CA).

Statistical analyses

Experimental differences were tested for statistical significance using ANOVA and Students' t-test. p -value of < 0.05 or < 0.01 was considered to be significant.

RESULTS

Paclitaxel-mediated tumor cell death was reduced by HIF-1 α stabilization.

Given that actin-sequestering protein, thymosin beta 4 (TB4), induced a similar hypoxia condition and tumor cell density was increased by treatment with TB4 proteins (Oh *et al.*, 2006; Oh *et al.*, 2008), we investigated whether tu-

mor cell density could be affected by paclitaxel treatment under hypoxia. HIF-1 α stabilization increased under our experimental hypoxia condition (Fig. 1A). To examine the effect of hypoxia on paclitaxel-mediated tumor cell death, we incubated HeLa cells in the presence of paclitaxel under normoxia or hypoxia condition. A time-dependent increase in paclitaxel-induced hypodiploid cell formation was twice as much lower under hypoxia conditions compared to normoxia (Fig. 1B). When we also measured HeLa cell density rate in response to paclitaxel treatment by MTT assay, it was 40% higher under hypoxia conditions relative to normoxia (Fig. 1C). In addition, paclitaxel-mediated tumor cell death was inhibited by treatment with DMOG, chemical HIF-1 α stabilizer, in a dose-dependent manner (Fig.

Fig. 2. Chemical HIF-1 α stabilizer, dimethyloxallyl glycine (DMOG), inhibited paclitaxel-mediated tumor cell death. (A) Cells were incubated in the presence or absence of DMOG in normoxia condition. Cell lysates were prepared and HIF-1 α was detected by western blot analysis. (B) Cells were incubated with various concentrations of paclitaxel for 48 h in the presence or absence of DMOG under normoxia condition. Cell density was measured by MTT assay. Data represent mean $\pm$ SED. * $p < 0.05$, ** $p < 0.01$, cell density in paclitaxel-treated group was significantly different from control. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, cell density in DMOG-untreated group was significantly different from DMOG-treated group.

Fig. 3. Hypoxia increased ROS production. (A) Cells were incubated in hypoxic condition for 1 h in the presence or absence of NAC, ROS scavenger. Then, cells were incubated with DCF-DA for 30 min to detect intracellular ROS. Cells were analyzed with flow cytometry. (B) Cells were incubated for 24 h in the presence or absence of 10 nM H_2O_2 under normoxia condition. Cell density was measured by MTT assay. Data represent mean $\pm$ SED. * $p < 0.05$, cell density in H_2O_2 -treated group was significantly different from control.

2). These findings suggest that resistance to paclitaxel-mediated tumor cell death could be induced by HIF-1 α stabilization under hypoxia condition.

ROS scavenger, NAC, aggravated paclitaxel-mediated tumor cell death under hypoxia

Given that hypoxia-induced mitochondrial ROS have been shown to be necessary for the stabilization of HIF-1 in hypoxic cells (Guzy *et al.*, 2005; Kaelin, 2005; Mansfield *et al.*, 2005), we examined if ROS could be produced under hypoxia. As shown in Fig. 3A, intracellular ROS production was increased under hypoxia, which was decreased by pretreatment with N-acetylcysteine (NAC). Our previous data showed that tumor cell density was de-

Fig. 4. HIF-1 α level was increased by treatment with H_2O_2 . (A) Cells were incubated with 40 μ M H_2O_2 for an appropriate time. (B) Cells were incubated with various concentration of H_2O_2 for 1 h. Then, cell lysates were prepared and HIF-1 α was detected by Western blot analysis.

creased by the incubation with 20 mM or 40 mM NAC (Kim *et al.*, 2008). Fig. 3B showed that tumor cell density was increased when cells were treated with 10 nM H_2O_2 . In contrast, tumor cell density was decreased in response to H_2O_2 treatment at concentrations higher than 10 μ M H_2O_2 (data not shown). We also examined the effect of H_2O_2 on HIF-1 α stabilization. As shown in Fig. 4A, 40 μ M H_2O_2 enhanced HIF-1 α level in a time-dependent manner. HIF-1 α level was also increased by treatment with various concentration of H_2O_2 (Fig. 4B). When cells were pretreated with NAC, HIF-1 α stabilization was reduced (Fig. 5A). It implicates that paclitaxel-mediated tumor cell death could be affected by hypoxia-induced ROS production through HIF-1 α stabilization.

To examine the effect of hypoxia-induced ROS on paclitaxel-mediated cell death, cells were incubated with various concentrations of paclitaxel in the presence or absence of NAC under hypoxia. As shown in Fig. 5B, paclitaxel-mediated tumor cell death was aggravated by the incubation with NAC. These findings suggest that paclitaxel-resistance could be established by hypoxia-induced ROS production and a consequent HIF-1 α stabilization.

DISCUSSION

Hypoxia occurs naturally in physiological settings, as

Fig. 5. Hypoxia-induced paclitaxel-resistance was reduced by treatment with NAC. (A) Cells were pretreated with 10 mM NAC for 12 h and then incubated under hypoxic condition for 1 h in the presence or absence of NAC. Cell lysates were prepared and HIF-1 α was detected by Western blot analysis. (B) Cells were pretreated with 10 mM NAC for 12 h and then incubated with paclitaxel for 48 h in the presence or absence of NAC under hypoxia condition. Cell density was measured by MTT assay. Data represent mean $\pm$ SED. * $p < 0.05$, cell density in paclitaxel-treated group was significantly different from control. [#] $p < 0.05$, cell density in paclitaxel- and NAC-treated group was significantly different from paclitaxel-treated but NAC-untreated group.

well as in pathophysiological conditions (Simon, 2006). Hypoxia in solid tumors is not only a major problem for radiation therapy but also leads to resistance against most anticancer drugs (Hockel and Vaupel, 2001; Chan *et al.*, 2007; Selvendiran *et al.*, 2009). Adaptations of cancer cells to hypoxia are critical for the establishment of a primary tumor and its progression to the lethal phenotype (Semenza, 2000) by stimulating HIF-1 and by activating transcription of target genes (Zhong *et al.*, 1999). Previous reports showed that physiological or pathophysiological hypoxia increased ROS production and HIF-1 α stabilization (Simon, 2006; Guzy *et al.*, 2008). Here, we inves-

tigated the effect of hypoxia on paclitaxel-resistance induction in HeLa cervical tumor cells through ROS production. We found that HeLa cells increased HIF-1 α level (Fig. 1A) and produced ROS (Fig. 3) under hypoxia. ROS-mediated HIF-1 α stabilization was re-affirmed by treatment with NAC under hypoxia (Fig. 5A). Our data also showed that hypoxia could play a role in tumor cell resistance to anti-cancer agents like paclitaxel through ROS-mediated HIF-1 α stabilization.

A few explanations are possible for the role of hypoxia-induced ROS on paclitaxel-resistance through HIF-1 α stabilization. One explanation is that hypoxia-induced ROS could reduce the apoptosis by paclitaxel treatment. This was supported by the data that 10 nM H₂O₂ increased tumor cell density (Fig. 3B) and NAC treatment reduced cell density (Kim *et al.*, 2008). The other explanation is that ROS activate NF- κ B-dependent signaling pathway and directly increased not only HIF-1 α expression (Jung *et al.*, 2003; Belaiba *et al.*, 2007) but also other genes inducing drug-resistance. Nuclear translocation of NF- κ B can be triggered by exposure to H₂O₂ (Janssen-Heininger *et al.*, 2000) and facilitated by conditions associated with increased intracellular oxidative stress (Toledano and Leonard, 1991). NF- κ B can be activated by Ras/ERK/NF- κ B pathways (Han *et al.*, 2006). Therefore, hypoxia-induced ROS production may be involved in Ras/ERK/NF- κ B pathways to enhance HIF-1 α expression. PI3K/AKT pathway is another candidate to increase HIF-1 α expression. Hypoxia induces HIF-1 α mRNA expression via the PI3K/AKT pathway and activation of NF- κ B (Belaiba *et al.*, 2007). It could not be ruled out that ROS also affects other molecular pathways in addition to HIF-1 α stabilization. p70S6K1 may be another molecule that is activated by ROS produced from treatment with EGF or insulin (Liu *et al.*, 2006; Zhou *et al.*, 2007). Paclitaxel-resistance was also induced by thymosin beta 4 (TB4) gene-overexpression (Oh *et al.*, 2006) and TB4 proteins induced HIF-1 α stabilization (Oh *et al.*, 2008). Therefore, HIF-1 α stabilization under all hypoxia conditions may interfere with the effects of anti-tumor agents like paclitaxel and induce the drug-resistance through ROS production.

In conclusion, even though we could not explain all of the mechanisms responsible for the regulation of cell death decreased by hypoxia-mediated ROS production in regards to paclitaxel-resistance induction, our data clearly demonstrate that ROS stabilized HIF-1 α , which inhibits paclitaxel-mediated tumor cell death and may have induced anti-tumor drug-resistance. These findings suggest that hypoxia-induced ROS could, in part, control tumor cell death through an increase in HIF-1 α level.

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