

Original Article

Effects of NF- κ B and hypoxia on the biological behavior of Y79 retinoblastoma cells

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Abstract: We aimed to investigate the influence of nuclear factor- κ B (NF- κ B) on the biological behavior of Y79 retinoblastoma cells exposed to hypoxia and its possible mechanism. The cells were administrated with hypoxia, and/or 5 μ M pyrrolidine dithiocarbamate (PDTC) (a selective NF- κ B inhibitor) to inhibit the NF- κ B activity, expressions of NF- κ B was measured by western blot, and the translocation of NF- κ B was detected. To examine the proliferation of Y79 cells, MTT assay was applied. Transwell assay was used to detect the invasion and migration ability of cells. The expressions of molecules involved in invasion was analyzed including HIF-1 α , MMP-2, 9, and VEGF. We found that hypoxia significantly activated NF- κ B activity. While once the NF- κ B was inhibited, the proliferation, invasion and migration ability of Y79 cells were also blocked. Interestingly, the expressions of invasion-involved molecules elevated by hypoxia induction were also decreased when NF- κ B was inhibited. Hypoxia could significantly change the adhesive and invasive ability of Y79 retinoblastoma cells, NF- κ B signal might be one of the main mediators for these hypoxia induced cell changes of biological behavior via downregulation of HIF-1 α and the invasion related molecules, and the mechanism still needs further investigation.

Keywords: Hypoxia, NF- κ B, biological behavior, retinoblastoma, Y79 cells

Introduction

Retinoblastoma (RB) is a common intraocular malignancy tumor of infancy and childhood with an incidence of 1/15,000 to 1/20,000 births, representing approximately 4% of all pediatric malignancies [1, 2]. Most retinoblastomas are caused by sporadic somatic mutations in the RB1 gene while about one-third arise in infants with germline mutations. Progressive advances in the understanding of retinoblastoma pathogenesis continue to lead treatment strategies. Treatment approaches have changed over the past decade, and the current therapies for Rb include intravenous chemoreduction, enucleation, transpupillary thermotherapy, cryotherapy, thermotherapy, laser photocoagulation, brachytherapy, plaque radiotherapy, orbital exenteration, external beam radiotherapy and chemotherapy (systemic and local delivery) [3-6], however, unfavorable therapeutic response is still a quest in retinoblastoma. Children with Rb are at risk for

three life-threatening problems, including metastasis of Rb, intracranial neuroblastic malignancy (trilateral Rb), and second primary tumors [7].

Hypoxia is a key feature of tumor and inflammation identified as a significant step in the tumor progression and a novel target for future treatments, and it contributes to poor prognosis and resistance to conventional therapy. Hypoxic tumor microenvironment is one of the factors that determine the therapeutic response in many tumors including retinoblastoma [8, 9]. Some studies have identified a relationship between tumor hypoxia and distant metastatic disease, and previous research revealed that RB with invasion of the choroid, optic nerve, and/or orbit strongly correlates with increased oxidative stress [10, 11]. Nuclear Factor kappa B (NF- κ B) is a key regulator of cellular processes involved in the immune response, differentiation, cell proliferation, and apoptosis. NF- κ B acts as 'first responder' to various types of cel-

NF- κ B and hypoxia on the biological behavior of Y79 cells

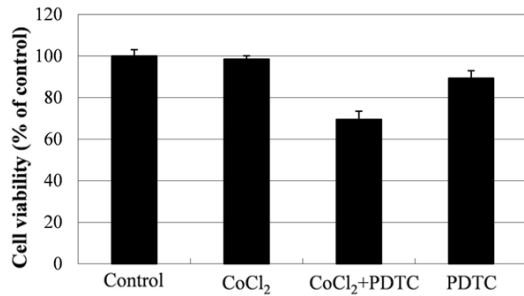


Figure 1. Effects of hypoxia and NF- κ B inhibition on cell viability of Y79 cells.

ular stress as a proinflammatory transcription factor [12]. And NF- κ B was previously found to be over-expressed in solid tumors and to be activated by hypoxia [13]. However, whether hypoxia-induced NF- κ B activation contributes to tumor cell biological behavior change (like adhesive and invasive ability change) is still unknown.

In this study, we aimed to investigate the influence of nuclear factor- κ B (NF- κ B) and hypoxia on the biological behavior of Y79 retinoblastoma cells and the possible mechanism in vitro.

Materials and methods

Cell line and cell culture

Human retinoblastoma cell lines Y79 (American Type Culture Collection, USA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin-streptomycin (100 IU/ml-100 μ g/ml), and 10 mM Hepes buffer, and 2.5 μ g/mL amphotericin B at 37°C in a humid atmosphere (5% CO₂-95% air).

MTT assay for cell proliferation

Cell viability was measured by MTT assay. Briefly, cells were seeded into 96-well plates (3.0×10^3 /well) and cultured in the presence or absence of CoCl₂ (200 μ M, Sigma, St. Louis, MO, USA) and/or PDTC (5 μ M) (control group, CoCl₂ group, PDTC group, and CoCl₂ + PDTC group). MTT solution (5 mg/mL) was added to each well for 4 h. Then 150 μ L DMSO was added to dissolve the formazan precipitate before absorbance was measured at 570 nm using a THERMO^{max} microplate reader. Cell viability was expressed as the percentage to the untreated control.

Transwell assay

A 24-well transwell chamber (Corning, NY, USA) was used to evaluate the motility and invasive ability of Y79 cells exposed to different treatment. The upper surface of polycarbonate filters with 8- μ m pores was coated with 100 μ g of the Matrigel (Collaborative Biomedical, USA). Cancer cells were submitted to different treatment for 24 h at 37°C in a CO₂ incubator and then detached and resuspended in serum-free RPMI-1640 medium. A suspension of cells (1×10^4 cells/100 μ l) was placed in the upper chambers. The lower chambers were filled with 500 μ l of RPMI-1640 medium. After 8 h of incubation, cells remaining on the upper surface of the filters were removed by wiping with cotton swabs, and the cells which had migrated onto the lower surface were stained with Giemsa. The number of cells on the lower surface of the filters was counted under a microscope at a magnification of $\times 200$ (Olympus IX51, Olympus Corporation, Japan), the average number of five random selected field was taken as the number of invasion and metastasis. Triplicate experiments with triplicate samples were performed [13].

Western blotting analysis

Y79 cells were lysed in buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, and 1:500 protease inhibitor mixture (Sigma-Aldrich, USA). Samples of 30 μ g of protein per lane were fractionated by 10% SDS-PAGE. The proteins were electro-transferred onto PVDF membranes and then protein levels were detected using dilutions of the primary antibodies. The primary antibodies were washed in 0.05% Tween-20/PBS and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Millipore, USA), and quantified by densitometry using a ChemiDoc XRS + image analyzer (Bio-Rad, USA). Densitometric analyses of bands were adjusted with β -actin as loading control. Triplicate experiments with triplicate samples were performed.

Immune-fluorescence

Y79 cells were cultured in 96-well plates (Corning Costar, Tewksbury, MA, USA). At the

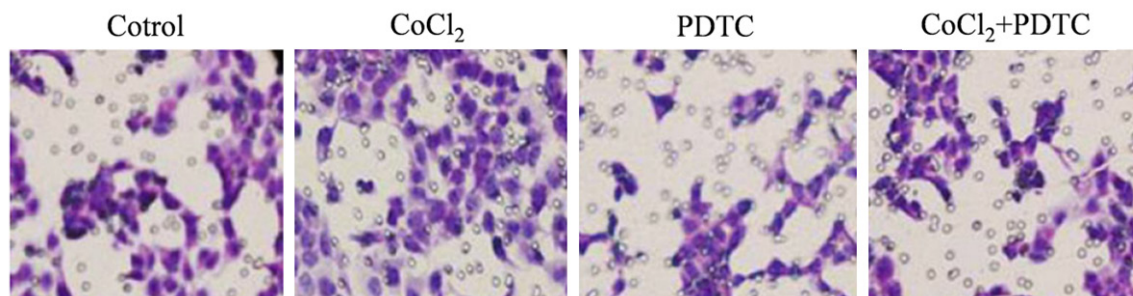


Figure 2. Effects of hypoxia and NF-κB inhibition on invasion and migration ability of Y79 retinoblastoma cells. The number of cells passing through the filters was counted after staining with Giemsa (magnification × 200).

Table 1. Number of invasion and metastasis in each group (mean ± SD)

Group	Control	CoCl ₂	PDTC	CoCl ₂ + PDTC
Number of invasion and metastasis	102 ± 10	135 ± 15**	83 ± 9*.#	98 ± 7

*P < 0.05 vs control; **P < 0.01 vs control; #P < 0.05 vs CoCl₂ + PDTC group.

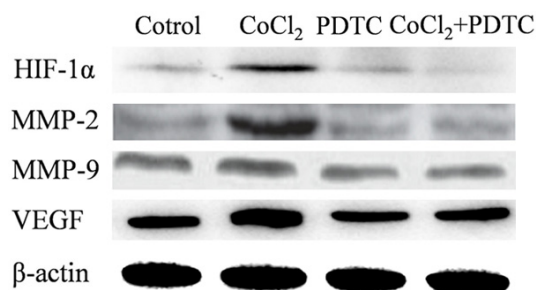


Figure 3. Western blot analysis for HIF-1α, MMP-2, MMP-9 and VEGF in Y79 retinoblastoma cells. The levels of HIF-1α, MMP-2, MMP-9 and VEGF were elevated under hypoxia condition, PDTC neutralized the abnormal upregulation of these molecular.

end of the treatment, cells were fixed with ice-cold methanol/acetone for 20 minutes, permeabilized for 25 min, blocked for 30 min and incubated with primary antibodies against NF-κB p65 (1:100 dilution; Cell Signaling Technology) overnight at 4°C. Then cells were incubated with FITC-conjugated secondary antibody (1:500 dilution; ZSGB-BIO, Beijing, China) at 37°C for 1 h and Hoechst 33258 for 10 min. Cells were finally observed through an inverted fluorescence microscope (Olympus IX70, Tokyo, Japan).

Statistical analysis

Data was described as the mean ± S.D and analyzed by one-way ANOVA. A p value < 0.05 was considered statistically significant. Stati-

tical analysis was performed using the SPSS/Win13.0 software (SPSS, Inc., Chicago, IL).

Results

Detection of retinoblastoma cell growth

To study the effect of hypoxia and NF-κB inhibition on the growth of cancer cells, Y79 cells were exposed to CoCl₂ (200 μM) and/or PDTC (5 μM) and their proliferations were estimated by MTT assay. There is no significant difference between hypoxia and control group (P > 0.05). PDTC treatment alone slightly inhibited cell growth compared to control group (P < 0.05), while under hypoxia condition the growth inhibition effect of PDTC was significantly elevated, and the cell viability of CoCl₂ + PDTC group was the lowest among the four groups (**Figure 1**).

Cell invasion and migration

The effect of hypoxia and NF-κB on invasion and migration of Y79 cells was examined using an invasion assay with Matrigel-coated filters, and the number of cells pass through the basement membrane represents the activity of invasion and migration of cancer cells. As shown in **Figure 2** and **Table 1**, the activity of invasion and migration of Y79 cells was markedly elevated under hypoxia condition (CoCl₂ 200 μM). And our results also showed that PDTC could inhibit cell invasion and migration both under hypoxia and normal condition.

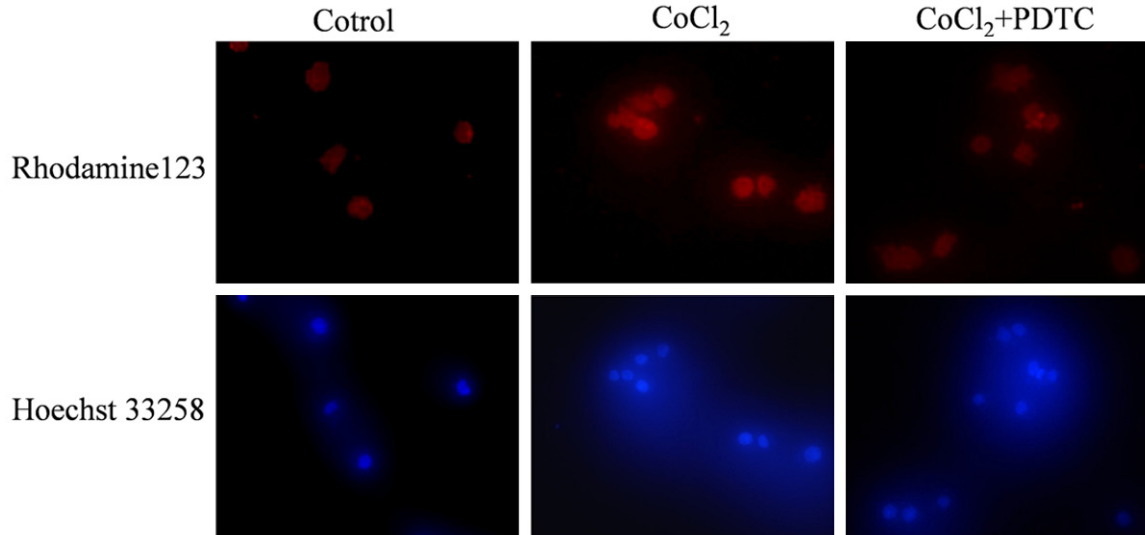


Figure 4. PDTC inhibited the CoCl₂-hypoxia induced nuclear translocation of NF- κ B p65. The sub-cellular localization of NF- κ B p65 was investigated by immune-fluorescence through primary antibodies to NF- κ B p65 and Rhodamine123-conjugated secondary antibody (above). Nuclear was stained with Hoechst 33258 (below) (magnification \times 200).

Molecular analysis by western blotting

The effects of hypoxia and NF- κ B on the expression of HIF-1 α , MMP-2, 9 and VEGF was also evaluated using Western blot assay in Y79 cells exposed to CoCl₂ and/or PDTC treatment. Oxidative stress has been extensively implicated in the process of tumor development, in which process HIF-1 α is an important mediator. As shown in **Figure 3**, HIF-1 α was significantly elevated under hypoxia condition (276.4% of control, $P < 0.05$), and the expression level was then decreased when the specific NF- κ B inhibitor PDTC was added (91.8% of control, $P > 0.05$).

MMP-2, 9 and VEGF are also important cytokines in the development, invasiveness and migration process, which play key roles in cancer cell metastasis. In this study, we observed that the levels of MMP-2, MMP-9 and VEGF in Y79 cells were significantly increased by oxidative stress stimulation (296.9%, 187.6%, 217.8% of control, respectively, $P < 0.05$), and PDTC could decrease the elevated protein levels.

Translocation of NF- κ B

NF- κ B p65 was measured by immuno-fluorescence (control, CoCl₂, and CoCl₂ + PDTC group). In Y79 cells treated with 200 μ M CoCl₂ for 24

hours, NF- κ B p65 signal located in the nucleus was stronger than control group; the nuclear NF- κ B was significantly elevated. While in control cells, the NF- κ B signal was weaker and was mostly found in the cytoplasm (**Figure 4**). Then, to inhibit the activation of NF- κ B in Y79 cells under CoCl₂-hypoxia conditions, Y79 cells were treated with PDTC, and the results showed that when PDTC was added, nucleus-located NF- κ B was decreased again.

Discussion

This study is the first to demonstrate that inhibition of NF- κ B could significantly reduce hypoxia and retinoblastoma cell invasion and migration ability. The key findings of our study include: (a) hypoxia significantly activated NF- κ B activity; (b) once the NF- κ B was inhibited, the proliferation, invasion and migration ability of Y79 cells were also blocked; (c) the expressions of invasion-involved molecules elevated by hypoxia induction such as HIF-1 α , MMP-2, 9 and VEGF were also decreased when NF- κ B was inhibited.

Hypoxia is a frequently encountered feature of the cellular microenvironment in tumor development and metastasis, the presence of hypoxia can significantly affect the process of tumor progression, metastasis and angiogenesis and other physiological processes [14]. However,

the mechanism relating hypoxia to tumor characteristics still remains unclear. As previously reported, hypoxia is one of the fundamental biological phenomena that are intricately associated with the development and aggressiveness of a variety of solid tumors [15]. In this research we observed that hypoxia stimulation did not affect cell viability or proliferation of Y79 cells, but increase cell invasion and migration ability.

Hypoxia-inducible factor-1 (HIF-1) is a key transcription factor activated by low oxygen tensions. NF- κ B is also a critical transcription factor in various cancers that regulates genes associated with a variety of cellular functions. Both HIF-1 α and NF- κ B are transcription factors very frequently activated in tumors during hypoxia that regulates tumor growth, progression and resistance to chemotherapy by regulating transcription of many genes. It has been reported that there is a positive correlation between HIF-1 α and NF- κ B in surgical colorectal cancer specimens and gastric tumor [16-18]. The results of the present study showed that hypoxia activated NF- κ B as evidenced by translocation of NF- κ B assay, in which NF- κ B p65 signal located in the nucleus was much stronger under hypoxia condition compared to normal condition. When PDTC was used to block NF- κ B, we found that the expression of HIF-1 α was also inhibited which is consistent with previous reports. So we confirmed the correlation between HIF-1 α and NF- κ B in retinoblastoma cells under hypoxia condition.

Mechanisms of hypoxia-related angiogenesis are important for tumor metastasis. Factors that are related to angiogenesis during hypoxia include VEGF, MMPs, and other cytokines [19, 20]. VEGF is the most important vascular endothelial growth factor to activate angiogenesis and enhance vascular permeability in the process of tumor invasion and metastasis. The expression and the activity of MMP-2 and MMP-9 in the malignant tumors are particularly important for the metastatic spread of cancer cells among members of the MMP family. MMP-2 and MMP-9 primarily degrade type IV collagen which is a major component of the basement membrane during cell metastasis [21]. It was found that HIF-1 α and NF- κ B activation was significantly correlated with VEGF protein expression, and NF- κ B could also promote expression of several matrix metalloproteinases

(MMPs), including MMP-2, -3, and -9 [17, 22]. The cross talk of MMPs and VEGF and hypoxia has also been discussed previously [23, 24]. In this study, we evaluated effects of hypoxia and NF- κ B on the expression of MMP-2, MMP-9 and VEGF in retinoblastoma cells. Our results showed that hypoxia stimulation can increase the levels of MMP-2, 9 and VEGF, and the capacity of invasion and migration of cancer cells was also increased. Further examination revealed that when NF- κ B was blocked by its specific inhibitor PDTC, the levels of MMP-2, 9 and VEGF, and the capacity of invasion and migration of Y79 cells were inhibited, too. Our results confirmed that NF- κ B was a mediator of tumor invasion and metastasis under hypoxia condition by regulating MMP-2, MMP-9 and VEGF.

In summary, our data demonstrated that hypoxia could significantly change the invasion and migration ability of Y79 retinoblastoma cells, NF- κ B signal might be one of the main mediators for these hypoxia induced cell changes of biological behavior via downregulation of HIF-1 α and the invasion related molecules, and it may provide potential target and possible treatment strategy for clinical diagnosis and therapeutic regimen for retinoblastoma.

Disclosure of conflict of interest

None.

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NF- κ B and hypoxia on the biological behavior of Y79 cells

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