Endostar inhibits hypoxia-induced cell proliferation and migration via the hypoxia-inducible factor-1α/vascular endothelial growth factor pathway *in vitro*

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Abstract. Endostar, a recombinant human endostatin, is recognized as one of the most effective angiogenesis inhibitors. The angiogenesis inhibitory effects of Endostar suggest a possible beneficial role of Endostar in choroidal neovascularization (CNV), which is predominantly induced by hypoxia. In our previous study, it was reported that Endostar may inhibit the proliferation and migration of RF/6A choroid-retinal endothelial cells. However, the inhibitory effect of Endostar on hypoxia-induced cell proliferation and migration in RF/6A cells has not yet been elucidated. Therefore, the present study investigated the effect of Endostar on hypoxia-induced cell proliferation and migration in RF/6A cells and the possible mechanisms underlying this effect. Under chemical hypoxia conditions, cell viability was increased to 114.9±10.1 and 123.6±9.6% in cells treated with 100 and 200 μm $CoCl_2$, respectively, compared with the control (P<0.01). Pretreatment with 10-100 μg/ml Endostar significantly inhibited $CoCl_2$-induced cell proliferation (P<0.05), and pre-treatment with 10 μg/ml Endostar for 24, 48 and 96 h attenuated $CoCl_2$-promoted cell migration by 60.5, 48.3 and 39.6%, respectively, compared with the control (P<0.001). In addition, pretreatment with 10 μg/ml Endostar reversed the cell cycle arrest at S phase and the increased expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) mRNA in RF/6A cells treated with 200 μM $CoCl_2$. These data indicate that Endostar inhibited $CoCl_2$-induced hypoxic proliferation and migration, and limited cell cycle progression *in vitro* possibly through the HIF-1α/VEGF pathway.

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Introduction

Angiogenesis is a complex process involving angiogenic factor secretion, proteolytic enzyme secretion and activation, extracellular matrix degradation, endothelial cell activation, migration, proliferation, growth, sprouting and lumen formation, new vessel differentiation and maturation, and vasoganglion remodeling (1). Choroidal neovascularization (CNV) is one of the most important intraocular neovascular manifestations and is correlated with numerous ocular diseases, including age-related macular degeneration (AMD), which is the primary cause of vision loss among people >60 years of age in developed countries (2,3), as well as idiopathic chorioretinitis, ocular histoplasmosis, high myopia macular degeneration, ophthalmic tumors and ocular injury.

CNV is caused by fibrous vascular tissue formed by choroidal neovascular buds passing through the Bruch's membrane and proliferating in the subretinal space (4). The mechanisms underlying CNV are diverse and complex, involving numerous cellular factors and signal transduction pathways that regulate the incidence and development of CNV. Hypoxia-inducible factor (HIF), angiopoietin (Ang) and numerous cytokines, including vascular endothelial growth factor (VEGF), have been found to have an important role in CNV (2,5). Several recent studies found that hypoxia/ischemia has an important role in CNV, and HIF-1α and VEGF are key regulators of CNV under hypoxic conditions (6,7).

Recently, an angiogenesis inhibitor has been developed as a novel strategy for the treatment of CNV (8). Endostatin, a 20 kD potential angiogenesis inhibitor, has been found to exert powerful effects preventing endothelial vascular formation and tumor development *in vitro* and *in vivo*, mainly in advanced solid tumors and human umbilical vein endothelial cells (9,10). Furthermore, Mori *et al* (11) compared the similarities between tumor angiogenesis and CNV, and found that endostatins inhibit occular neovascularization, and that the occurrence and development of CNV were negatively correlated with the serum endostatin level. In addition, they also confirmed that systemic application of endostatin inhibited intraocular neovascularization. Tatar *et al* (12) found that endostatin was expressed in 92% of CNV samples obtained

from patients with AMD using immunohistochemical analysis. Our previous study reported that Endostar was able to inhibit cell proliferation and migration in normal RF/6A choroid-retinal endothelial cells through regulating the expression of growth factors and inflammatory factors in a dose- and time-dependent manner (13). Although Endostar may effectively inhibit angiogenesis and tumor growth, its specific role and mechanism in regulating CNV inhibition have not been well defined. In the present study, RF/6A cells were cultured under hypoxic conditions to simulate vascular endothelial cell growth, proliferation and migration *in vitro,* and were used to examine the possible mechanisms underlying the inhibitory effects of Endostar on cell proliferation and migration.

Materials and methods

Cell viability assay. RF/6A rhesus choroid retinal endothelial cells obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) were cultured in Eagle's minimum essential medium (EMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Bioengineering Material Co., Ltd., Hangzhou, China). Hypoxia was induced by exposing the cells to $CoCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) at various concentrations (100-800 μM) in EMEM medium with 0.5% FBS for 24 h, and the cell viability was determined by a methylthiazol tetrazolium (MTT) assay (Sigma-Aldrich). The absorbance at 570 nm was measured with a Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). The data were reported as percentages of the absorbance in the control cells.

Wound-healing assay. When the cells had been cultured to a monolayer, the cells were wounded with 200 μl plastic pipette tips and incubated in EMEM containing 0.5% FBS and 200 μM $CoCl_2$, in the absence or presence of Endostar (0.5, 1 or 10 μg/ml; Simcere Pharmaceutical Group, Nanjing, China). Images were captured at 0, 24, 48 and 96 h using an Olympus IX-81 inverted microscope (Olympus Corp., Tokyo, Japan). Migration was quantified by counting the number of cells that had advanced into the cell-free space from the initial wound border at 0 h.

Cell cycle analysis. Following treatment, the cells were washed with ice-cold phopshate-buffered saline (PBS), trypsinized, resuspended in PBS supplemented with 0.2% Triton X-100 and 1 mg/ml RNase A (Sigma-Aldrich), and incubated with propidium iodide (PI; Sigma-Aldrich) for 30 min at room temperature in the dark for DNA staining. The cells were then analyzed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA, USA) with cell quest software (Becton Dickinson).

Determination of the levels of secreted VEGF using ELISA. Following overnight serum starvation, the cells in the 24-well culture plates were pretreated with 0, 0.5, 1 or 10 μg/ml Endostar, for 1 h and then treated with 200 μM $CoCl_2$ for 24 h. The culture medium (200 μl) was collected by centrifugation at 100 x g for 10 min and the VEGF content was detected using a commercial human VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and calculated as pg/ml protein.

Reverse transcription-polymerase chain reaction (RT-PCR). To identify the mRNA expression levels of HIF-1α and VEGF, the total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse-transcribed into cDNA in a 20 μl reaction, which was incubated at 42˚C for 60 min, and then heated to 72˚C for 10 min to inactivate the reverse transcriptase. cDNA was then used as a template in a 20-μl PCR system under the following conditions: Denaturation at 94˚C for 2 min followed by 35 cycles of denaturation at 94˚C for 10 sec, annealing at 55˚C for 10 sec and elongation at 72˚C for 10 sec, and a final incubation at 72˚C for 10 min. The amplified products were examined on 2% agarose gels and densitometrically analyzed with a UVP gel analysis system (Bio-Rad). The primer sequences were as follows: Forward: 5'-CAT TAG AAA GCA GTT CCG CAA GC-3' and reverse: 5'-CAG TGG TAG TGG TGG CAT TAG C-3' for human HIF-1α; forward: 5'-GAG CCT TGC CTT GCT GCT CTA C-3' and reverse: 5'-CAC CAG GGT CTC GAT TGG ATG-3' for human VEGF; and forward: 5'-TCA ACG GAT TTG GTC GTA TT-3' and reverse: 5'-CTG TGG TCA TGA GTC CTT CC-3' for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were synthesized by Sangon Biotech (Shanghai, China).

Statistical analysis. The data are expressed as the mean ± standard deviation and were statistically analyzed by one-way analysis of variance using Prism 4 software (GraphPad software Inc., San Diego, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Induction of hypoxia with $CoCl_2$. Treatment with $CoCl_2$ at doses <400 μM for 24 h was able to induce cell proliferation , as measured by an MTT assay. The cell viability was 114.9±10.1 and 123.6±9.6% in cells treated with 100 and 200 μM $CoCl_2$, respectively ($P<0.01$). However, at a concentration >400 μM, $CoCl_2$ significantly inhibited cell proliferation (Fig. 1).

Effect of Endostar on the viability of $CoCl_2$-treated RF/6A cells. The viability of RF/6A cells treated with 200 μM $CoCl_2$ for 24 h was significantly increased compared with the untreated control cells. Pre-treatment with Endostar at concentrations of 1-500 μg/ml significantly attenuated $CoCl_2$-induced increase in cell viability ($P<0.05$); however, treatment with 500 μg/ml Endostar resulted in a decline in cell viability of RF/6A cells compared with that of the untreated cells (Fig. 2). This therefore suggested that concentrations of Endostar >100 μg/ml may have toxic effects on these cells.

Effect of Endostar on the migration of $CoCl_2$-treated RF/6A cells. The effect of Endostar on the migration of $CoCl_2$-treated RF/6A cells was examined using a wound-healing assay *in vitro*. It was identified that $CoCl_2$ treatment for 24 h

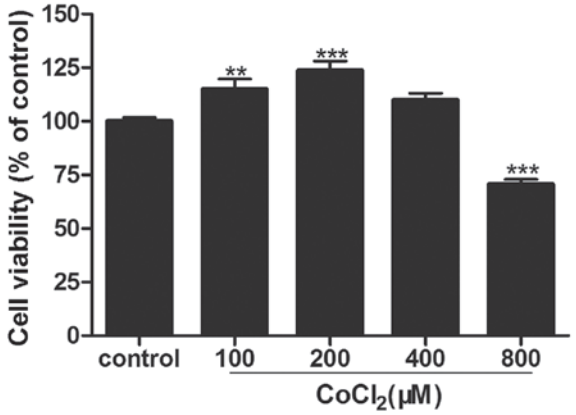

Figure 1. Effect of different concentrations of $CoCl_2$ on choroid-retinal endothelial (RF/6A) cell growth as measured by a methylthiazol tetrazolium assay. The control cells did not receive any treatment. Data are expressed as the mean ± standard deviation (n=18). **P<0.01 and ***P<0.001 vs. the control.

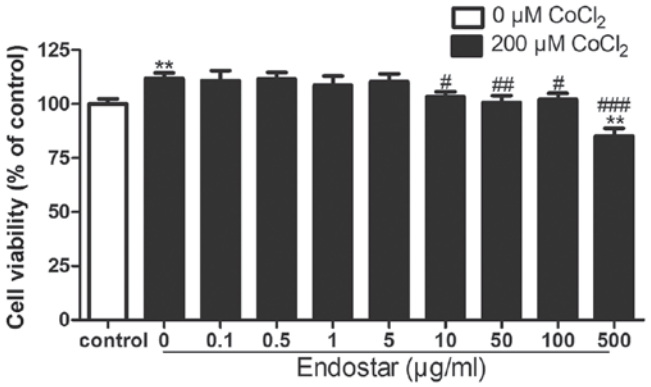

Figure 2. Inhibitory effect of Endostar on $CoCl_2$-induced choroid-retinal endothelial (RF/6A) cell proliferation as determined by a methylthiazol tetrazolium assay. The control cells did not receive any treatment. Data are expressed as the mean ± standard deviation (n=18). ***P<0.001 vs. the control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the cells treated with $CoCl_2$ only.

significantly promoted cell migration, which was inhibited by Endostar pretreatment at different time points (Fig. 3). As shown in Fig. 4, cell migration was inhibited by Endostar treatment in a time and dose-dependent manner. Under hypoxic conditions, the cell migration was increased nearly 2-fold compared with the level in the control group after 24 h, by 92.0% compared with the control group after 48 h, and by 80.4% after 72 h. Compared with the group treated with $CoCl_2$ alone, cell migration was decreased to 70.0, 60.9 and 60.5% following 24 h, 67.8, 62.9 and 48.3% after 48 h, and 65.3, 57.1 and 39.6% after 72 h, following treatment with 0.5, 1 and 10 μg/ml Endostar, respectively.

Effect of pretreatment with Endostar on the cell cycle of $CoCl_2$-treated RF/6A cells. The DNA content of $CoCl_2$-treated RF/6A cells was used for cell cycle analysis. The results demonstrated that $CoCl_2$ arrested the cell cycle at S phase and Endostar pretreatment was able to reverse this effect (Fig. 5). It was observed that treatment with 200 μM $CoCl_2$ increased the percentage of cells in S phase from 19.1±0.9 to 27.8±1.5%, but decreased the percentage of cells in G0/G1 phase from 73.5±1.3 to 62.8±1.1%. However, pretreatment with 10 μg/ml Endostar completely reversed these effects, and the percentage of cells in S phase and G0/G1 phase returned to 16.3±3.5 and 73.9±5.8%, respectively.

Effect of pretreatment with Endostar on the expression of HIF-1α and VEGF in $CoCl_2$-treated RF/6A cells. HIF-1α and VEGF mRNA levels were quantified with RT-PCR, normalized to the internal control GAPDH and compared. Compared with the control group, 200 μM $CoCl_2$ treatment induced a 4-fold increase in HIF-1α expression, which was then attenuated by Endostart in a concetration-dependent manner (0.5-10 μg/ml) (Fig. 6A and B). In addition, 200 μM $CoCl_2$ increased VEGF mRNA expression by 50%, which was then downregulated in a concentration-dependent manner following treatment with 0.5-10 μg/ml Endostar (Fig. 6A and C). Furthermore, the amount of VEGF protein release into the medium was consistent with that of the mRNA expression of VEGF (Fig. 6D).

Discussion

Hypoxia/ischemia-associated retinopathy and optic neuropathy is the major cause of blindness, central/branch retinal vein occlusion, diabetic retinopathy and retinopathy of prematurity, which appear to always be accompanied with retinal and choroidal neovascularization at advanced stages, causing vitreous hemorrhage, proliferative retinopathy, retinal detachment and severe visual impairment (14,15). RF/6A cells were isolated from the choroid retina of a healthy rhesus fetus and confirmed as endothelial cells by morphology, growth pattern and immunohistochemistry. RF/6A cells, as a choroidal endothelial cell line, were used to investigate the pathogenesis and prevention of CNV-associated diseases and have been proven to be a reliable *in vitro* model for the formation of CNV. CNV formation is a complex process (16,17), affected by a variety of etiological factors, including hypoxia, which has an important role in angiogenesis (6). In the present study, $CoCl_2$, a chemical reagent used to establish a hypoxia cell model, induced cell proliferation and migration in a certain dose range. The concentration of 200 μM $CoCl_2$ was determined to be appropriate for treating RF/6A cells and simulating CNV formation *in vitro*.

Endostar, a novel recombinant human endostatin, was synthesized in China and approved as an anticancer drug by the State Food and Drug Administration in 2005 (18). Endostar is easier to purify and has more clinical advantages than Endostatin, demonstrating stable physicochemical characteristics and higher water solubility (19). Endostar exhibits anti-angiogenic effects and has been used to treat numerous types of cancer, including non-small lung, breast and gastric cancer (20-22). However, its applications in the field of ocular disease and the possible underlying molecular mechanisms of its effects have not been well investigated.

The present study demonstrated that $CoCl_2$ treatment promoted the proliferation and migration of RF/6A cells and arrested more cells in the S phase, leaving fewer cells in the G0/G1 phase, while pretreatment with Endostar significantly reversed all $CoCl_2$-mediated effects in RF/6A cells. This suggested that the effects of Endostar on the proliferation and migration of $CoCl_2$-induced RF/6A cells may occur due to the inhibition of RF/6A cell transition from G0/G1 phase to S phase.

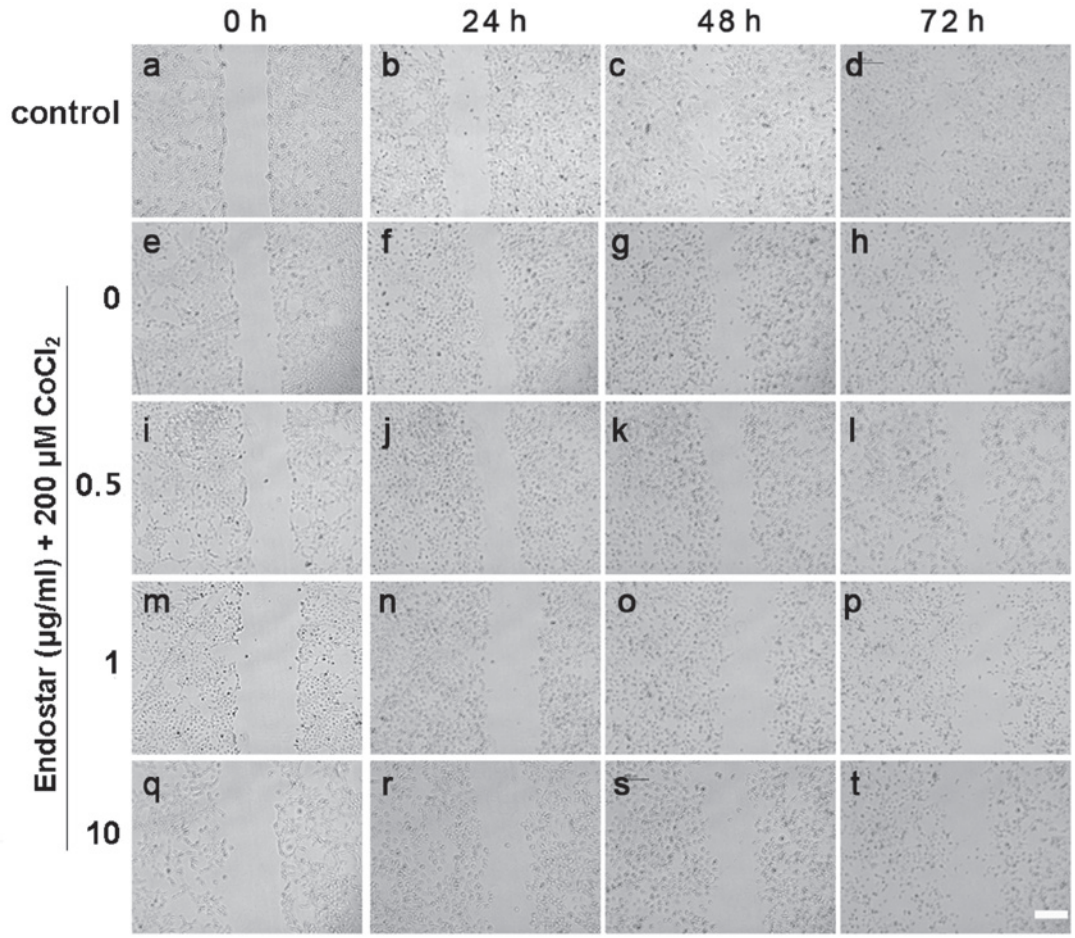

Figure 3. Inhibitory effect of Endostar on the migration of hypoxic choroid-retinal endothelial cells (RF/6A). (a-t) The cells as measured by a wound healing assay *in vitro*. The migration of cells without any treatment at (a) 0, (b) 24, (b) 48 and (d) 72 h; treated with $CoCl_2$ at (e) 0, (f) 24, (g) 48 and (h) 72 h; pretreated with 0.5 μg/ml Endostar and treated with $CoCl_2$ at (i) 0, (j) 24, (k) 48 and (l) 72 h; pretreated with 1 μg/ml Endostar and treated with $CoCl_2$ at (m) 0, (n) 24, (o) 48 and (p) 72 h; pretreated with 10 μg/ml Endostar and treated with $CoCl_2$ at (q) 0, (r) 24, (s) 48 and (t) 72 h. Scale bar, 100 μm.

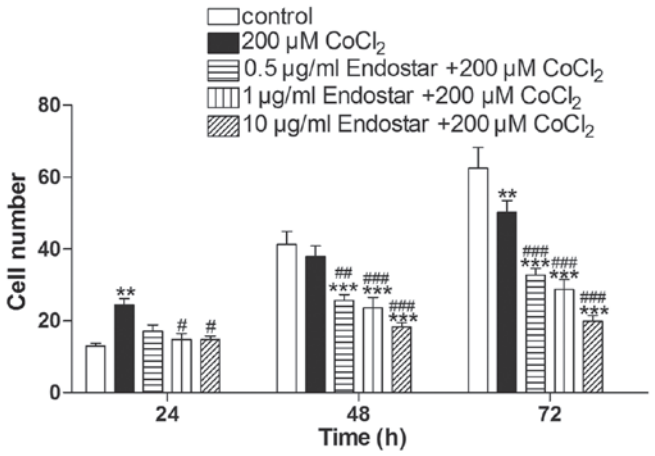

Figure 4. Endostar inhibits $CoCl_2$-induced cell migration. The control cells did not receive any treatment. Data are expressed as the mean ± standard deviation (n=9). **P<0.01 and ***P<0.001 vs. the control at the corresponding time, $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. 200 μM $CoCl_2$ at the corresponding time.

HIF-1, a DNA binding protein, is an important transcription factor regulating hypoxia (23). It has been found to be overexpressed under hypoxic conditions, implying that hypoxia may increase the content of HIF-1α to regulate the expression of its downstream genes, including VEGF (7,23-25). In addition, HIF-1 may directly or indirectly regulate the expression of numerous genes, such as VEGF, placental growth factor and TGF-β1 (26), in myocardial cells, fibroblasts and smooth muscle cells by binding to its binding site in their promoters (27).

VEGF is a necessary stimulator for retinal and choroidal neovascularization. Numerous agents that bind VEGF or block VEGF receptors may suppress retinal and choroidal neovascularization (28,29). Hypoxia is the main factor leading to CNV, and the subsequent simultaneous increase in VEGF and HIF-1α expression (30). These studies indicated that hypoxia may enhance the expression of HIF-1α, which subsequently regulates the expression of VEGF. In the present study, it was identified that $CoCl_2$ enhanced the expression of HIF-1α and VEGF in RF/6A cells *in vitro*.

Endostatin has been demonstrated to exert anti-angiogenenic effects in a HIF-1α-dependent manner (31). In human lung adenocarcinoma cancer cells, Endostar was able to suppress HIF-1α and VEGF expression and radiotherapy-induced angiogenesis (32). The present study demonstrated that Endostar inhibited $CoCl_2$-induced HIF-1α and VEGF expression in RF/6A cells, suggesting that Endostar may affect cell proliferation and migration through regulating the HIF-1α/VEGF pathway.

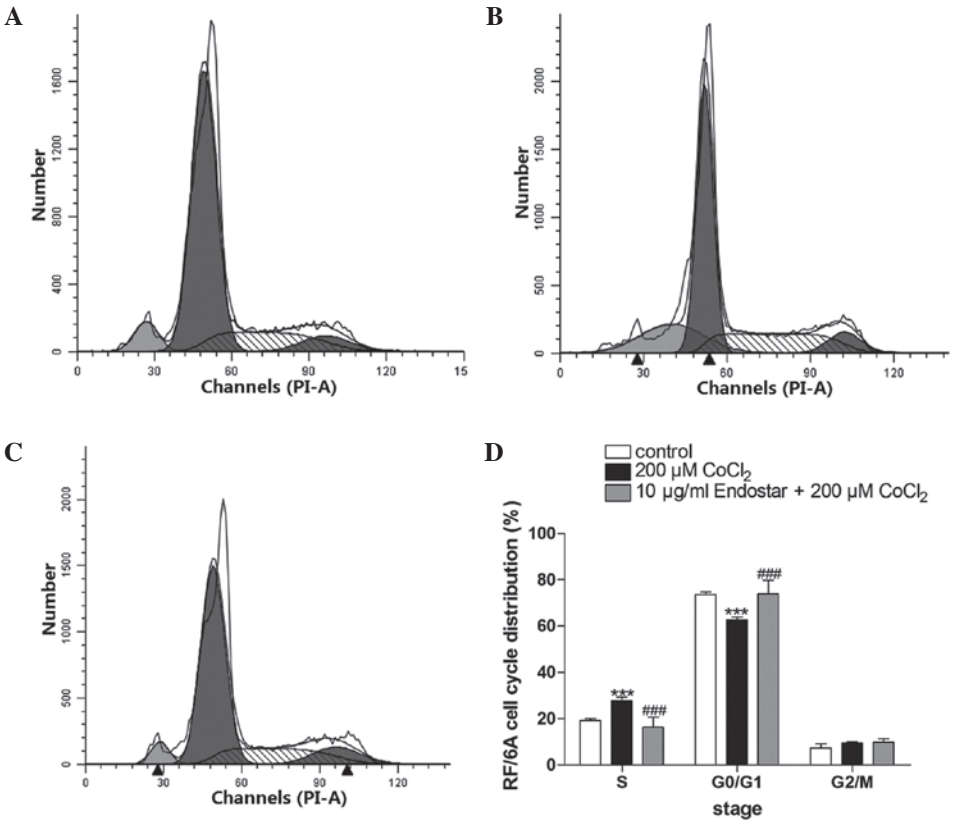

Figure 5. Effect of Endostar on cell cycle progression of hypoxic choroid-retinal endothelial cells (RF/6A) cells. (A) Representative flow cytometric analysis of RF/6A cells without any treatment (control). (B) Representative flow cytometric analysis of RF/6A cells exposed to 200 μM $CoCl_2$. (C) Representative flow cytometric analysis of RF/6A cells exposed to 200 μM $CoCl_2$ following pretreatment with Endostar (10 μg/ml). (D) There was a significant difference in the percentage of cells in S, G0/G1 and G2/M phases among the control cells, $CoCl_2$-treated cells and $CoCl_2$-treated cells following pretreatment with Endostar (10 μg/ml). Data are expressed as the mean ± standard deviation. ***P<0.001 vs. the control; $^{###}$P<0.001 vs. 200 μM $CoCl_2$.

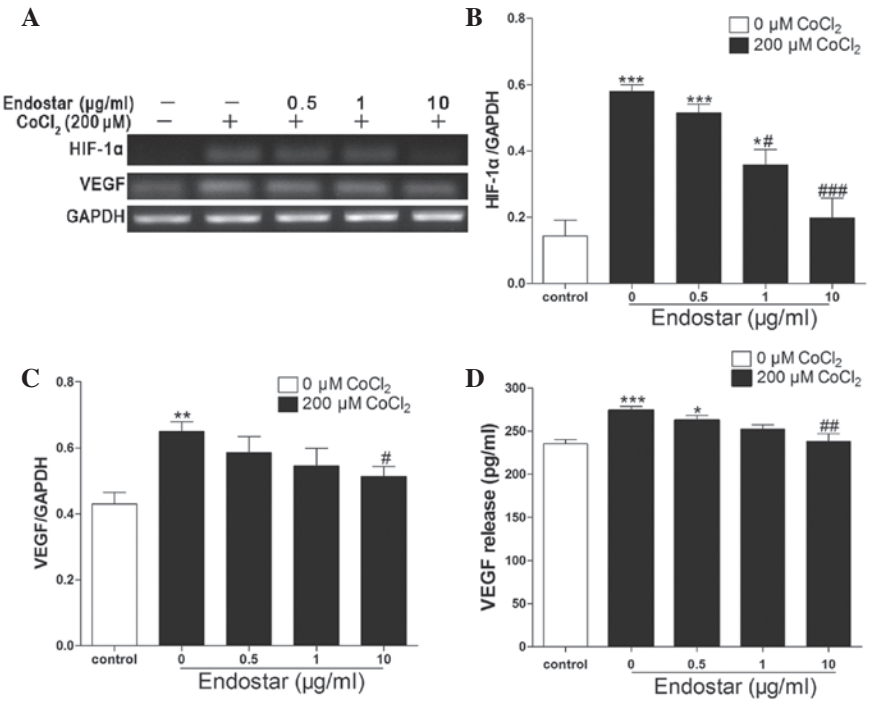

Figure 6. Endostar inhibits HIF-1α and VEGF expression in hypoxic choroid-retinal endothelial cells (RF/6A). (A) After the cells were incubated for 24 h with 200 μM $CoCl_2$ in the absence or presence of 0.5, 1 and 10 μg/ml Endostar, mRNA levels of HIF-1α and VEGF were detected by RT-PCR. (B and C) Densitometric analyses of RT-PCR are presented as the mean ± standard deviation of three independent experiments performed in triplicate. (D) After the cells were treated with 200 μM $CoCl_2$ in the absence or presence of Endostar (0.5, 1, and 10 μg/ml), the cell supernatant was used to analyze the secreted VEGF protein using ELISA. *P<0.05, **P<0.01 and ***P<0.001 vs. the control; $^{#}$P<0.05, $^{##}$P<0.01 and $^{###}$P<0.001 vs. cells only treated with $CoCl_2$. HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcription-polymerase chain reaction.

In conclusion, Endostar, a recently introduced recombinant human endostatin, is able to inhibit $CoCl_2$-induced RF/6A cell proliferation and migration possibly by downregulating HIF-1α and secondarily inhibiting VEGF expression. These results indicate that Endostar may have an important role in hypoxia-induced CNV, which highlights its significant potential for clinical application.

Acknowledgements

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