Pigment epithelium-derived factor inhibits angiogenesis and growth of gastric carcinoma by down-regulation of VEGF

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Abstract. Previous studies have shown that pigment epithelium-derived factor (PEDF) is an antitumor candidate with anti-oxidative, anti-inflammatory and anti-angiogenesis properties. However, the effect of PEDF on gastric carcinoma has not been elucidated. MTT assay and Annexin V/PI staining were performed. Immunohistochemistry was applied to detect microvessel density (MVD) of a xenograft model. The protein levels of vascular endothelial growth factor (VEGF) were examined by Western blot analysis and hypoxia-inducible factor-1α (HIF-1α) translocation was investigated by immunofluorescence. Results showed that growth and angiogenesis of gastric carcinoma were suppressed after PEDF injection. PEDF could not directly suppress proliferation or induce apoptosis of gastric carcinoma cells. However, the expression of VEGF both in tumor tissues and gastric carcinoma cells was down-regulated by PEDF. The amount and nuclear translocation of HIF-1α, the transcription factor of VEGF, was also inhibited by PEDF. In conclusion, PEDF suppresses angiogenesis and growth of gastric carcinoma by down-regulating HIF-1α and VEGF and may have potential for gastric carcinoma treatment.

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Introduction

Gastric carcinoma is a common malignancy, and the second cause of cancer-related deaths (1). There are about 800,000 annual deaths worldwide (2). The five-year survival rate for gastric carcinoma with distant metastasis is much lower than that for localized malignancies (3).

Angiogenesis, proliferating new vessels from pre-existing capillaries, has an important role in the metastasis and prognosis of gastric carcinoma (4). VEGF, secreted by tumor cells, is a main angiogenesis inducer, supporting tumor growth and enhancing metastasis (5). Therefore, anti-angiogenesis therapy may be a strategy in gastric carcinoma therapy.

Pigment epithelium-derived factor (PEDF), a 50-kDa glycoprotein present in most tissues of the body, is a member of the serpin family (6). It is a multifunctional molecule that binds to a cell membrane receptor, performing neuroprotective, neurotrophic, anti-oxidative and anti-inflammatory activities (7,8). Additionally, PEDF is an active endogenous angiogenic inhibitor and tumor treatment candidate (9). Previous studies elucidated that PEDF may induce endothelial cell apoptosis and suppress tumor growth (10-13). However, whether PEDF could inhibit gastric carcinoma growth has not been investigated. In this study, the anti-tumor effect of PEDF was investigated in animal experiments, and the intrinsic mechanism was explored.

Materials and methods

Cell culture and reagents. Human gastric carcinoma SGC7901 cells were purchased from the Cell Bank of Sun Yat-sen University (Guangzhou, China) and maintained in RPMI-1640 medium (R1640) supplemented with 10% (v/v) FBS (Gibco-BRL, Gaithersburg, MD) and incubated under normal conditions (at 37˚C with 5% CO_2). Human umbilical vein endothelial cells (HUVECs), isolated from umbilical vein cords of normal pregnancies (14), were cultured in M199 medium (Gibco-BRL) with 15% FBS and endothelial cell growth supplement (ECGS) (BD Biosciences, CA), and cultured at 37˚C with 5% CO_2.

HUVECs at passages 2-6 were used in all the experiments. PEDF protein was acquired as previously described (15). Briefly the PEDF cDNA was inserted into the pET30a plasmid (Novagen). Soluble PEDF was purified using the His-Tag affinity method (Novagen) as recommended by the manufacturer. The recombinant PEDF was confirmed by Western blot analysis using a PEDF antibody produced as previously described (16).

Animal model. Suspensions (0.1 ml) of SGC7901 cells ($5x10^7$ cells/ml) in R1640 were injected subcutaneously in the dorsal site of male athymic mice (BALB/c nu/nu, 18-22 g, Center of Experimental Animal, Sun Yat-sen University). When the volume of tumors reached 50 mm^3, the mice were randomly separated into PEDF and PBS groups (n=5 in each group). The PEDF group received an intraperitoneal injection of 5 mg/kg PEDF per mouse every other day. The overall dosage per mouse was 15 mg/kg. The control group was treated with the same volume of PBS. The length and width of the tumors were measured every second day. The tumor volume was calculated by the equation: volume = (length x $width^2$)/2. The tumors were excised four weeks after the first injection and weighed. The inhibition ratio of PEDF was calculated by the following equation: Inhibition ratio (%) = [(C-T)/C] x 100%, where C and T are the average tumor weight of the PBS and PEDF group, respectively. All animal experiments were carried out according to the US Public Health Service Guide for the Care and Use of Laboratory Animals.

Cell proliferation assay. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) assay. SGC7901 cells or HUVECs at a density of $2x10^4$cells/well were seeded into 24-well plates in triplicate. After adherence, the medium was changed to M199 with 2% FBS for HUVECs or to serum-free R1640 for SGC7901 cells with various concentrations of PEDF (0, 40, 80, 160, 320 and 640 nmol/l). After treatment for another 72 h, 100 μl fresh medium with 5 mg/ml MTT was added into each well and the cells were cultured for another 4 h. The medium was then aspirated and 1 ml dimethyl sulfoxide (DMSO) (Sigma) was added into each well. The absorbance was measured at 570 nm. Data are expressed as the percentage of viable cells compared to controls. The anti-proliferation effects of PEDF on the cells were determined from three independent tests.

Annexin V/PI analysis. About $1x10^5$ cells per well were seeded into 6-well plates. Twenty hours later, cells were cultured for another 72 h with 320 nmol/l PEDF. Then the cells were trypsinized, washed twice with PBS, then resuspended in 1X binding buffer with 5 μl Annexin V and 5 μl propidium iodide (PI) from the Annexin V/PI kit (Keygen, China) following the protocol recommended by the manufacturer. The apoptosis ratio of the 25 μmol/l colchicine-treated group was used as a positive control. The apoptotic cells were subsequently counted by flow cytometry.

Western blot analysis. SGC7901 cells were cultured in 90-mm plates until 70% confluence. The cells were then incubated with serum-free medium containing different concentrations of PEDF (0, 40, 80, 160, 320 and 640 nmol/l) under normoxia (20% O_2, v/v) or hypoxia (1% O_2, v/v) at 37˚C for the indicated time. The cells were lysed and the protein concentrations of the samples were detected using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Western blot analysis was performed with 60 μg of cell lysates as previously described (17). The primary antibodies used were anti-VEGF (1:2,000, Abcam, USA); anti-Fas (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-HIF-1α (1:1,000) and anti-FasL (1:1,000, BD Biosciences). The β-actin antibody (1:10,000, Sigma) was used as a control and was probed on the same membrane after stripping.

Immunofluorescence. SGC7901 cells were cultured in 6-well plates with coverslips and incubated overnight at 37˚C. The medium was then changed into serum-free R1640 with or without PEDF (320 nmol/l) and the cells were incubated under normoxia (20% O_2, v/v) or hypoxia (1% O_2, v/v) for another 9 h. After washing twice with PBS, the cells were fixed with 4% paraformaldehyde (Sigma) buffered in PBS for 15 min. The cells were then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 15 min, followed by blocking for 30 min with 3% FBS in PBS. The cells were then incubated with HIF-1α (BD Biosciences) antibody (1:50 in 3% FBS) overnight at 4˚C. After washing twice with PBS, the cells were incubated in the dark with Alexa Fluor 594 anti-mouse IgG (20 μg/ml, Invitrogen, USA) for 1 h at room temperature. After rinsing with DAPI (Vector Laboratories, Burlingame, CA), the cells were then analyzed using fluorescent microscopy (Olympus, JAP) using the IPP software (Image Plus Pro 6.0, Bethesda, MD, USA).

Immunohistochemistry. Heterotopic tumors were fixed in formalin and embedded in paraffin. To detect microvessel formation, 5 μm histological sections were prepared. After endogenous peroxidase was blocked, the sections were incubated with normal goat serum for 1 h at room temperature. Antibodies against CD34 (1:100, Abcam, UK), and VEGF (1:50) were used and incubated overnight at 4˚C. After washing twice in PBS, sections were covered with biotinylated secondary antibody for 20 min at room temperature, washed twice, and then incubated with HRP-labeled streptavidin for another 20 min. Finally, the results were visualized by a peroxidase reaction with diaminobenzidine (DAB) and quantified by Weidner's method (18). The amount of VEGF was indicated by integral optical density (IOD) using IPP (Image Plus Pro).

Statistical analysis. All data were analyzed by the Student's t-test using the SPSS 13.0 software. The results are expressed as the mean ± SD. Statistical significance was set at $P<0.05$.

Results

To evaluate the effect of PEDF on gastric carcinoma growth, a heterotopic tumor xenografted model was established. Since the 12th day after the first injection, the average tumor size was significantly suppressed by PEDF (Fig. 1A-C; $P<0.05$, n=5). The average tumor weight of the PEDF group was notably lower than that of the control group (Fig. 1C). The results demonstrate that PEDF can effectively suppress gastric carcinoma growth *in vivo*.

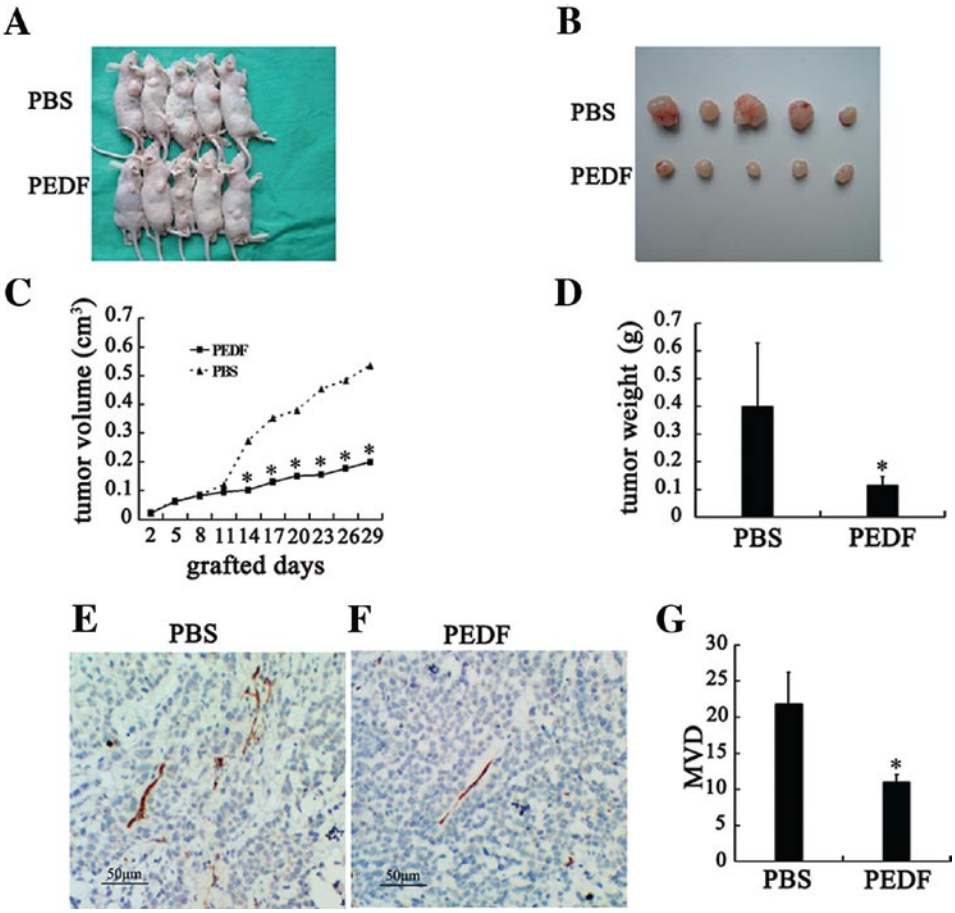

Figure 1. SGC7901 cell heterotopic transplanted tumors were developed as described in Materials and methods. Mice received an intraperitoneal injection of PEDF 7 days after transplantation. (A and B) Tumor growth was monitored, and tumor tissues were collected and weighed 4 weeks after the first injection of PEDF. (C) Tumor growth curves, volumes of PEDF- vs. PBS-treated group on days indicated. Data are presented as the mean ± SD, n=5. Microvessel density (MVD) in tumor tissues were determined by immunohistochemical staining by an endothelial-specific antibody CD34. (D) Tumor weight at day 28 after treatment. An average of 71.1% suppression of primary tumor growth was observed. MVD of (E) PBS group and (F) PEDF group. (G) MVD quantitation. Data are presented as the mean ± SD (*P<0.05).

To evaluate the effect of PEDF in tumor tissue, immunostaining of CD34, which exists in vascular endothelium and is a marker of angiogenesis, was performed. The amount of CD34-stained capillaries in the PEDF group was reduced compared with the PBS group (Fig. 1E and F). Microvessel density (MVD) of the PEDF group was markedly less than that of the PBS group (Fig. 1G, P<0.05). These results clearly demonstrate that PEDF could effectively inhibit neovascularization of gastric carcinoma in xenografted model.

Our previous report suggested that PEDF dose-dependently promotes apoptosis of HUVECs (17). The IC_{50} was ~80 nmol/l. However, in this study, as observed in the MTT assay, PEDF had no anti-proliferative effect on SGC7901 cells, even at a high concentration of 640 nmol/l (Fig. 2A). Annexin V-PI staining showed that the PEDF (320 nmol/l) could not induce apoptosis of SGC7901 cells (Fig. 2B). Western blotting demonstrated that there is no difference in the protein levels of Fas and Fas-L between the PEDF and control groups, even after 72 h of treatment (Fig. 2C). Therefore, PEDF had no apparent inhibitory effect on SGC7901 cells.

Western blot analysis showed that PEDF (40-640 nmol/l) dose-dependently down-regulated the expression of VEGF after 24 h of treatment (Fig. 3A) under hypoxia. In xenografted tumor tissues, the VEGF level in the tumor tissue of the PEDF group was lower than that of the PBS group (Fig. 3B), which suggests that PEDF decreases the expression of VEGF *in vivo*. Therefore, VEGF expression could be down-regulated by PEDF both *in vitro* and *in vivo*.

HIF-1α is the main regulator of VEGF expression. In order to elucidate the role of HIF-1α in the PEDF-induced down-regulation of VEGF expression, immunofluorescence and Western blotting were performed to analyze the localization and the amount, respectively, of HIF-1α in SGC7901 cells after PEDF treatment. HIF-1α protein levels were elevated prominently in hypoxic condition, and PEDF remarkably reduced HIF-1α expression in SGC7901 cells (Fig. 4A). Immunofluorescence analysis showed that hypoxia significantly enhanced the stability and HIF-1α nuclear translocation and PEDF notably decreased the translocation and expression of HIF-1α under hypoxic conditions (Fig. 4B).

Discussion

Angiogenesis is essential for solid tumor growth and metastasis (6,19). Anti-angiogenesis therapy provides a novel approach for cancer therapy (6). Angiogenesis provides a pathway for nutrient transportation and metastasis of gastric carcinoma. Studies have suggested that anti-angiogenic agents may be a

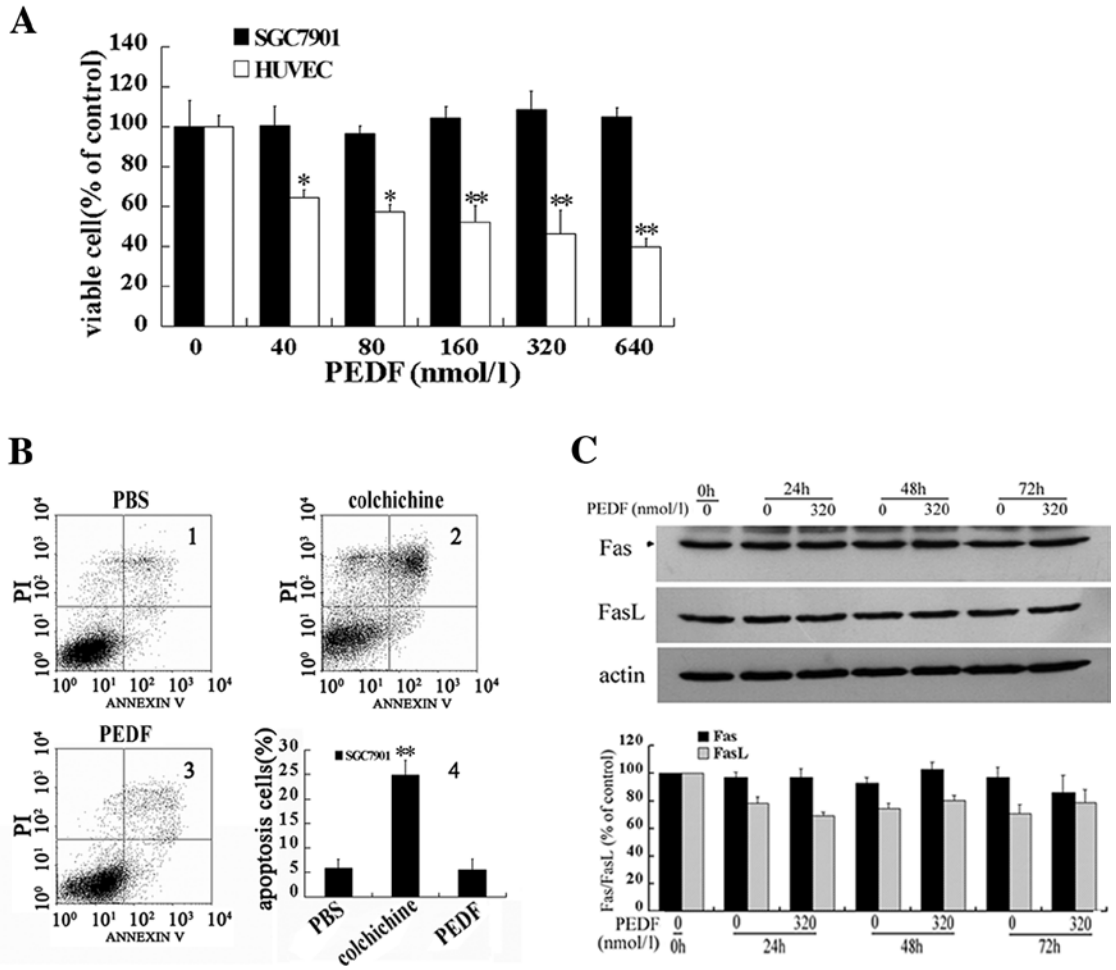

Figure 2. Primary HUVECs and SGC7901 cells were treated with PEDF at the indicated concentrations for 72 h. (A) The viable cells were counted by the MTT assay (means ± SD, n=3). (B) Quantitative analysis of SGC7901 cell apoptosis after treatment with PEDF for 72 h. Apoptotic cells were quantified by flow cytometric analysis. 1, PBS as a negative control; 2, colchicine as a positive control; 3, cells treated with PEDF at 320 nmol/l; 4, statistical analysis. (C) Fas and FasL were detected at different times after PEDF treatment. Values significantly higher than control are indicated (*P<0.05, **P<0.01).

new method for gastric carcinoma treatment (20-22). This is the first research to elucidate that PEDF could inhibit gastric carcinoma growth *in vivo*. The MVD results showed that angiogenesis in tumor tissues was inhibited by PEDF (Fig. 1E and F). However, the MTT assay and apoptosis experiments performed on cell models suggested that PEDF had no effect on the proliferation and apoptosis of SGC7901 cells (Fig. 2) or another gastric carcinoma cell line, MGC 803 (data not shown), even at a high concentration of 640 nmol/l and after 72 h of treatment. The observation that PEDF could not induce gastric carcinoma cell apoptosis was further confirmed. Studies have shown that the Fas-FasL pathway may be involved in tumor apoptosis of human osteosarcoma and melanoma cells treated with PEDF (23). However, PEDF treatment did not alter the protein levels of Fas or Fas-L in the present study (Fig. 2C). These results suggest that the mechanism by which PEDF suppresses gastric carcinoma growth is mainly through blocking of angiogenesis instead through a directly pro-apoptotic effect on tumor cells. Y79 is a widely used human retinoblastoma cell line. Though the Fas-FasL signaling pathway was activated after PEDF treatment, the hypermethylation of caspase-8 gene made this cell line resistant to apoptosis (24). On the other hand, in the hepatocellular carcinoma cell line HepG2, PEDF was reported to exert an anti-apoptotic effect via inhibition of Bcl-xL degradation in the lysosome (25). Collectively the data suggest that the effect of PEDF on tumor apoptosis is cell-type dependent, but the detailed mechanism remains to be elucidated.

VEGF is the most potent factor of pro-angiogenesis and is secreted by almost all types of tumor cells (26). In pathological neovascularization, angiogenesis stimulators such as VEGF, were up-regulated, while inhibitors, for example PEDF, were down-regulated (27). PEDF can restore this balance by restoring pro-angiogenic factor expression (28). VEGF is highly expressed in gastric carcinoma (29,30). However, whether PEDF can balance VEGF protein levels in gastric carcinoma has not been investigated. We revealed that VEGF protein was significantly down-regulated both in SGC7901 cells and gastric carcinoma tissue treated with PEDF (Fig. 3).

HIF-1α is a major transcription factor up-regulating VEGF expression (31,32), and is significantly expressed in gastric carcinoma (30). Attenuation of HIF-1α protein inhibited VEGF expression and suppressed tumor growth (33-35). We further examined if HIF-1α was regulated by PEDF in SGC7901 cells

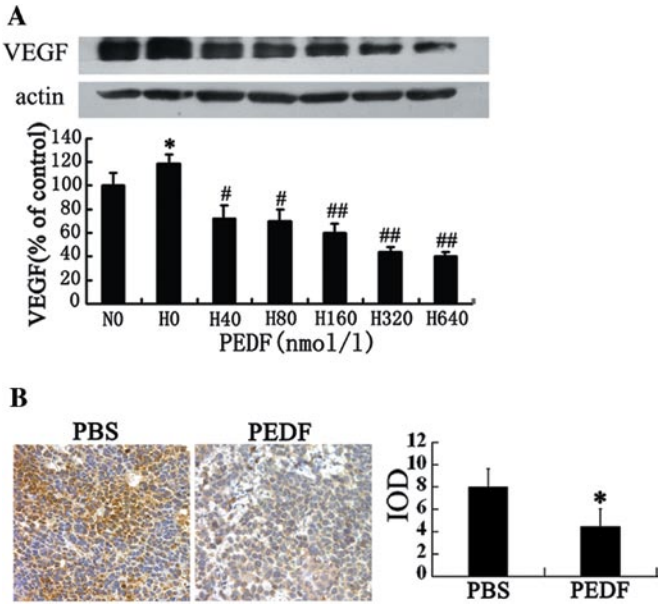

Figure 3. SGC7901 cells were treated with PEDF at the indicated concentrations for 24 h under normoxic or hypoxic conditions with 1% oxygen. (A) VEGF protein levels were measured by Western blot analysis and the results were normalized to β-actin. (B) The VEGF amount was investigated in heterotopic tumors by immunohistochemistry. *P<0.05 compared with normoxia; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with hypoxia. IOD, integral optical density.

and in xenograft tissue. HIF-1α levels significantly increased under hypoxia compared to normoxia in the cell model. PEDF suppressed HIF-1α protein levels and decreased its nuclear translocation (Fig. 4). These results suggest that PEDF inhibits VEGF expression via suppressing HIF-1α protein.

In conclusion, the present study reported for the first time that PEDF suppresses gastric carcinoma growth by anti-angiogenesis. PEDF may have potential for the treatment of gastric carcinoma.

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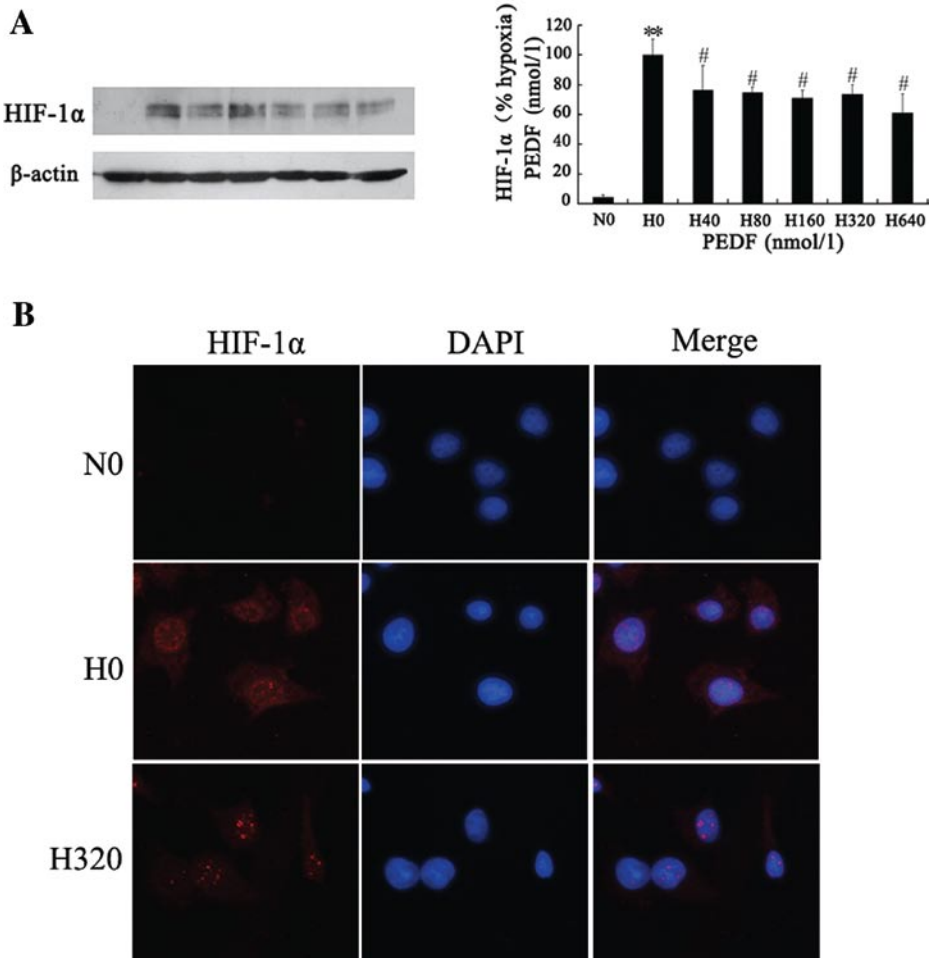

Figure 4. SGC7901 cells were treated with PEDF at the indicated concentrations for 9 h under normoxic or hypoxic conditions with 1% oxygen. The cells were then subjected to (A) immunoblotting or (B) immunofluorescence analysis. **P<0.01 compared with normoxia; $^{\#}$P<0.05 compared with hypoxia.

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