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PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases

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Abstract

We examined how pigment epithelium derived factor (PEDF), an effective endogenous antiangiogenic protein, decreases survival of primary cultures of human umbilical vein endothelial cells (HUVECs) in a low serum environment supplemented with the endothelial cell growth factor (VEGF). We provide evidence that induction of apoptosis by PEDF is associated with activation of p38 followed by cleavage of caspases 3, 8, and 9 by treatment with PEDF, and PEDF's actions are caspase dependent. A key mediator in the executioner effects of PEDF is p38 since the inhibition of p38 activity blocked apoptosis and prevented cleavage of caspases 3, 8, and 9. Although PEDF-induced phosphorylation of JNK1, the inhibition of JNK1 had no effect on apoptosis, even though it prevented phosphorylation of JNK1 by PEDF. Based on these findings, we propose that the antiangiogenic action of PEDF is dependent on activation of p38 MAPkinase which regulates cleavage of multiple caspases cascades.

Keywords: Pigment epithelium derived factor; Endothelial cell growth factor; Endothelium; p38; JNK1; Caspase 3; Caspase 8; Caspase 9; Apoptosis; Low serum

Proliferation, migration, and adhesion of endothelial cells are essential events to angiogenesis. Vascular endothelial growth factor (VEGF) is considered the key molecule controlling many of these important processes required for neovascularization [1–3]. VEGF promotes both physiological and pathological angiogenesis and plays a key role in the development and progression of tumors and several blinding eye disorders including diabetic retinopathy and age related macular degeneration.

Apart from promoting migration, proliferation, and permeability of endothelial cells, one of the major actions of VEGF is to prevent apoptosis of endothelial cells even in conditions of serum starvation [4,5]. Many of the actions of VEGF on endothelial cells, however, can be specifically counteracted by pigment epithelium derived factor (PEDF), a 50 kDa protein synthesized in most tissues of the body [6–8]. Although the antiangiogenic effect of PEDF on VEGF-induced neovascularization has been clearly demonstrated in several in vitro and in vivo models, there are no data on the actions of PEDF on VEGF-mediated protection of endothelial cells and little on the specific mechanisms by which PEDF inhibits the growth of these cells.

There is some evidence that PEDF mediates apoptosis in HUVECs by increasing the expression of Fas/FasL [9]. However, it is still unclear whether other pathways are involved in PEDF signaling of apoptotic events in HUVECs since PEDF can still inhibit neovascularization in mice lacking either Fas or FasL [10].

For this reason, we examined the molecular actions of PEDF on HUVEC cells in the presence and absence of the survival and proliferation signals from VEGF. We provide strong evidence that PEDF induces death of endothelial cells through the activation of both receptor-mediated and mitochondria-mediated pathways of caspase

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activation and that activation of these pathways is p38-dependent.

Materials and methods

Cell culture. HUVECs were isolated from discarded tissue in accordance with a protocol approved by Yale University Human Investigations Committee. The cells were serially diluted and cultured on gelatin-coated (0.1%) (J.T. Baker Inc.) plates as previously described [11]. Cells were starved in 0.2% FBS medium for 8 h before treatments with 2 ng/ml VEGF (R&D Systems, Minneapolis, MN), 100 ng/ml PEDF (Bioproduts MD, LLC, Maryland), both factors, or the specific pharmacological inhibitors of caspase (ZVAD-fmk) (10 μM) (R&D Systems, Minneapolis, MN), p38 MAP Kinase (SB203580) (25 μM) and the JNK1 inhibitor (SP601125) (2 μM) (Calbiochem, La Jolla, CA). Cells used in this study were obtained between passages 3 and 6.

Cell viability assay. HUVECs were seeded at 3×10^4/well in 0.1% gelatin-coated 96-well plates and grown overnight in M199 supplemented with endothelial cell growth supplement (50 μg/ml) (BD Biosciences, Bedford, MA) and heparin (100 μg/ml). Cells were washed with PBS, cultured for 8 h in M199 containing 0.2% FBS, then incubated in medium with or without VEGF, PEDF, both factors, or a specific pharmacological inhibitor for an additional 24 h. The plates were then washed, freeze-thawed, and analyzed for DNA content using a CyQuant assay (Invitrogen Corporation, Carlsbad, California). Fluorescence intensity was measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Data were obtained from three separate experiments using six replicate wells for each. The fluorescences density in each treated group was compared with the value in the group before treatment, so the percentages mean cell survival.

Cell proliferation assay. Measurement of cell proliferation was obtained from analysis of DNA incorporation of the thymidine analog 5′-bromo-2′-deoxyuridine (BrdU) as described by the manufacturer (Roche Diagnostics Corporation, Indianapolis, IN). HUVECs were seeded at 1×10^4/cm^2 on 0.1% gelatin-coated tissue culture slides chambers. The cells were allowed to attach to the slides overnight then washed and incubated for 8 h in M199 prior to treatment with or without VEGF, PEDF, or both factors for 24 h. BrdU (10 μM) was subsequently added and incubation of the cultures continued for 1 h at 37 °C. The samples were then processed for BrdU immunocytochemistry as described by the manufacturer. Cell proliferation was calculated using the percentage obtained from positive cells in each field (400×) from at least 5 different fields for each treatment and control groups. HUVEC proliferation was also measured by Western blot analysis using an antibody to proliferating cell nuclear antigen (PCNA) (Sigma–Aldrich, St. Louis, MO).

Apoptosis detection assay. To determine the percentage of cell death occurring in the cultures, terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) was carried out on some of the cultures treated as described above using an in situ cell death detection kit (Roche Diagnostics Corporation, Indianapolis, IN). After 24 h, the cells were washed with PBS, fixed for 1 h at room temperature with 4% paraformaldehyde, and cell membranes permeabilized with Triton X-100 (0.1%). Samples were then incubated with the TUNEL reaction mixture, counterstained with DAPI, and then examined by fluorescence microscopy. The percentage of apoptotic cells was calculated from the positively labeled cells in each field (400×) from at least 5 fields for each group.

Western blotting. Changes in the phosphorylation of specific signal molecules between the various treatment groups were determined using Western blot analysis. Cells from the experimental and control groups were scraped into lysis buffer [12], rotated for 1 h at 4 °C and the lysates collected by centrifugation for 15 min at 12,000 rpm at 4 °C. Western blots were prepared as previously described [13]. The membranes were incubated with primary antibody: anti-PCNA antibody PCNA, anti-β-actin antibody (Sigma–Aldrich, St. Louis, MO), anti-cleaved caspases 3, 8, 9 antibodies, anti-phospho-p38 antibody, anti-p38 antibody (Cell Signaling Technology, Inc., Danvers, MA), anti-phospho-JNK antibody, and anti-JNK antibody (Santa Cruz Biotechnology, Santa Cruz, California). Densitometric data of positively reacting antigen–antibody complexes on the membranes are expressed as fold induction of the target protein in the experimental group compared with the non-treated controls. Signals of phosphorylated proteins were normalized using signals from the non-phosphorylated protein.

p38 MAPK kinase assay. This assay was performed using a commercial kit (Cell Signaling Technology, Inc., Danvers, MA) to determine activation of the p38 kinase in the HUVEC cultures after the various treatments. Anti-phospho-p38 (Thr-180/Tyr-182) was used to immunoprecipitate p38 from cell lysates, and the phosphorylation at Thr-71 of ATF-2, a known substrate for activated p38, was measured by Western blot analysis using phospho-ATF-2 (Thr-71) antibody.

Immunocytochemical staining for cleaved caspase 3. To determine the effect of PEDF on caspase 3 cleavage and activation, 40,000 HUVECs/well were cultured and treated with the various factors as described above but for only 3 h were fixed using 4% paraformaldehyde in PBS. The cell membranes were permeabilized with 0.2% Triton X-100 in PBS, and non-specific immunobinding sites were blocked with preimmune goat serum before reaction with a polyclonal antibody that specifically recognizes the cleaved form of caspase-3 followed by labeling with a cy-3 goat anti-rabbit IgG (Cell Signaling Technology, Inc., Danvers, MA). Cells that were positive for the cleaved form of caspase 3 were visualized by epifluorescence microscopy.

Data analysis and statistics. All data represent means ± SD of at least three independent experiments. Statistical analysis was performed by one-way ANOVA assay. Differences were considered significant at $p < 0.05$.

Results

PEDF blocks VEGF-mediated survival of HUVECs by regulating cell death not cell proliferation

When endothelial cells are cultured in low serum (0.2% FBS) they progressively undergo loss of cells within 24 h. In our assay, 50 ± 15% cells grown in medium containing 0.2% serum remained viable compared with cultures maintained in medium supplemented with 20% FBS (Fig. 1A). The effects of serum starvation on the viability of HUVECs can be counteracted by addition of low concentrations of VEGF to these cultures [5,14], a finding which we have confirmed. The addition of 2 ng/ml VEGF to the low serum environment leads to survival of 81 ± 8% of the cells when compared to the non-VEGF-treated cultures (Fig. 1A). When we added 100 ng/nl PEDF to cultures grown in low serum alone the number of viable HUVECs decreased to 21 ± 15% below the control cultures after 24 h. Addition of the same concentration of PEDF to low serum cultures supplemented with VEGF reduced cell viability by 26 ± 20% (Fig. 1A).

We next examined whether the opposing effects of VEGF and PEDF on cell viability are due to regulation of the proliferation rate of endothelial cells. For this study, we measured cell proliferation using both BrdU labeling (Fig. 1B) and expression of PCNA (Fig. 1C) after the cells were treated with VEGF, PEDF, or both for 24 h. In the low serum control cultures we detected 10 ± 8% BrdU labeled cells. There was no significant difference in either BrdU labeling or PCNA expression between the controls and any of the treatment groups. These results indicate that the regulation of cell viability by VEGF and PEDF was not