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Original Paper

Pigment Epithelium-Derived Factor and its Phosphomimetic Mutant Induce JNK-Dependent Apoptosis and P38-Mediated Migration Arrest

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Key Words
PEDF • phosphomimetic mutations • angiogenesis inhibitor • MAPK • JNK • p38

Abstract
Background/Aims: Pigment epithelium-derived factor (PEDF) is a potent endogenous inhibitor of angiogenesis, and a promising anticancer agent. We have previously shown that PEDF can be phosphorylated, and that distinct phosphorylations differentially regulate its physiological functions. We also demonstrated that triple phosphomimetic mutant (EEE-PEDF), has significantly increased antiangiogenic activity, and is much more efficient than WT-PEDF in inhibiting neovascularization and tumor growth. The enhanced antiangiogenic effect was associated with a direct ability to facilitate apoptosis of tumor-residing endothelial cells (EC), and subsequently, disruption of intratumoral vascularization. In the present report, we elucidated the molecular mechanism by which EEE-PEDF exerts more profound effects at the cellular level. ***Methods:*** Here we used Western blotting, as well as *in vitro* binding, proliferation, apoptosis and migration assays to follow the signaling components responsible for the PEDF and EEE-PEDF effects. ***Results:*** We found that EEE-PEDF suppresses EC proliferation due to caspase-3-dependent apoptosis, and also inhibits migration of the EC much better than WT-PEDF. Although WT-PEDF and EEE-PEDF did not affect proliferation and did not induce apoptosis of cancer cells, these agents efficiently inhibited cancer cell motility, with EEE-PEDF showing stronger effect. The stronger activity of EEE-PEDF was correlated to a better binding to laminin receptors. Furthermore, the proapoptotic and antimigratory activities of WT-PEDF and EEE-PEDF were found respectively regulated by differential activation of two distinct MAPK pathways, namely JNK and p38. We show that JNK and p38 phosphorylation is much higher in cells treated with EEE-PEDF. JNK leads to apoptosis of ECs, while p38 leads to antimigratory effect in both EC and cancer cells. ***Conclusion:*** These results reveal the molecular signaling mechanism by which the phosphorylated PEDF exerts its stronger antiangiogenic, antitumor activities.

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Introduction

Pigment epithelium-derived factor (PEDF) is a 46-kDa secreted glycoprotein that can act either as a neurotrophic or an antiangiogenic factor [1-3]. PEDF has been originally identified in the retina, however, it has now become evident that it is expressed throughout the human body and is constantly present in the systemic circulation [4, 5]. Studies in the past years have implicated PEDF in the pathophysiology of a wide range of angiogenesis-associated disorders, including diabetic complications [6, 7], macular degeneration [8, 9], Osteogenesis Imperfecta Type VI [10, 11], and the growth of many types of solid tumors [12-16]. Some of these roles may be mediated by the effect of PEDF on stem cells as was shown in the brain, muscle, and eye [11]. Furthermore, the natural antiangiogenic activity of PEDF, which is far greater than that of any other known endogenously produced factor [2, 17], has turned it into a highly potent therapeutic agent for the inhibition of pathological neovascularization and, therefore, a promising tumor suppressor [18]. Indeed, a number of studies have shown that exogenous administration of PEDF results in tumor regression, decreased intratumoral microvessel density and prolonged survival in various animal cancer models [19-22]. The underlying molecular mechanism of anticancer activity of PEDF has been under extensive investigation recently [5, 23]. Yet, the signaling pathways that mediate the effects of PEDF are not fully delineated and its exact mechanism of action remains to be elucidated.

Previous studies by our group revealed that plasma PEDF is a phosphoprotein that can be phosphorylated by casein kinase 2 (CK2) on Ser24 and Ser114, and by protein kinase A (PKA) on Ser227 [24, 25]. Using double and triple phosphorylation site mutants, we have identified the physiological significance of these phosphorylations by demonstrating that variable phosphorylation states of PEDF differentially regulate its biological mode of activity [26]. In these studies, mutants mimicking either CK2 or the accumulative CK2 plus PKA phosphorylation (S24E114E227A, EEA-PEDF and S24E114E227E, EEE-PEDF) exhibited much stronger antiangiogenic activity than their wild-type (WT) counterpart [24-26]. More recently, we have also shown that phosphomimetic mutants of PEDF, particularly EEE-PEDF, are significantly more efficient than WT-PEDF in inhibiting tumor growth and neovascularization in human breast, colon cancer and glioblastoma xenograft models [27]. Immunohistochemical analysis revealed that PEDF and its mutants affect mainly tumor-residing endothelial cells (ECs) and prevent the formation of intratumoral vascular network by facilitating EC apoptosis. On the other hand, PEDF and its mutants did not affect survival of cancer cells, indicating that the antiangiogenic activity of these agents is the foremost element of the observed antitumor effect.

In the current study, we aimed to uncover mechanistic aspects of the enhanced antiangiogenic and anticancer activity of the phosphomimetic PEDF, by examining the physiological effects and signaling cascades induced by WT-PEDF and EEE-PEDF. We found that EEE-PEDF functions more potently than WT-PEDF in suppressing EC proliferation due to induction of caspase-3-dependent apoptosis, and also in inhibiting migration of these cells. In addition, we demonstrate that despite having no effect on cancer cell proliferation, WT-PEDF, and more so, EEE-PEDF efficiently inhibit cancer cell migration. The enhanced EEE-PEDF effects are correlated to a better binding to laminin receptor. The proapoptotic and antimigratory activities of WT-PEDF and EEE-PEDF are further shown to be differentially and independently regulated by JNK and p38 MAPKs. As compared to WT-PEDF, EEE-PEDF induces much stronger signaling via p38 and JNK, and we propose that this stronger signaling effect is the reason for the enhanced antiangiogenic activity of this PEDF mutant.

Materials and Methods

Materials

MEK1/2 inhibitor U0126 and p38α/β inhibitor SB203580 were purchased from Calbiochem, and JNK1-3 inhibitor SP600125 was from Biomol. Recombinant bFGF was purchased from Sigma. The following primary antibodies were used: anti-MKK3/6, anti-phospho MKK3/6, anti-MKK4, anti-MKK7 from Santa-Cruz Biotechnology, anti-cleaved caspase-3, anti phospho JNK1-3, anti-phospho MKK4, anti-phospho MKK7, anti-phospho c-Jun, anti-phospho MAPKAPK2, anti-pSer473-AKT (pAKT), anti-general-AKT (gAKT), anti-GST and anti-GFP from Cell Signaling, anti-tubulin, anti-ERK1/2, anti-phospho ERK1/2, anti-JNK, ant-p38α, anti-phosphor-p38, anti-c-Jun and anti-MAPKAPK2 from Sigma, and anti-GFP from Roche. Propidium iodide was purchased from Sigma-Aldrich (St Louis, MO, USA). SB202190, RWJ67567, and BI78D3, inhibitors were from Tocris Bioscience (Ellisville, MO, USA). antibodies were from Cell Signaling (Beverly, MA, USA).

Cell culture

U87-MG (human glioblastoma) and BAEC (bovine aorta ECs) were cultured in 4.5 g/l D-Glucose DMEM (Invitrogen), supplemented with 2 mM L-glutamine, antibiotics and 10% fetal calf serum (FCS). HCT116 (human colorectal carcinoma) were grown in McCoy's 5A medium (Sigma) with the same supplements and MDA-MB-231 (human invasive breast carcinoma) were grown in RPMI medium (Invitrogen) with the same supplements plus 1mM sodium pyruvate. HUVEC (human umbilical vein ECs) were cultured (passages 4-9) on gelatin-coated plates in M199 medium (Sigma) with 20% FCS, L-glutamine, antibiotics, 0.1 mg/ml heparin and 0.025 mg/ml endothelial cell growth supplement (ECGS, Biomedical Technologies, MA, USA). COS7 (African green monkey kidney) cells were cultured in 4.5 g/l D-Glucose DMEM, supplemented with 2 mM L-glutamine, antibiotics and 10% fetal calf serum (FCS). All cells were maintained at 37°C in a humidified atmosphere and 5% CO_2.

PEDF production

WT-PEDF and EEE-PEDF (from human origin) were cloned from pBlueScriptSKII(+)/PEDF, and pcDNA3/EEE-PEDF [24, 26] respectively into pRSET(A) (Invitrogen), and expressed in *E. coli* BL21. Bacterial cells were grown at 30°C to OD_{600} = 0.5-0.6, and the expression of recombinant proteins was induced by 0.5 mM IPTG for 4-5 h. Pelleted bacterial cells were lysed in ice-cold Ni-NTA binding buffer (containing 300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0) supplemented with 10 μg/ml leupeptin, 1 mM PMSF, 10 mM beta-mercaptoethanol and 1 mg/ml lysozyme followed by sonication. Lysates were then cleared by centrifugation at 12,000Xg at 4°C for 15 min. Batch purification of recombinant proteins was performed using ion metal affinity chromatography with Ni-NTA His-Bind resin (Novagen) according to the manufacturer's protocol. Elution fractions were resolved on SDS-PAGE followed by silver staining and immunoblotting with anti-PEDF antibody. The identity of recombinant WT-PEDF and its mutants was verified by mass spectroscopy. To remove the excess of imidazole, eluates that exhibited > 90% purity were dialyzed overnight at 4°C against PBS. Endotoxin content was evaluated by LAL turbidimetric assay and was < 2 EU/μg protein.

Immunoblotting (Western blotting)

Cells were grown to subconfluency and then serum-starved (0.1% FCS for all cells except of HUVEC, which were serum-starved with 0.5% FCS) for 16 h. After incubation with indicated treatments, cells were rinsed twice with ice-cold PBS and scraped into RIPA buffer (0.2 ml/6 cm plater). Extracts were then obtained by centrifugation at 15,000Xg for 15 min, 4°C. Aliquots of cellular extracts were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Tamar, Israel) by electroblotting. Membranes were incubated overnight at 4°C with the corresponding primary antibody, followed by incubation with either HRP- or AP-conjugated secondary antibody (Jackson). Membranes were developed either using EZ-ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel). Each experiment was performed at least 3 times to test for reproducibility and obtain statistically significant data. Quantification was performed using QuantityOne software (Bio-Rad).

Proliferation assay

The effect of WT-PEDF and EEE-PEDF on cell proliferation was determined using Methylene Blue assay. For this purpose, cells were incubated for 48 h in 1% FCS-containing medium with the indicated amounts of recombinant proteins. Following treatment, cells were fixed in 4% paraformaldehyde at room temperature (RT). Cells were then washed once in 0.1 M sodium borate buffer, pH 8.5 and, thereafter, incubated with 1% Methylene Blue in 0.1 M sodium borate buffer, pH 8.5 for 10 min. Excess of stain was washed out with double distilled water (DDW) and the stain was extracted with 0.1 M HCl (0.4 ml/well) for 2h at RT with shaking. Thereafter, aliquots of each sample were transferred into 96-well plate and OD_{595} was determined using ELISA reader.

In vitro migration assays

Transwell assay*:* the migration of BAEC, HUVEC and MDA-MB-231 cells was assayed in 24-well Transwell plates (Corning, NY, USA). For ECs, the upper surface of the polycarbonate filters with 8 μm pores was coated with 0.2% gelatin in PBS. A suspension of cells (5-8X10^4 cells/100 μl) was placed in the upper chambers. The lower chambers were filled with 600 μl of the corresponding medium containing 20 ng/ml bFGF. Cells were allowed to migrate for 16-20 h in the presence of different treatments. Thereafter, cells were removed from the upper compartment of the filter with a cotton swab. Cells that reached the lower surface of the filter were fixed with paraformaldehyde (PFA, 3.5%, 20 min), stained with 0.1% Crystal violet and images were then captured using digital camera coupled with a microscope. Relative migration was quantified after stain extraction with methanol and OD_{540} measurement in ELISA reader.

"Wound healing" assay: BAEC and MDA-MB-231 cells were grown to 95-100% confluence. Thereafter, a cell scratch spatula was used to create a fixed-width "wound" in a cell monolayer, followed by incubation with medium containing 1% FCS in the presence of bFGF and/or other treatments for 12-16 h. Wound closure was monitored by capturing the images using digital camera coupled with a microscope and following the advancement of the migrating front at defined time intervals.

Apoptosis assay

Apoptosis was evaluated using terminal deoxinucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) kit (Roche). After indicated treatments, cells grown on 18 mm cover slips were washed and fixed with 3% paraformaldehyde in PBS. Thereafter, apoptotic cells were identified using manufacturer's (Roche) protocol and quantified. DeltaVision OMX fluorescent microscope supplied with a digital camera and SoftWorx software (Applied Precision) was used to process the slides.

In vitro binding assay

Laminin receptor (LR) and patatin-like phospholipase domain-containing protein 2 (PNPLA2) sequences were amplified from HUVECs cDNA using specific primers flanked by *SalI* (forward) and *BamHI* (reverse) restriction sites. To create GFP-LR and GFP-PNPLA2 constructs, amplicons were respectively cloned between *SalI* and *BamHI* sites of pEGFP-C1 (Clontech). Thereafter, COS-7 cells were transiently transfected with 0.5 μg of either GFP-LR or GFP-PNPLA2 plasmid DNA using PEI (poliethilenimine). Forty-eight hours after transfection cells were lysed and GFP-LR or GFP-PNPLA2 were immunoprecipitated using anti-GFP antibody pre-linked to A/G agarose beads (Santa-Cruz). Thereafter, immunoprecipitate-bound A/G beads were washed twice with ice-cold washing buffer containing 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 150 mM NaCl and 0.5% Triton X-100. Following second wash beads were resuspended in PBS and aliquoted. Each aliquot was combined with 4 μg of recombinant GST-WT-PEDF or GST-EEE-PEDF in PBS and incubated for 4 h at 4°C with rotation. Thereafter, beads were washed three times with washing buffer, resuspended in 1XSB and boiled. Resolved proteins were analyzed by immunoblotting with anti-PEDF antibody. To confirm equal amounts of GFP-LR or GFP-PNPLA2, membrane was then re-probed with anti-GFP antibody.

FACS analysis

Cells were plated in 60mm dish, starved overnight in 1% FCS media and then either preincubated with Z-VAD.fmk (BD biosciences; 10 μM, 30 min) or left untreated. Cells were then treated with PEDFs (WT-PEDF and EEE-PEDF, 20 nM) for 48 h, after which they were harvested in Buffer W 0.1% sodium citrate, 0.1% Triton X-100. Cell cycle profiles and apoptosis were evaluated by DNA staining with 500 μl solution of 50

μg/ml propidium iodide in Buffer W for 30 minutes at 23°C. Samples were analyzed with a FACScan flow cytometer (LSR II, Becton Dickinson), using Mod FitLT program.

GST-PEDF purification

WT-PEDF and EEE-PEDF were cloned from pBlueScriptSKII(+)/PEDF, and pcDNA3/EEE-PEDF [24, 26] respectively into pGJ41-GST-8His-TEV (provided by Dr. Ghil Jona, Protein Purification Unit, Weizmann Institute of Science) and expressed in *E. coli* BL21-pLysS. Bacterial cells were grown at 37°C to $OD_{600\,nm}$ = 0.5-0.6, and the expression of recombinant proteins was induced by 0.5 mM IPTG for 4 h. Pelleted bacterial cells were lysed in ice-cold PBS supplemented with 10 μg/ml leupeptin, 1 mM PMSF, 1 mM DTT and 1 mg/ml lysozyme followed by sonication. Lysates were then cleared by centrifugation at 12, 000Xg at 4°C for 15 min. Purification of recombinant proteins was performed using glutathione sepharose beads (GE Healthcare) according to the manufacturer's protocol. The identity of recombinant constructs was verified by blotting with anti-PEDF antibody and mass spectroscopy.

GST pull-down assay

COS-7 cells were transiently transfected with 0.5 μg of either GFP-LR plasmid DNA using PEI (polyethylenimine). Forty-eight hours after transfection cells were lysed and aliquots of cell lysates were combined with 0.5 μg of recombinant GST-WT-PEDF, GST-EEE-PEDF or GST alone in PBS. Samples were incubated overnight at 4°C with rotation. Thereafter, equal amounts of glutathione sepharose beads were added to each sample and incubated for additional 2 h at 4°C with rotation. Beads washed three times with washing buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 150 mM NaCl, 0.5% Triton X-100), resuspended in 1XSB and boiled. Resolved protein complexes were analyzed by immunoblotting with anti-GFP antibody. To confirm equal amounts of GST-WT-PEDF, GST-EEE-PEDF or GST in each sample, membrane was then re-probed with anti-GST antibody.

Statistical analysis

Data are expressed as mean ± S.E. Statistical evaluation was carried out using functional analysis (ANOVA) and Student's t-test (two-tailed), to test for differences between the control and experimental results. Values of $P < 0.05$ were considered statistically significant.

Results

The effect of WT-PEDF and EEE-PEDF on endothelial and cancer cell proliferation

We have previously shown that when administered to mice bearing MDA-MB-231, HCT116 or U87-MG xenografts, WT-PEDF and its phosphomimetic mutant EEE-PEDF predominantly affect ECs of the tumor vasculature, rather than cancer cells themselves [27]. Thus, we undertook to further study the mechanisms of action of WT-PEDF and of EEE-PEDF, which caused the most profound inhibition of xenograft growth, in cultured endothelial and cancer cells. First, we examined the effect of WT-PEDF and EEE-PEDF on proliferation of bovine aorta ECs (BAEC) by methylene blue assay. We found that nanomolar concentrations of both PEDF constructs suppress their proliferation in a dose-dependent manner, where EEE-PEDF had a significantly stronger effect than WT-PEDF (Fig. 1A). On the other hand, the same concentrations of the PEDF constructs didn't affect proliferation of MDA-MB-231 (Fig. 1B), HCT116 (Fig. 1C), U87-MG (Fig. 1D), as well as HeLa, DU145 and MCF-7 (not shown) cells. These results suggest that the inhibitory effect of PEDF constructs on tumor growth is mostly indirect.

The effect of WT-PEDF and EEE-PEDF on endothelial cell apoptosis

According to our previous results [27], the antiproliferative effect of PEDF towards ECs was associated with an enhanced ECs apoptosis. To verify it, we first compared the rate of WT-PEDF- and EEE-PEDF-induced apoptosis by TUNEL assay. In these experiments, we observed a time- and dose-dependent induction of apoptosis in BAEC by both WT-PEDF and EEE-PEDF, where the apoptotic effect of EEE-PEDF was almost 3-fold higher than that of WT-PEDF in

all tested concentrations (Fig. 2A & 2B). These results were supported by a dose-dependent activation of caspase-3 upon treatment with both WT-PEDF and EEE-PEDF, as manifested by the appearance of 17- and 19-kDa caspase-3 cleavage products (Fig. 2C). In contrast, WT-PEDF as well as EEE-PEDF failed to induce apoptosis of MDA-MB-231 cells as evaluated by TUNEL over 48 h period [27]. For further information on the apoptosis, we used a FACS-based cell cycle analysis. By this method, we found that a large fraction of cells treated with WT-PEDF and more so EEE-PEDF are in sub-G1 phase (hypodiploid nuclei), without an arrest in G1 or G2 phases of the cell cycle (Fig. S1 (For all supplemental material see www.karger.com/10.1159/000492990)). This effect was completely abolished by the general caspase inhibitor Z-VAD, clearly confirming again the apoptotic effect the PEDFs.

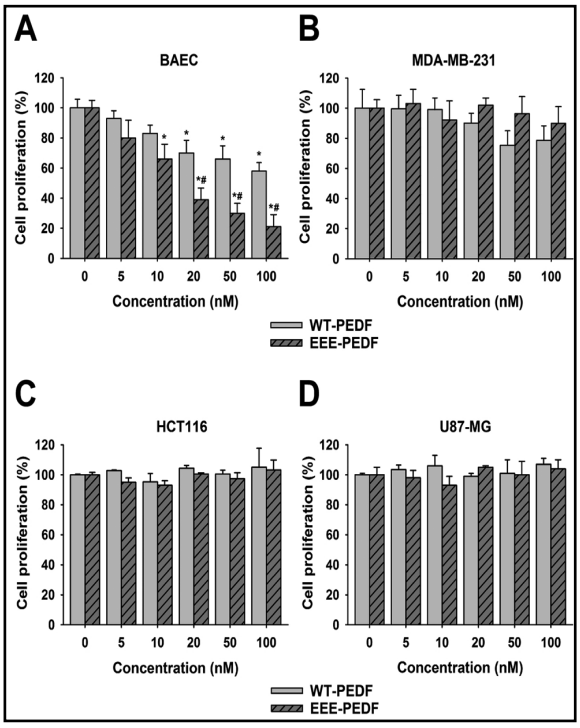
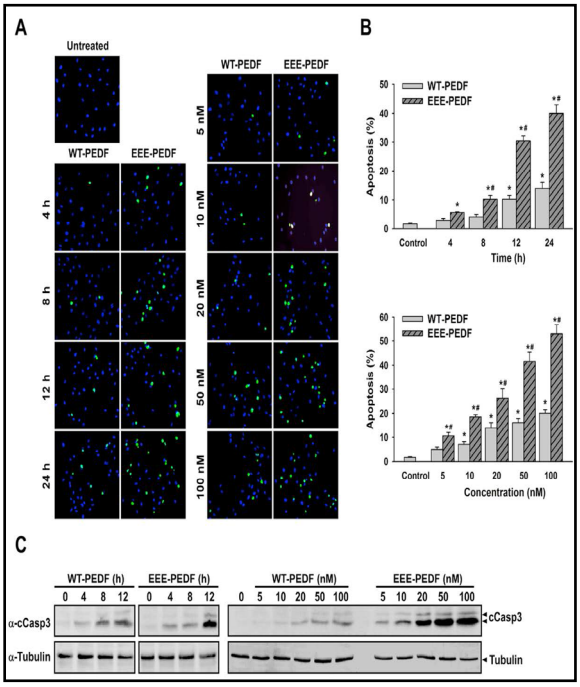


Fig. 1. The effect of WT-PEDF and EEE-PEDF on proliferation rate of endothelial and cancer cells. The rate of BAEC (A), MDA-MB-231 (B), HCT116 (C) and U87-MG (D) cell proliferation following treatment for 48 h with the indicated concentrations of WT-PEDF or EEE-PEDF was analyzed using Methylene Blue colorimetric assay. Data represent mean ± S.E. (N = 3) *, P<0.05, treated vs. control; #, P<0.05, EEE-PEDF vs. WT-PEDF.

Fig. 2. The effect of WT-PEDF and EEE-PEDF on apoptosis of cultured endothelial cells. A. Representative TUNEL-labeled X20 fields of BAEC treated with WT-PEDF or EEE-PEDF as indicated. Left: time-course of WT-PEDF- and EEE-PEDF-induced apoptosis (PEDF constructs used at concentration of 20 nM). Right: dose-dependent effect of WT-PEDF and EEE-PEDF on BAEC apoptosis (12 h treatment). Green, TUNEL; blue, DAPI. B. Quantification of the time-course (upper) and dose-response (lower) of the relative apoptotic rate in BAEC treated with WT-PEDF or EEE-PEDF. Data represent mean ± S.E. (N = 4) *, P<0.05, treated vs. control; #, P<0.05, EEE-PEDF vs. WT-PEDF. C. Time-course (left panel) and dose-response (right panel) effect of the PEDF constructs on caspase-3 cleavage as assessed by immunoblotting with anti-cleaved caspase-3.



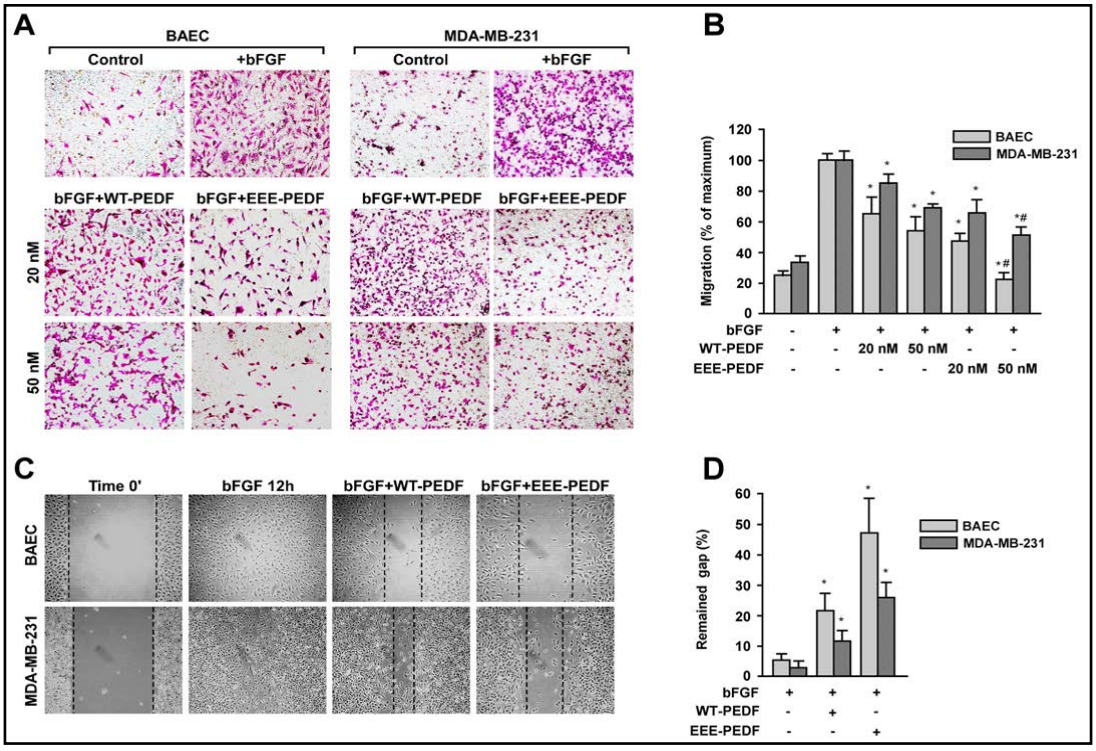


Fig. 3. The effect of WT-PEDF and EEE-PEDF on endothelial and cancer cell migration. A. Migration of BAEC and MDA-MB-231 cells in the presence of bFGF (20 ng/ml) and either WT-PEDF or EEE-PEDF as evaluated by Transwell assay. Shown are representative photographs of Crystal violet-stained X20 fields of migrated cells taken from the bottom side of the polycarbonate membranes. B. Following visualization, stain was extracted with methanol and migration was quantified by OD_{540} measurement. Data shown represent mean ± S.E. (N = 3) * P<0.05, treated vs. control; #, P<0.05, EEE-PEDF vs. WT-PEDF. C. Migration of BAEC and MDA-MB-231 cells in the presence of bFGF (20 ng/ml) and either WT-PEDF or EEE-PEDF (both at 20 nM) as evaluated by "wound healing" assay. D. Quantification of the results in C. Data represent mean ± S.E. (N = 3) * P<0.01, treated vs. control.

The effect of WT-PEDF and EEE-PEDF on endothelial and cancer cell motility

It is well known that the establishment of tumor vascular network depends on the motility of actively proliferating ECs, and the migration of cancer cells generally serves as a parameter for the degree of their invasive behavior [28]. Thus, the effect of the PEDF constructs on cell migration was examined in both BAEC and MDA-MB-231 cells. For this purpose, we first employed an *in vitro* Transwell assay, where chemoattractant-driven cell migration through the polycarbonate membranes in the presence of either WT-PEDF or EEE-PEDF was followed over 16 h. Both PEDF constructs dose-dependently inhibited bFGF-induced BAEC migration, and the ability of EEE-PEDF to attenuate cell migration was more substantial than that of WT-PEDF (Fig. 3A & 3B). Notably, unlike their lack of effect on apoptosis, WT-PEDF and more so EEE-PEDF did affect the migration of MDA-MB-231 cells although this effect was not as marked as in BAEC (Fig. 3A & 3B). These results were corroborated using "wound healing" assay, in which a fixed-width scratch in a cell monolayer was created and the advancement of the migrating front was followed in the presence of either WT-PEDF or EEE-PEDF (Fig. 3C). "Wound healing" experiments performed in HCT116 and U87-MG cells gained similar results as with MDA-MB-231 (data not shown), confirming that the effect on cancer cells is general. Taken together, these observations indicate that EEE-PEDF is more efficient than WT-PEDF in inhibiting different angiogenic activities of cultured ECs, such as proliferation and migration, and also, to some extent, in the inhibition of cancer cell migration.

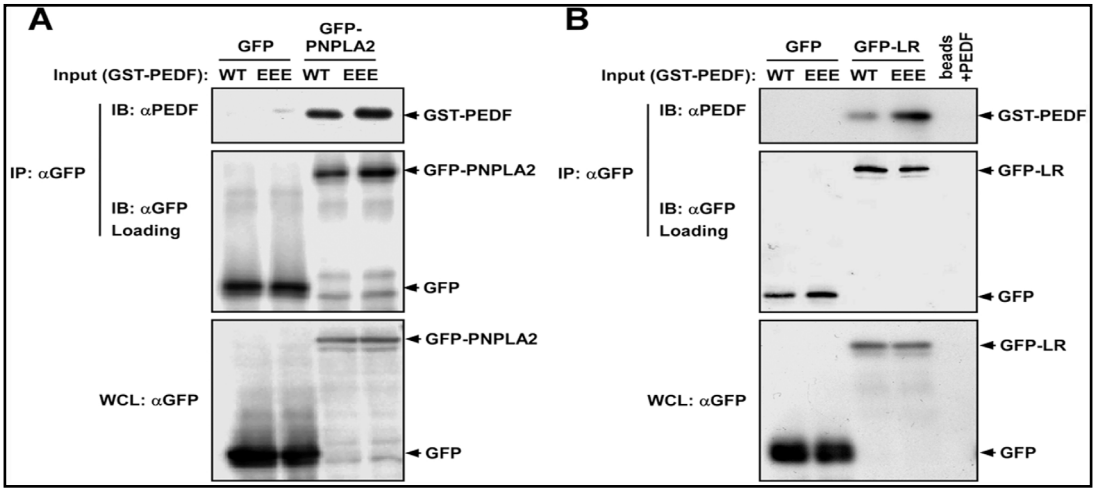


Fig. 4. Binding of WT-PEDF and EEE-PEDF to recombinant PNPLA2 and LR in in vitro binding assay. COS-7 cells were transfected with either GFP-PNPLA2 (A) or GFP-LR (B). Forty-eight hours after transfection cells were lysed and recombinant proteins were immunoprecipitated using anti-GFP antibody. Thereafter, aliquots of washed immunoprecipitate-bound A/G beads were incubated with 4 μg of recombinant GST-WT-PEDF or GST-EEE-PEDF. Following incubation, beads were boiled and resolved protein complexes were analyzed by immunoblotting with anti-PEDF antibody. WCL – Whole Cell Lysate.

Enhanced EEE-PEDF interaction with laminin receptor (LR) but not with patatin-like phospholipase domain-containing protein 2 (PNPLA2)

Next, we undertook to study the molecular signaling mechanism(s) by which EEE-PEDF exert its stronger antiangiogenic activity. As a first step, we examined the ability of the PEDF constructs to bind to two putative receptors that were recently published, namely LR [29] and PNPLA2 [30]. For this purpose, we transfected COS7 cells with either GFP-LR or GFP-PNPLA2. Forty-eight hours after transfection cells were lysed and Co-immunoprecipitation was performed with anti-GFP antibody. The immunoprecipaitated proteins were briefly washed, incubated with recombinant WT-PEDF or EEE-PEDF, and subjected to immunoblotting that revealed that while EEE-PEDF and WT-PEDF interacted with PNPLA2 equally well, the binding of EEE-PEDF to LR was much stronger than that of WT-PEDF (Fig. 4). This result was confirmed using a GST-pulldown that showed again a much better binding of EEE-PEDF to LR (Fig. S2). Interestingly, in a previous study [17], we found that WT-PEDF and EEE-PEDF bind to plasma membrane in similar affinities. In view of the relatively week binding of PNPLA2 and the changes in binding to LR, this similar binding to membranes may indicate that the PEDF and its phosphomimetic mutants bind to additional proteins on the cell surface, which may lead to distinct functions. However, since LR seems to be the main PEDF receptor that leads to its antiangiogenic effects [29], it is likely that the effect of EEE-PEDF is mediated by this receptor, and not by PNPLA2 or any of the other putative binding proteins.

Time-dependent activation of distinct MAPK cascades by WT-PEDF and EEE-PEDF

Since LR is probably not the only PEDF receptor, and the mechanism of its action are not clear yet, it was important to elucidate the intracellular regulatory mechanisms that mediate the effects of WT-PEDF and EEE-PEDF in endothelial and cancer cells. Hence, we examined whether the PEDF constructs affect the activity of MAPK cascades, ERK, p38 and JNK, which are known to play an important role in survival, apoptosis and cellular response to stress [31]. In BAEC, EEE-PEDF induced marked and sustained elevation in p38α and JNK1/2 phosphorylation up to 270 min of incubation, while the activation of ERK1/2 peaked at 15 min and declined back to basal level within 120 min (Fig. 5A, 5B & 5C). WT-PEDF had a similar trend of activation of all three MAPKs, although the fold changes of p38α and JNK1/2

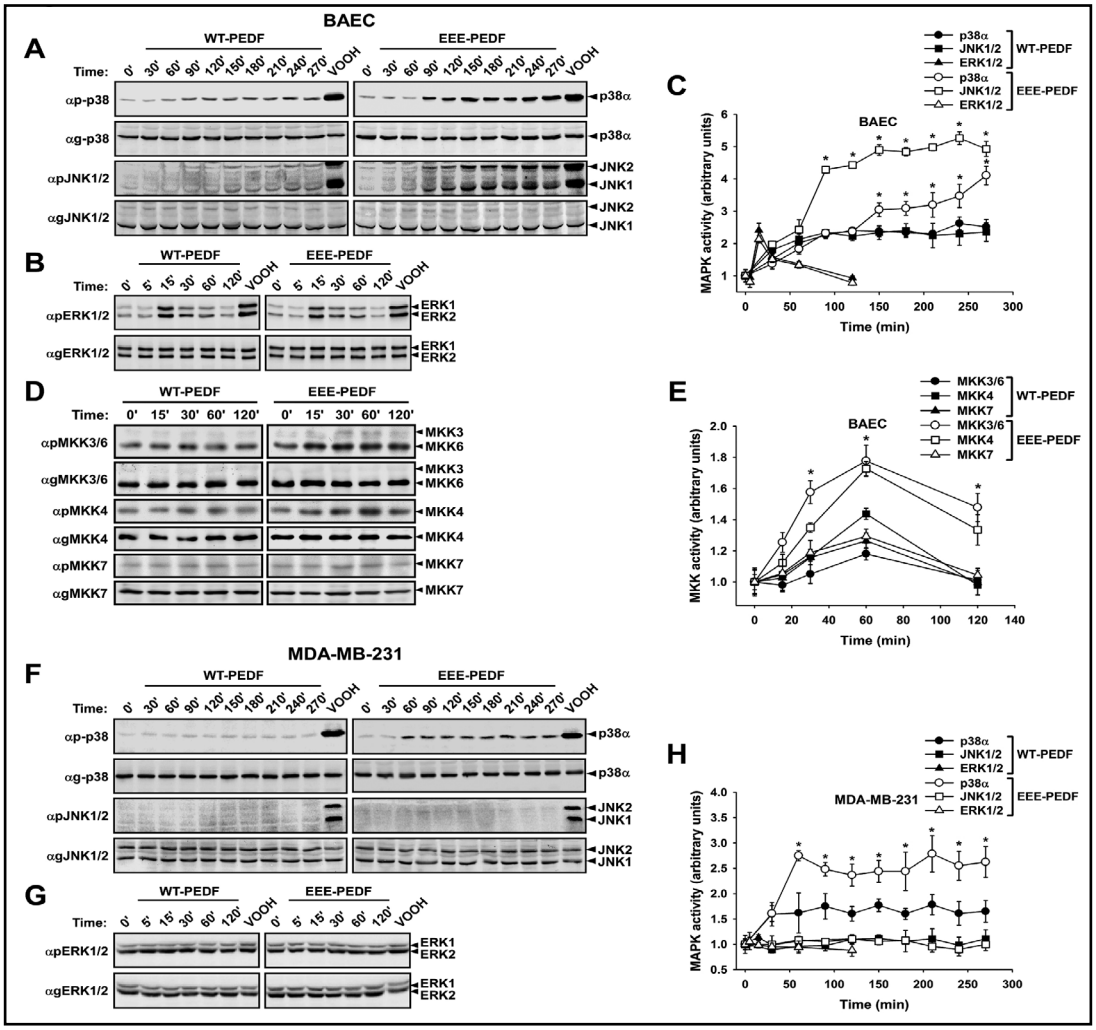


Fig. 5. The effect of WT-PEDF and EEE-PEDF on the activity of MAPK cascades in BAEC and MDA-MB-231 cells. A – E. The effect of WT-PEDF and EEE-PEDF (20 nM) on p38α, JNK1/2 (A), ERK1/2 (B), MKK3, MKK6, MKK4 and MKK7 (D) phosphorylation in BAEC was analyzed by immunoblotting with anti-phospho-specific antibodies. Time-course of WT-PEDF- and EEE-PEDF-induced MAPKs (C) and MAP2Ks (E) phosphorylation in BAEC was quantified from 3 independent immunoblotting experiments. *, P<0.05, EEE-PEDF vs. WT-PEDF. F – H. The effect of PEDF constructs on p38α, JNK1/2 (F) and ERK1/2 (G) phosphorylation in MDA-MB-231 cells was analyzed by immunoblotting with anti-phospho-specific antibodies. Time-course of WT-PEDF- and EEE-PEDF-induced MAPKs phosphorylation in MDA-MB-231 cells (H) was quantified from 3 independent immunoblotting experiments. *, P<0.05, EEE-PEDF vs. WT-PEDF. In all experiments, peroxyvanadate (VOOH; 200 μM H_2O_2, 100 μM Vanadate) was used as a positive control of MAPK/MAPKK activation.

phosphorylation were significantly lower than that observed with EEE-PEDF. The changes in p38α and JNK1/2 activity were preceded by the activation of the corresponding kinases at the MAP2K level (Fig. 5D & 5E). WT-PEDF and more so EEE-PEDF induced MKK3/6 and MKK4 phosphorylation, known to be the respective activators of p38 and JNK [32], but almost didn't affect phosphorylation of MKK7, which also acts upstream of JNK [33]. Both WT-PEDF and EEE-PEDF didn't affect JNK1/2, but did elevate slightly p38α activity in MDA-MB-231 cells (Fig. 5F & 5H). In this cell line, known to harbor K-Ras activating mutation [34] and high basal levels of ERK1/2 activity, both PEDF constructs failed to induce further increase in ERK1/2 phosphorylation (Fig. 5G & 5H). In order to study the possibility of synergism with other growth factors on the activation of p38 and JNK, we added low amount of bFGF that

did not cause any significant change of the MAPK phosphorylation. Simultaneous addition of PEDF resulted in the expected activation of the MAPKs with no significant change by the bFGF in BAEC cells (Fig. S3). Furthermore, short-term AKT phosphorylation was affected neither by WT-PEDF nor by EEE-PEDF in the cell lines examined (Fig. S4). These results indicate that PEDF-induced signaling is not likely affected by or interfering with angiogenic/proliferation/survival-regulating signal transduction pathways. Overall, WT-PEDF, and more so its phosphomimetic mutant, induce diverse signaling in different cell lines, supporting our findings on preferential susceptibility of ECs to PEDF action.

Differential regulation of the apoptotic and antimigratory activities of WT-PEDF and EEE-PEDF by JNK and p38 MAPKs

Next, we undertook to examine whether the signaling events described above are involved in the apoptotic and the antimigratory activities of WT-PEDF and EEE-PEDF. For this purpose, we first followed the effects of the PEDF constructs in BAEC and MDA-MB-231 cells pretreated with selective pharmacological inhibitors of JNK1-3 (SP600125), p38α/β (SB203580) or MEK1/2 (U0126). The specificity of p38α/β and JNK1-3 inhibitors was validated in BAEC, where these agents correspondingly and without interfering with effects of each other inhibited WT-PEDF- and EEE-PEDF-induced phosphorylation of the respective p38α and JNK1/2 substrates, MAPKAPK2 and c-Jun (Fig. S5A). Similarly, MEK1/2 inhibitor prevented WT-PEDF- and EEE-PEDF- induced ERK1/2, but not p38α or JNK1/2 phosphorylations (Fig. S5B & S5C). We found that JNK1/2 appear to be the main executors of PEDF-induced apoptosis in BAEC, since pretreatment with JNK1-3 inhibitor almost completely attenuated the apoptotic effect of both PEDF constructs in these cells, while pretreatment with the p38α/β inhibitor or the MEK1/2 inhibitor did not (Figures 6A & 6B). Also, JNK1-3, but not p38α/β inhibition resulted in significant decline of WT-PEDF- and EEE-PEDF-induced caspase-3 cleavage (Fig. 6C). Conversely, the antimigratory activity of the

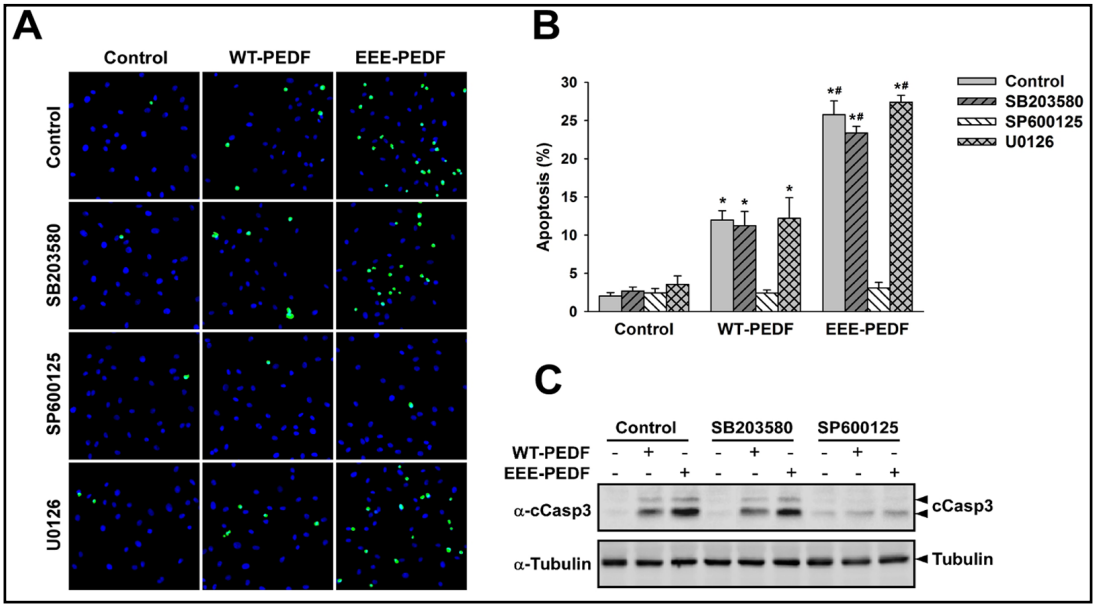


Fig. 6. The proapoptotic activity of WT-PEDF and EEE-PEDF is mediated by JNK but not by p38α/β or ERK. A. Representative TUNEL-labeled X20 fields of BAEC after pretreatment with either p38 α/β (SB203580, 10 μM), JNK1-3 (SP600125, 5 μM) or MEK1/2 (U0126, 5 μM) inhibitors for 1 h followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for 12 hours. Green, TUNEL; blue, DAPI. B. Quantification of the relative apoptotic rate in TUNEL-labeled BAEC treated as described in (A). Data shown are mean ± S.E. (N = 4) *, P<0.05, treated vs. control; #, P<0.05, EEE-PEDF vs. WT-PEDF. C. The effect of p38 α/β (SB203580, 10 μM) and JNK1-3 (SP600125, 5 μM) inhibitors on WT-PEDF and EEE-PEDF (20 nM)-induced caspase-3 cleavage in BAEC, as evaluated by immunoblotting with anti-cleaved caspase-3 antibody.

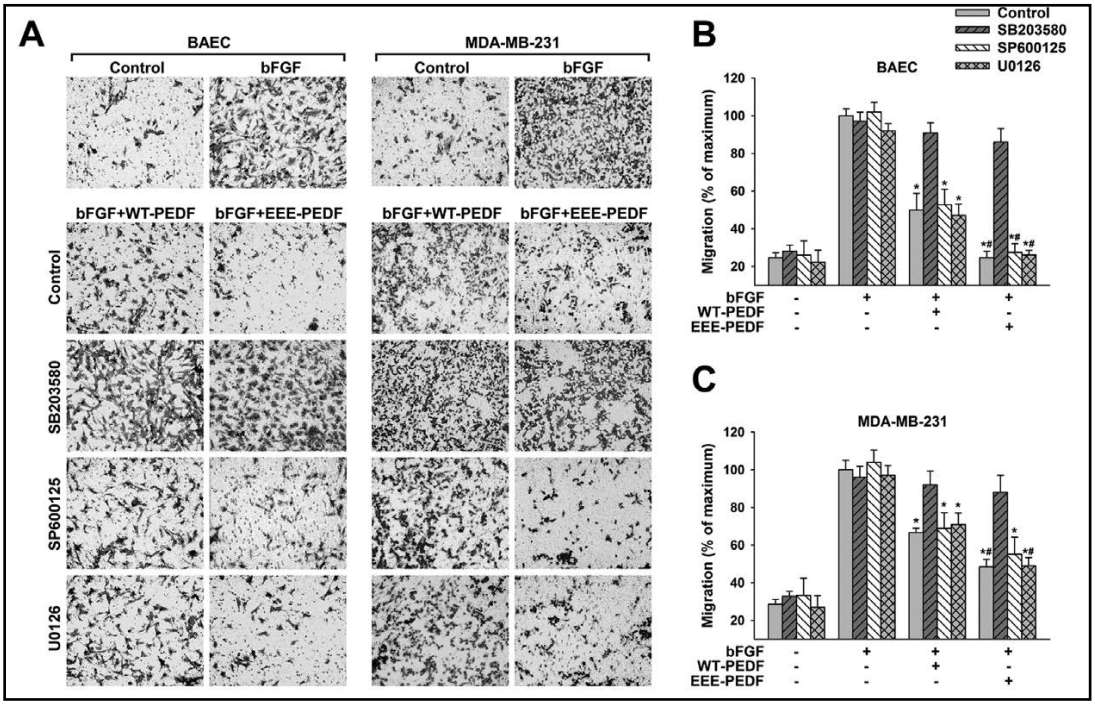


Fig. 7. The antimigratory activity of WT-PEDF and EEE-PEDF is mediated by P38α/β, but not by JNK or ERK. A. Migration of BAEC and MDA-MB-231 in the presence of bFGF (20 ng/ml) after pretreatment with p38α/β (SB203580, 10 μM), JNK1-3 (SP600125, 5 μM) or MEK1/2 (U0126, 5 μM) inhibitors for 1 h followed by incubation with either WT-PEDF or EEE-PEDF (20 nM) for 16 h, as evaluated by Transwell assay. Shown are representative photographs of Crystal violet-stained X20 fields of migrated cells taken from the bottom side of the polycarbonate membranes. B & C. Following visualization, stain was extracted and migration was quantified for BAEC (B) and MDA-MB-231 (C) by OD_{540} measurement. Data represent mean ± S.E. (N = 3) * P<0.05, treated vs. maximal migration; #, P<0.05, EEE-PEDF vs. WT-PEDF.

PEDF constructs towards BAEC appears to be mediated by p38α, because p38α/β inhibitor, but not the inhibitors of the JNK1-3 and ERK1/2 cascades, significantly reduced the ability of the PEDF constructs to inhibit BAEC migration (Fig. 7A, 7B, and S6). Inhibition of p38α/β produced similar outcome in MDA-MB-231 cells, where only pretreatment with the p38α/β inhibitor prevented the antimigratory effects of WT-PEDF and EEE-PEDF (Fig. 7A, 7C, and S6). The results obtained above were confirmed by additional selective JNK (BI78D3) and p38 (SB202190 and RWJ67657) inhibitors, that gave very similar results to that obtained with SP600125 and SB203580 respectively (Fig. S7, S8, & S9). Thus, p38α and JNK1/2 appear to have distinct regulatory functions in the antiangiogenic and antitumor activities of WT-PEDF and EEE-PEDF, where JNK1/2 executes EC apoptosis, while p38α executes the inhibition of migration.

PEDF has been previously reported to exert opposite effects on endothelial cells of distinct phenotypes cultured under different conditions [35]. Therefore, we validated the observed effects in human umbilical vein endothelial cells (HUVEC). HUVEC's culturing capacity is known to be different from that of BAEC, and these cells represent a suitable model for young endothelial cells. In low-passage HUVEC, WT-PEDF and EEE-PEDF induced signaling events similar to those observed in BAEC, i.e. activation of ERK1/2, p38α and JNK1/2, with EEE-PEDF causing much stronger effect on p38 α and JNK1/2 than WT-PEDF (Fig. 8A). Pharmacological inhibition of WT-PEDF- and EEE-PEDF-induced JNK1/2 and p38α activities in HUVEC confirmed their respective roles in the induction of EC apoptosis (Fig. 8A & 8B) and the inhibition of EC migration (Fig. 8C & 8D). These results clearly show that WT-PEDF- and EEE-PEDF-induced effects persist in EC populations of different phenotypes and, therefore, substantiate the generality of the observed effects towards ECs.

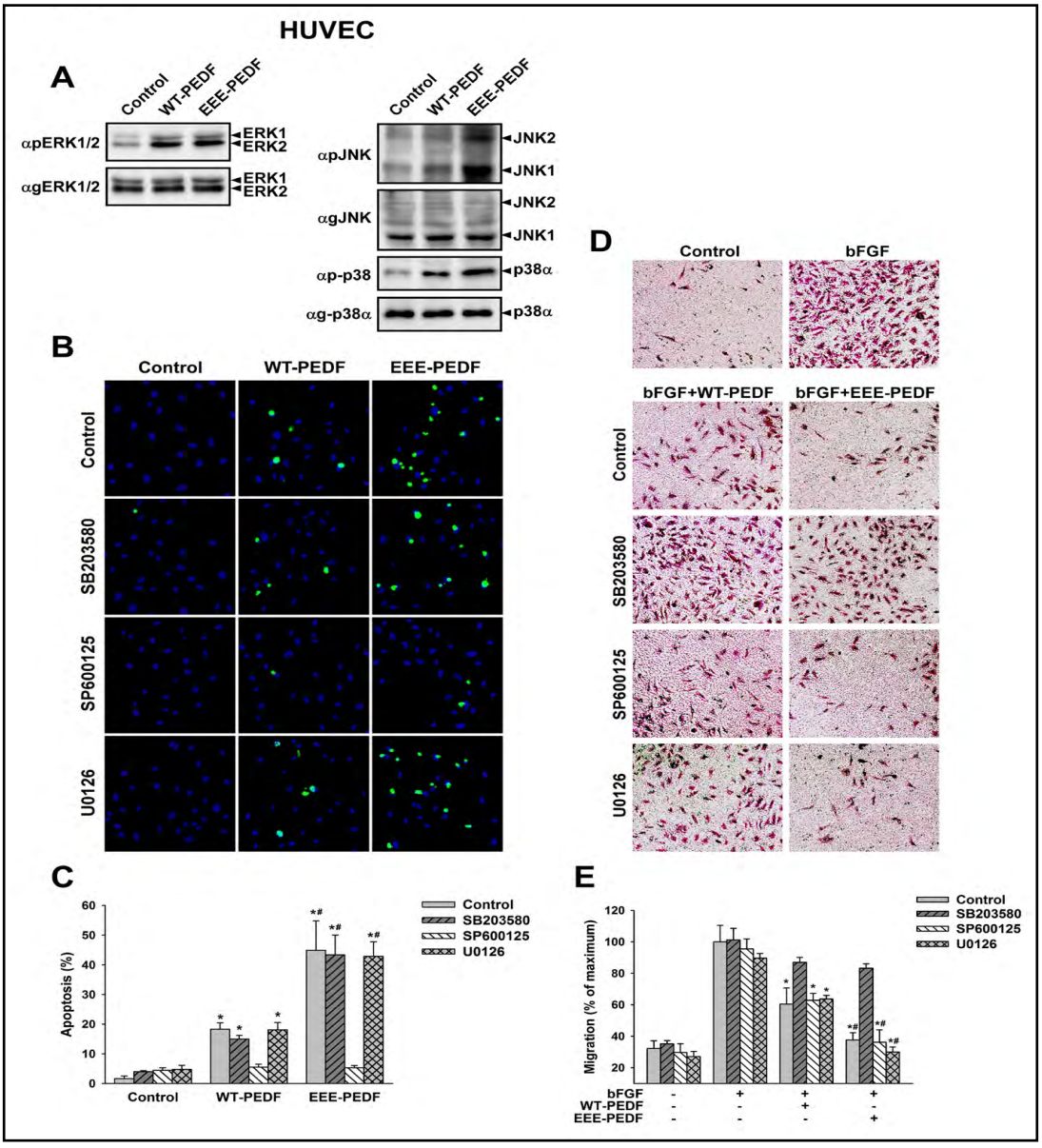


Fig. 8. The effects of WT-PEDF and EEE-PEDF on MAPKs activity, apoptosis and migration in HUVEC. A. The effect of WT-PEDF and EEE-PEDF (20 nM) on ERK1/2 (right), p38α and JNK1/2 (left) phosphorylation in HUVEC as analyzed by immunoblotting with anti-phospho-specific antibodies. For the detection of ERK1/2 phosphorylation, HUVEC were stimulated with PEDF constructs for 15 min, whereas p38 α and JNK1/2 phosphorylation was tested after 120 min. B. Representative TUNEL-labeled X20 fields of HUVEC after pretreatment with either p38 α/β (SB203580, 10 μM), JNK1-3 (SP600125, 5 μM) or MEK1/2 (U0126, 5 μM) inhibitors for 1 h followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for 24 hours. Green, TUNEL; blue, DAPI. C. Quantification of the relative apoptotic rate in TUNEL-labeled HUVEC treated as described in (B). Data shown are mean ± S.E. (N = 4) *, P<0.05, treated vs. control; #, P<0.05, EEE-PEDF vs. WT-PEDF. D. Migration of HUVEC in the presence of bFGF (20 ng/ml) after pretreatment with p38 α/β (SB203580, 10 μM), JNK1-3 (SP600125, 5 μM) or MEK1/2 (U0126, 5 μM) inhibitors for 1 h followed by incubation with either WT-PEDF or EEE-PEDF (20 nM) for 24 h, as evaluated by Transwell assay. Shown are representative photographs of Crystal violet-stained X20 fields of migrated cells taken from the bottom side of the polycarbonate membranes. E. Following visualization, stain was extracted and migration of HUVEC was quantified by OD_{540} measurement. Data represent mean ± S.E. (N = 3) * P<0.05, treated vs. maximal migration; #, P<0.05, EEE-PEDF vs. WT-PEDF.

Discussion

The initial isolation of PEDF was based on its ability to promote and support the growth of neuronal cells and therefore it was first described as a neurotrophic factor [1, 36]. Later on, it was found that PEDF is also a very potent endogenous inhibitor of angiogenesis, turning it into a promising agent for the inhibition of neovascularization-dependent tumor growth [2, 19, 37]. The anticancer activity of PEDF has been suggested to involve both indirect and direct antitumor effects. Indirectly, reduction in tumor growth is achieved through the antiangiogenic action of PEDF, in which the selective targeting of newly-formed vasculature without harming the existing blood vessels is of a particular importance [38]. This effect was suggested to involve inhibition of activity and/or expression of vascular endothelial growth factor (VEGF) [5, 39], as well as the proapoptotic activity of PEDF towards immature and migrating endothelial cells (ECs) [5, 39, 40]. Yet, in our previous study that employed three different xenograft models to test the anticancer effect of WT-PEDF and its mutants, these agents did not affect VEGF expression and activity *in vivo*, implying direct action on tumor ECs as a major determinant of the observed anticancer activity [27]. Though still controversial, it has been reported that PEDF also exerts a direct antitumor effect, possibly by inducing either antiproliferative or prodifferentiation activities towards cancer cells [39, 41].

We have previously shown that physiological functions of PEDF are differentially regulated by phosphorylation, and mimicking the fully phosphorylated state of PEDF significantly enhances its antiangiogenic activity [24, 26]. In a recent study we have examined the antitumor activity of the phosphomimetic mutants of PEDF and found that EEE-PEDF mutant can serve much more potent anticancer agent than WT-PEDF [27]. The observed antitumor effect of the phosphomimetic PEDF was associated with more profound reduction in intratumoral microvessel density than that caused by its wild-type form. We have also found that inhibition of tumor neovascularization by WT-PEDF and its mutants occurs independently of VEGF downregulation and is rather a result of their direct effect towards tumor ECs [27]. Our present study shows that this direct effect involves inhibition of EC migration and induction of EC apoptosis, where EEE-PEDF is much more efficient than WT-PEDF in inducing both these activities. In addition, we demonstrate that WT-PEDF and EEE-PEDF do not affect cancer cell proliferation, but nevertheless inhibit cancer cell motility (see Fig. 9 for a model). Thus, we provide here an answer to the widely debatable question of whether PEDF exerts a direct effect on cancer cells and what is the nature of this effect. Our findings are in agreement with another study that showed lack of PEDF effect on proliferation of cultured HeLa cells, but efficient eradication of HeLa xenografts *in vivo* [42]. The lack of the proapoptotic effect of PEDF towards cancer cells could be explained by the higher basal activity of survival pathways or the presence of a distinct, less-specific receptor. However, existence of a putative PEDF receptor is still obscure. Overall, our results indicate that the antiangiogenic activity of PEDF and its mutants, e.g. their ability to affect actively proliferating and migrating ECs is the main component of their antitumor action, although the antimigratory activity towards cancer cells is likely to contribute to such profound anticancer effect as well. Furthermore, the latter suggests that PEDF and more so, phosphomimetic PEDF may be beneficial for the inhibition of tumor invasiveness and metastatic potential. Thus, in contrast to exclusively antiangiogenic agents, PEDF exerts a combined antitumor effect, which may have important implications for its further development as an anticancer drug.

Our results indicate that PEDFs inhibit the proliferation of ECs but not cancer cells. As discussed in our previous article (19), the reason for this intriguing effect is not clear yet. One possibility to explain it might have been the expression of different PEDF receptors with distinct signaling machinery in the different cells. Since LR, which was reported to be the main antiangiogenic receptor [21], is expressed in both types of cells, this is probably not the main differential mechanism, However, it is still possible that different co-receptors

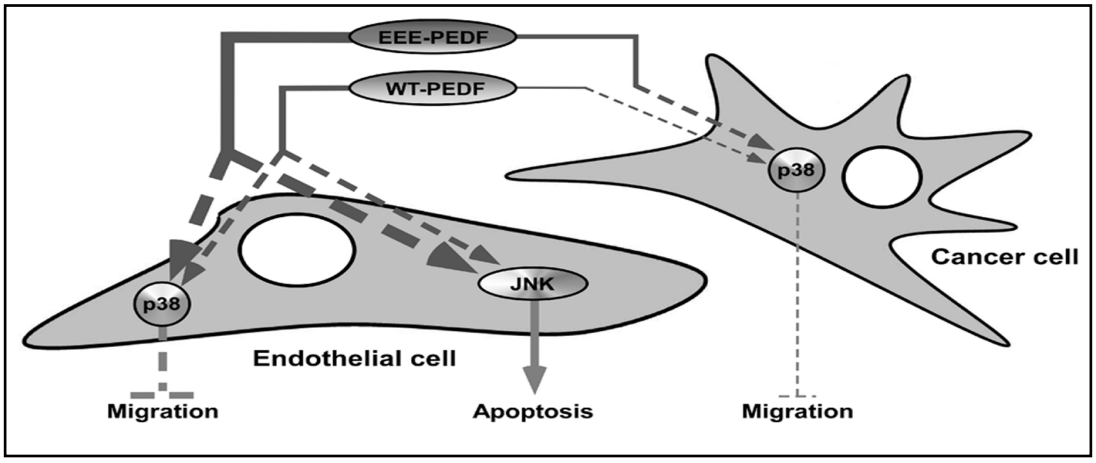


Fig. 9. Schematic representation of the molecular mechanism of the proapoptotic and antimigratory effect of WT-PEDF and EEE-PEDF towards endothelial and cancer cells.

are expressed in the different cells and those modulate the PEDF's effects. Another possibility is that cancer cells are resistant to PEDF-induced apoptosis by the induction of a higher basal activity of survival factors. One of them might be AKT that exhibit much higher basal activity in MDA-MB-231 than in BAEC (Fig. S4, and data not shown). However, additional studies are needed to further clarify these points.

Detailed examination of the molecular mechanism by which WT-PEDF and EEE-PEDF exert their effects in endothelial and cancer cells revealed a regulation by differential activation of two distinct MAPK cascades. We found that JNK1/2 and p38α respectively and independently of each other, regulate apoptotic and antimigratory activities of WT-PEDF and its phosphomimetic mutant (Fig. 9), where much stronger activation of p38α and JNK1/2 by EEE-PEDF results in the enhanced antiangiogenic and, therefore, anticancer properties of this mutant. The involvement of JNK1-3 in apoptosis has been extensively elaborated in a variety of cell systems [43], including apoptosis of ECs in response to PEDF [44] or other stimuli [45, 46]. Accordingly, p38 MAPKs have been implicated in the regulation of cell migration [47]. However, our study here is unique in showing such distinct and independent activities of these two stress-related signaling cascades when simultaneously activated by the same agent.

We further show that WT-PEDF and EEE-PEDF also cause transient increase in ERK1/2 phosphorylation, which is not required for their apoptotic and antimigratory activities and doesn't interfere with these effects. Since the ERK1/2 cascade is known to induce survival in various cells [48], the molecular mechanisms that prevent such effect in ECs treated with PEDF constructs are not clear but may be dependent on the decline of ERK1/2 phosphorylation before JNK1/2 and p38α reach their peak of activity. PEDF-induced ERK1/2 phosphorylation has been previously reported by us [26] and others that linked it to the cytoprotective properties of PEDF [49, 50]. Indeed, our previous report shows that WT-PEDF and EEE-PEDF exhibit same degree of neurotrophic and neuroprotective activity [26]. Accordingly, our present results indicate that WT-PEDF and EEE-PEDF induce similar levels of ERK1/2 phosphorylation.

A number of independent studies have demonstrated the involvement of different signaling pathways in the biological activity of PEDF. It has been shown, for example, that PEDF-induced apoptosis of ECs can be mediated by the activation of Fas-FasL system [40] and by PPARα signaling to p53 [51]. Furthermore, PEDF has been reported to block mitogenic effects of growth factors on ECs through the modulation of PI3K/Akt pathway [52], which is known to be critical for EC survival. Modulation of MAPK signaling module by PEDF has been also suggested to play an important role in its physiological activities [52]. PEDF-induced ERK1/2 phosphorylation has been largely implicated in the prosurvival

effects of PEDF towards neural cells [49, 50]. On the other hand, the ability of PEDF to block growth of ECs and to reduce angiogenesis has been associated with upregulation of p38 and JNK MAPKs [53, 54], and p38-dependent cleavage of multiple caspases [55]. In addition, work by Biyashev et al. demonstrated the involvement of ERK5 phosphorylation and the subsequent displacement of SMRT co-repressor in PEDF-induced activation of PPARα [56]. In our experiments, we did not observe any ERK5 activation by PEDF, while JNK and p38 were found to mediate respectively PEDF-induced EC apoptosis through caspase-3 activation and both endothelial and cancer cell migration arrest. We have also shown herein that ERK1/2 activation by PEDF and its mutant does not interfere with the above effects. These evidences suggest that antiangiogenic signaling by PEDF occurs mainly through the activation of p38 and JNK stress-related kinases, but the overall downstream outcome can be also influenced by additional signaling pathways that vary in different cell systems. Therefore, additional studies are needed to fully delineate the exact subsequence of intracellular signaling events that mediate the effects of PEDF.

Conclusion

Our study here unveils important mechanistic insights of the biological activity of PEDF and its phosphomimetic mutant, EEE-PEDF. In continuation to our previous studies, we are now showing that the enhanced antiangiogenic and anticancer properties of the phosphomimetic PEDF are achieved through significantly stronger binding to LR and stronger intracellular signaling effects, as compared to WT-PEDF. Additionally, we demonstrate that JNK and p38 MAPKs differentially regulate proapoptotic and antimigratory activities of PEDF. We believe that our findings will contribute to better understanding of the complexity of PEDF effects on endothelial and cancer cells.

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Disclosure Statement

No conflict of interests exists.

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