Pigment epithelium-derived factor (PEDF) is a potent endogenous inhibitor of angiogenesis and a promising antitumor agent. We show that PEDF modulates and inhibits tumor vascularization more profoundly than WT-PEDF, with a different phosphorylation mechanism by which the phosphorylated PEDF exerts its antiangiogenic and antitumor activities. We identified the retina; however, it has now become evident that it is expressed throughout the body and is constantly made aware of issues in Figs. 2A and 7A as well as supplemental Fig. S4. The 4-h EEE-PEDF panel was reused in the 10 nM EEE-PEDF panel in Fig. 2A. In Fig. 7A, the control panel for BAEC cells was reused in the SP600125 panel from BAEC cells in the presence of bFGF and EEE-PEDF. Lane 7 of the pAKT panel from BAEC cells was inappropriately manipulated in supplemental Fig S4. In the interest of maintaining accuracy in the published scientific literature, the authors wish to withdraw this article. However, the authors have full confidence in the findings and conclusions of this paper and have replicated the findings in subsequent work.
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 (LR). 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 with 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.

**EXPERIMENTAL PROCEDURES**

**Materials**

MEK1/2 inhibitor U0126 and p38α/β inhibitor SB203580 were purchased from Calbiochem, and JNK1–3 inhibitor SP600125 was from Biomol. Recombinant basic fibroblast growth factor (bFGF) was purchased from Sigma. The following primary antibodies were used: anti-MKK3/6, antiphospho-MKK3/6, anti-MKK4, anti-MKK7 from Santa Cruz Biotechnology; anticleaved caspase-3, antiphospho-JNK1–3, antiphospho-MAPKAPK2, and anti-GFP from Cell Science.

**Cell Culture**

U87-MG (human glioblastoma EC) were cultured in 4.5 g/liter D-glucose DMEM (Invitrogen) supplemented with 2 mM l-glutamine, antibiotics and 10% FCS. HCT116 (human colorectal carcinoma) were grown in McCoy’s 5A medium (Sigma) with the same supplements and 5% CO2. HCT116 (human colorectal carcinoma) were grown in RPMI medium (Invitrogen) with the same supplements and 5% CO2.

**PEDF Production**

WT-PEDF and EEE-PEDF (from human origin) were cloned from pBlueScriptSKII(+)/PEDF, and pcDNA3/EEE-PEDF (17, 18), respectively into pRSET(A) (Invitrogen), and expressed in *Escherichia coli* BL21. Bacterial cells were grown at 30 °C to an *A*600 = 0.5–0.6, and the expression of recombinant proteins was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 4–5 h. Pelleted bacterial cells were lysed in ice-cold nickel-nitrilotriacetic acid binding buffer containing 300 mM NaCl, 50 mM NaH2PO4, 20 mM imidazole, pH 8.0) supplemented with 10 μg/ml leupeptin, 1 mM PMSF, 10 mM β-mercaptoethanol, and 1 mg/ml lysozyme followed by sonication. Lysates were then cleared by centrifugation at 12,000 × g for 15 min. Batch purification of recombinant proteins was performed using ion metal affinity chromatography with nickel-nitrilotriacetic acid 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 in final protein formulations was evaluated by LAL turbidimetric assay and was <2 enzyme units/μg protein.

**Immunoblotting (Western blotting)**

Cells were grown to subconfluence and then serum-starved (0.1% FCS and 0.5% FCS for HUVEC) for 16 h. After incubation with indicated treatments, cells were rinsed twice with ice-cold PBS and scrapped into radioimmune precipitation assay buffer (0.2 ml/plate). Extracts were then obtained by centrifugation at 15,000 × g for 15 min at 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 horseradish peroxidase or alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch laboratories). Membranes were developed either using EZ-ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel) in Bio-Rad’s ChemiDoc XRS imaging station or with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Applied Science). Each experiment was performed at least three times to test for reproducibility and obtain statistically significant data. Quantification of the band intensities 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. 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, and the stain was extracted with 0.1 M HCl (0.4 ml/well) for 2 h at room temperature with shaking. Thereafter, aliquots of each sample were transferred into 96-well plate, and \( A_{595} \) was determined using an 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). 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 (5–8 ×10^4 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 3.5% paraformaldehyde, stained with 0.1% crystal violet, and images were then captured using a digital camera coupled with a microscope. Relative migration was quantified following stain extraction with methanol and A_{540} measurement in an ELISA reader.

**Apoptosis Assay**

Apoptosis was evaluated using a TUNEL kit (Roche Applied Science). After indicated treatments, cells grown on 18-mm coverslips were washed and fixed with 3% paraformaldehyde in PBS. Thereafter, apoptotic cells were identified using manufacturer’s protocol (Roche Applied Science) and quantified. A DeltaVision OMX fluorescent microscope station supplied with a digital camera and SoftWorx software (Applied Precision) was used to process the slides.

**In Vitro Binding Assay**

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 plasmids, using poly ethyl inositolamine. Forty eight hours after transfection, cells were

#### FIGURE 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 ×20 fields of migrated cells in polycarbonate membranes. **B**, following visualization, stain was extracted with methanol and migration was quantified following A_{540} measurement. Data shown represent mean ± S.E. (n = 3). *, p < 0.05, EEE-PEDF versus 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 shown represent mean ± S.E. (n = 3). *, p < 0.01, treated versus control.
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RESULTS

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 (19). 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 BAECs 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 alone. On the other hand, the same concentrations of these products did not affect proliferation of MDA-MB-231 (Fig. 1C), and U87-MG (data not shown), and MCF-7 (data not shown). These results suggest that the inhibitory effect of WT-PEDF and EEE-PEDF on endothelial cell proliferation is selective and that the inhibitory effect of PEDF toward ECs was associated with the suppression of EC apoptosis. To verify this, 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. 2, A and B). 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 to BAEC, WT-PEDF as well as EEE-PEDF failed to induce apoptosis of cultured MDA-MB-231 cells as evaluated by TUNEL assay over a 48-h period (19). For further information on the apoptotic effect, we next 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 any significant arrest in G1 or G2 phases of the cell cycle (supplemental Fig. S1). This effect was completely abolished by the general caspase inhibitor Z-VAD, clearly confirming the apoptotic effect of the PEDFs.

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 (20). Thus, the effect of the PEDF constructs on cell migration was examined in both BAEC and MDA-MB-231 cells. For this
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FIGURE 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 antiphospho-specific antibodies. The time course of WT-PEDF- and EEE-PEDF-induced MAPK (C) and MAP2K (E) phosphorylation in BAEC was quantified from three independent immunoblotting experiments. *, p < 0.05, EEE-PEDF versus 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 antiphospho-specific antibodies. Time course of WT-PEDF- and EEE-PEDF-induced MAPKs phosphorylation in MDA-MB-231 cells (H) was quantified from three independent immunoblotting experiments. *, p < 0.05, EEE-PEDF versus WT-PEDF. In all experiments, peroxyvanadate (VOOH; 200 μM H2O2, 100 μM vanadate) was used as a positive control of MAPK/MAPKK activation.

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. 3, A and B). Notably, unlike their lack of effect on apoptosis, WT-PEDF and
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FIGURE 6. The proapoptotic activity of WT-PEDF and EEE-PEDF is mediated by JNK but not by p38α/β or ERK1/2. A, representative TUNEL-labeled × 20 fields of BAEC after pretreatment with either p38α/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 h. Green, TUNEL; blue, DAPI. B, quantification of the proapoptotic rate in TUNEL-labeled BAEC treated as described in A. Data shown are mean ± S.E. (n = 4), *p < 0.05, treated versus control; †, p < 0.05, WT-PEDF versus WT-PEDF. C, the effect of p38α/β (SB203580, 10 μM) and JNK1–3 (SP600125, 5 μM) inhibitors on WT-PEDF and EEE-PEDF-induced caspase-3 cleavage (cCasp3) in BAEC, as evaluated by immunoblotting with anticleaved caspase-3 antibody (α-cCasp3).

more so EEE-PEDF did affect the migration of MDA-MB-231 cells, although this effect was not as marked as in BAEC (Fig. 3, A and B). These results were corroborated using a wound healing assay, in which a fixed-width scratch in a monolayer was created and the advancement of the wound was followed in the presence of either WT-PEDF or EEE-PEDF (Fig. 3C). Wound healing assays on HCT116 and U87-MG cells were also performed. No noticeable effect on MDA-MB-231 (data not shown) or other cancer cells was general. Taken together, these results indicate that EEE-PEDF is more effective than WT-PEDF in inhibiting different angiogenic activities in endothelial and cancer cells, such as proliferation and migration, and at some extent, in the inhibition of cancer cell migration.

Enhanced EEE-PEDF Interaction with LR but Not with PNPLA2—Next, we studied the molecular signaling mechanism(s) by which EEE-PEDF exerts 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 (21) and PNPLA2 (22). 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 immunoprecipitated proteins were briefly washed, incubated with recombinant WT-PEDF or EEE-PEDF, and subjected to immunoblotting that revealed that although 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 (supplemental 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 weak binding of PNPLA2 and the changes in affinity, it is possible that the relatively weak binding of PNPLA2 and the changes in binding of EEE-PEDF to LR are responsible for the stronger antiangiogenic effects of EEE-PEDF compared with WT-PEDF.

Time-dependent Activation of Distinct MAPK Cascades by WT-PEDF and EEE-PEDF—Because LR seems to be the main PEDF receptor and the mechanism of its action is 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 (23). In BAEC, EEE-PEDF induced marked and sustained elevation in p38α and JNK1/2 phosphorylation up to 270 min of incubation, whereas the activation of ERK1/2 peaked at 15 min and declined back to basal level within 120 min (Fig. 5, A–C). WT-PEDF had a similar trend of activation of all three MAPKs, although the fold changes of p38α and JNK1/2 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. 5, D and E). WT-PEDF and more so EEE-PEDF induced noticeable phosphorylation of MKK3/6 and MKK4, known to be the respective activators of p38 and JNK (24) but almost did not affect phosphorylation of MKK7, which also acts upstream of JNK, though is not critical when MKK4 is activated (25). Interestingly, both WT-PEDF and EEE-PEDF did not affect JNK1/2, but did elevate to some extent p38α activity in MDA-MB-231 cells, with EEE-PEDF...
showing stronger effect than WT-PEDF (Fig. 5, F and H). In this cell line, known to harbor K-Ras activating mutation (26) and high basal levels of ERK1/2 activity, both PEDF constructs failed to induce further increase in ERK1/2 phosphorylation (Fig. 5, G and H). Addition of bFGF simultaneously with the PEDF treatment did not significantly change the phosphorylation of p38 and JNK in BAEC cells (supplemental Fig. S3). Furthermore, the short term AKT phosphorylation was affected neither by WT-PEDF nor by EEE-PEDF in the cell lines examined (supplemental 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, these results indicate that WT-PEDF, and more so its phosphomimetic mutant, induces diverse intracellular signaling events in different cell lines and further support our previous findings on preferential susceptibility of ECs to PEDF action.

Differential Regulation of 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, 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 fields of migrated cells taken from the bottom side of the polycarbonate membranes. B and C, following visualization, stain was extracted and migration was quantified for BAEC (B) and MDA-MB-231 (C) by A540 measurement. Data represent mean ± S.E. (n = 3). *, p < 0.05, treated versus maximal migration; #, p < 0.05, EEE-PEDF versus WT-PEDF.
BAEC migration (Fig. 7, A and B, and supplemental Fig. S6). Inhibition of p38α/β produced a 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. 7, A and C, and supplemental Fig. S6). The results obtained above were confirmed by additional selective JNK (BI78D3) and p38 (SB202190 and RWJ67657) inhibitors, which gave very similar results to that obtained with
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SP600125 and SB203580, respectively (supplemental Figs. S7–S9) Thus, p38α and JNK1/2 appear to have distinct regulatory functions in controlling the antiangiogenic and antitumor activities of WT-PEDF and EEE-PEDF, where JNK1/2 executes EC apoptosis, whereas p38α is involved in the inhibition of endothelial and to some extent also of cancer cell migration.

PEDF has been previously reported to exert opposite effects on endothelial cells of distinct phenotypes cultured under different conditions (27). Therefore, we validated the observed effects in HUVECs. The HUVEC 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 p38α 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. 8, A and B) and the inhibition of EC migration (Fig. 8, C and D). 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 toward ECs.

DISCUSSION

The initial isolation of PEDF was based on its ability to promote and support the growth of neuronal cells and it was first described as a neurotrophic factor. It was found that PEDF is also an inhibitor of angiogenesis, turning it into a promising agent for antiangiogenic action of PEDF, in which selective targeting of newly-formed vasculature without harming the existing blood vessels is of a particular importance (30). This effect was suggested to involve inhibition of activity and/or expression of VEGF (4, 31) as well as the proangiogenic activity of PEDF toward immature and migrating ECs (4, 31, 32). Yet, in our previous study, we 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 (19). Though still controversial, it has been reported that PEDF also exerts a direct antitumor effect, possibly by inducing either antiproliferative or prodifferentiation activities toward cancer cells (31, 33).

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 (17, 18). In a recent study, we have investigated anticancer activity of the phosphomimetic PEDF (31) and found that EEE-PEDF mimics the antiangiogenic action of PEDF by significantly increasing tumor cell apoptosis (31, 33).

FIGURE 8. The effects of WT-PEDF and EEE-PEDF on MAPK 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 antiphospho-specific antibodies. For the detection of ERK1/2 phosphorylation, HUVEC were stimulated with PEDF constructs for 15 min, whereas p38α and JNK1/2 phosphorylation was tested after 120 min. B, representative TUNEL-labeled × 20 fields of HUVEC after pretreatment with either p38α/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 h. 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 versus control; #, p < 0.05, EEE-PEDF versus WT-PEDF. D, migration of HUVEC 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 24 h, as evaluated by Transwell assay. Shown are representative photographs of crystal violet-stained × 20 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 A540 measurement. Data represent mean ± S.E. (n = 3), *, p < 0.05, treated versus maximal migration; #, p < 0.05, EEE-PEDF versus WT-PEDF.

FIGURE 9. Schematic representation of the molecular mechanism of the proapoptotic and antimigratory effect of WT-PEDF and EEE-PEDF towards endothelial and cancer cells.
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 toward 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, is the expression of different PEDF receptors, with distinct signaling machinery, in the different cells. However, since LR seems to be expressed in both types of cells (21), this is probably not the main differential mechanism. Nonetheless, it is still possible that different co-receptors are expressed in the different cells and those modulate the effects of PEDF. Another possibility is that cancer cells are resistant to PEDF-induced apoptosis by the induction of a higher basal activity of survival factors. One such a survival factor might be AKT, which exhibits much higher basal activity in MDA-MB-231 than in BAEC (supplemental 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 ability to affect actively proliferating endothelial and cancer cells revealed a regulation of the activation of two distinct MAPK cascades, JNK1/2 and p38α independently, respectively, which is presumably required to regulate apoptotic and antimigratory activities of PEDF and its phosphomimetic mutant, EEE-PEDF. We demonstrate the enhanced antiangiogenic and, therefore, its anticancer properties of this mutant. The involvement of PEDF-induced apoptosis has been extensively evaluated in a variety of cell systems (35), including apoptosis of ECs in response to PEDF (36) or other stimuli (37,38). Accordingly, p38 MAPKs have been implicated in the regulation of cell migration (39). 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 does not interfere with these effects. Because the ERK1/2 cascade is known to induce survival in various cells (40), the molecular mechanisms that prevent such an 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 (18) and others that linked it to the cytoprotective properties of PEDF (41,42). Indeed, our previous report showed that WT-PEDF and EEE-PEDF exhibit the same degree of neurotrophic and neuroprotective activity (18). 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 (32) and by peroxisome proliferator-activated receptor γ signaling to p53 (43). Furthermore, PEDF has been reported to block mitogenic effects of growth factors on ECs through the modulation of PI3K/AKT pathway (44), 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 (44). PEDF-induced ERK1/2 phosphorylation has been largely implicated in the prosurvival effects of PEDF toward neural cells (41,42). On the other hand, the ability of PEDF to block growth of ECs and to reduce angiogenesis has been associated with up-regulation of p38 and JNK MAPKs (45,46) and p38-dependent cleavage of multiple caspases (47). In addition, recent work by Biyash et al. (48) demonstrated the involvement of ERK5 phosphorylation and the subsequent displacement of SMRT co-repressor in PEDF-induced activation of peroxisome proliferator-activated receptor γ. In our experience, ERK5 has been shown to mediate respectively, enhanced antiangiogenic and survival property through caspase-3 activation and reduction of cell migration arrest. We have also shown that ERK1/2 activation by PEDF and its phosphorylated mutant do not interfere with the above effects. These evidences suggest that antiangiogenic signaling by PEDF occurs through the activation of p38 and JNK stress-related cascades, 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.

In summary, our study 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 with 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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