Inhibition of Human Prostate Cancer Growth and Prevention of Metastasis Development by Antiangiogenic Activities of Pigment Epithelium-Derived Factor

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Citation

Abstract

Background: Human prostate mortality is associated with tumor invasion and metastasis. In this study, we examined the consequences of overexpression of pigment epithelium-derived factor (PEDF) on both prostate cancer primary tumor growth and metastasis development.

Methods:
In vivo, the prostate cancer cells DU145 with overexpression of PEDF were injected s.c. into SCID mice. The tumor volume (mm^3) was measured by applying the formula [volume = $0.52 \times$ (width)$^2 \times$ (length)] for approximating the volume of a spheroid, and lung metastases are evaluated using India ink staining. Intratumoral microvessel density (MVD) was detected by immunohistochemistry using mouse anti-human CD31 monoclonal antibody. Human microvessel endothelial cells (HMVEC) tube formation was assayed in vitro. Secreted VEGF was determined by ELISA.

Results: The growth of implanted tumor was significantly reduced in sizes, and the lung metastases were also completely inhibited. Compared to control, MVD decreased significantly in the mice transfected with PEDF [(31 ± 3.25) versus (14.25 ± 3.40) (p < 0.01)]. Furthermore, PEDF overexpression also greatly inhibited tube formation in vitro, and decreased production of VEGF in DU145 cells.

Conclusions: It was suggested that the effects of PEDF on primary tumor growth and lung metastasis appear associated with inhibition of angiogenic tumor response. PEDF-mediated inhibition of prostate cancer growth and metastases could thus have a major impact on existing therapies for prostate cancer.

INTRODUCTION

Prostate cancer is a leading cause of morbidity and mortality among males. The incidence and mortality of prostate cancer is mainly rest with invasion and metastasis of primary tumor. So far, it is no doubt that primary tumor and metastatic growth must be dependent on neovasculature formation [1]. Study indicates that angiogenesis correlates with disease stage and metastasis in prostate cancer [2], and occurs in those prostatic intraepithelial neoplasms [3] and latent carcinomas [4] with the potential for progression to invasive disease.

Pigment epithelium-derived factor PEDF 50-kDa glycoprotein initially isolated from retinal pigment epithelial cells, is initially identified as neuronal differentiation factor produced by cultured human retinal pigment epithelial cells [5]. In addition to its neurotrophic activity on the nervous system and retina, PEDF was recently found to be a strong inhibitor of angiogenesis in the eye and it might be responsible for maintenance of the avascular status of corneal tissue [6]. Presently, PEDF is known as one of most effective anti-angiogenic factors because it specifically inhibits proliferation and migration of endothelial cells [7].

Strong PEDF immunostaining was found in normal prostate epithelial cells and stromal cells, especially in smooth muscle cells. In contrast, the LNCaP, DU145 and PC-3 cancer cell lines secreted much less to no detectable PEDF [8]. Interesting, DU145 cells were found to have highly angiogenic activity in these cancer cell lines [9]. The fact that

DU145 cells secreted the very low PEDF may explain this finding, as the angioinductive activity was significantly increased when PEDF was blocked.

Guan et al previously found that PEDF decreased expression of VEGF and bFGF in glioma U251, and significantly inhibited primary tumor growth in SCID mouse in vivo ([10]). Here, we have chosen DU145 cells, in which PEDF levels are very low, to study whether the forced expression of this factor might influence the tumor growth and metastatic behavior by directly inhibiting angiogenesis. The data presented show that PEDF-suppressed tumor growth and metastasis is associated with anti-angiogenic activity in DU145 SCID mice.

MATERIALS AND METHODS
CELL CULTURE

Human DU145 cells were grown in Dulbecco's modified Eagle's media (DMEM) (Life Technologies, Inc.) supplemented with 10% FBS (Life Technologies, Inc.).

STABLE RE-EXPRESSION OF PEDF IN DU145 CELLS

DU145 cells were transfected with 2 μg of pcDNA3-PEDF plasmid DNA (kindly provided by Dr. Ming Guan, Center of Laboratory Medicine, Hua Shan Hospital, Fudan University, Shanghai, P. R. China) in Lipofectamine 2000 (Invitrogen), as instructed by the manufacturer. The cells were then selected in 0.8 mg/ml G418. PEDF-overexpressing clones were verified by immunoblot using anti-human PEDF antibody. Briefly, cells were washed three times in ice-cold phosphate-buffered saline, lysed with RIPA buffer, incubated on ice for 30 min, and then centrifuged at 13,000 × g for 15 min at 4 °C to remove debris. After determination of the protein concentration using the Bio-Rad protein assay, an equal amount of protein (50μg) from each sample was electrophoresed on 10% SDS-PAGE and transferred to PVDF membrane. Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBST). The membranes were then incubated overnight at 4°C in nonfat dry milk in TBST with polyclonal anti-PEDF (Upstate; working dilution- 1:800), monoclonal anti-actin (Sigma clone AC40; working dilution- 1:1000). Membranes were washed three times in TBST and incubated with either goat anti-rabbit (1:1,000) or anti-mouse (1:1,000) secondary antibody conjugated to horseradish peroxidase. Visualization of the protein bands was done using the enhanced chemiluminescence plus kit as recommended by the manufacturer (Roche).

TUMOR GROWTH AND METASTASIS ASSAY

PEDF transfectant DU145 and empty-vector transfectant DU145 were harvested by trypsinization and were resuspended at final concentration of 5×10^6 cells/0.1 ml in PBS. The viability of the cells was >90% as determined by trypan blue exclusion. Six-week-old female SCID mice were divided into two groups (5/each group) and injected subcutaneously (s.c.) into the flank regions with 5×10^6 DU145-PEDF and DU145-Vector respectively. Tumor volume (cubic millimeters) was measured by using a caliper, applying the formula [volume $= 0.52 \times$ (width)^2 x (length)] for approximating the volume of a spheroid by two people separately. Tumor burden per mouse was calculated by accumulating the tumor volume of every mouse. Four weeks later, the animals were sacrificed, and lungs were injected with ink solution and fixated in Fekete's solution (100 mL of 70% alcohol, 10 mL formalin, and 5 mL glacial acetic acid), and lung metastases (white dots) were counted.

TUMOR MICROVESSEL DENSITY

Tumors were extracted and fixed in 10% neutral buffered formalin for 6 h. After fixation, the tissue samples were processed into paraffin blocks. Sections were cut at 5 μm, placed on charged slides and allowed to dry overnight at room temperature. Sections were then deparaffinized, rehydrated and washed in PBS. Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS for 10 min. Tissue sections were subjected to immunostaining for CD31 (BD Pharmingen) following the manufacturer's recommendations with a few modifications as follows: The slides were incubated for 1 h with 10 μg/ml anti-CD31 or an isotype-matched control (10μg/ml rat IgG), washed with PBS/T (PBS + 0.05% Tween 20), then incubated for 30 min with biotinylated anti-rat Ig. After washing with PBS/T, the slide was incubated for 30 min with alkaline phosphatase conjugated streptavidin complex (DAKO, Carpinteria CA), washed with PBS/T, stained with a 5 min application of Fast Red resulting in a pink/magenta color reaction product. The slides were then counterstained with Hematoxylin and coverslipped with aqueous mountant. After the area of highest neovascularization (hotspots) was located by light microscopy at a total magnification of x40, microvessel density was determined by counting CD31-positive vessels in six different fields, using a x200 ocular microscope and average counts were recorded.

TUBE FORMATION ASSAY

Transfected DU145 cancer cells were generally cultured, and the medium of was changed with DMEM containing 1% FBS. Overnight, conditioned media (CM) were collected and filtered. Human microvascular endothelial cells (HMVEC, 1 x 10^4 cells) were seeded on a layer of previously polymerized Matrigel with CM in 96 wells. After overnight, changes of cell morphology were captured through a phase-contrast microscope and photographed. CM of DU145/Vector as control.

ELISA FOR VEGF

10^5 cells (DU145-PEDF or DU145-Vector) were seeded into 6-well plates with 2 ml/well of DMEM plus 1% FBS, incubated overnight, and then 200μl of conditioned media/well was assayed for VEGF using species-specific ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The assay was performed in duplicate, and results were confirmed in three independent experiments.

RESULTS

THE RE-EXPRESSION OF PEDF DECREASES TUMOR GROWTH .

In order to address whether PEDF was sufficient for inhibiting tumor growth and development, DU145 cells were transfected with cDNAs expressing human PEDF, or empty vector, as a negative control. In vitro, PEDF re-expression had no effect on proliferation of tumor cells (data not shown). Futhermore, DU145 cells (pcDNA3-PEDF) were injected s.c. in SCID mouse flanks, and then tumor formation were monitored. As shown in Fig. 1, vector-transduced cells formed rapidly growing tumors. In contrast, PEDF overexpression inhibited tumor growth of DU145 cells to a great extent throughout this experiment.

Figure 1

Figure 1: Growth rate of tumor in SCID mice. DU145 cells (pcDNA3-PEDF) were injected s.c. in SCID mouse flanks, and then tumor formation were monitored. Results indicated that PEDF significantly decreased growth of primary tumor at s.c. site. . *.
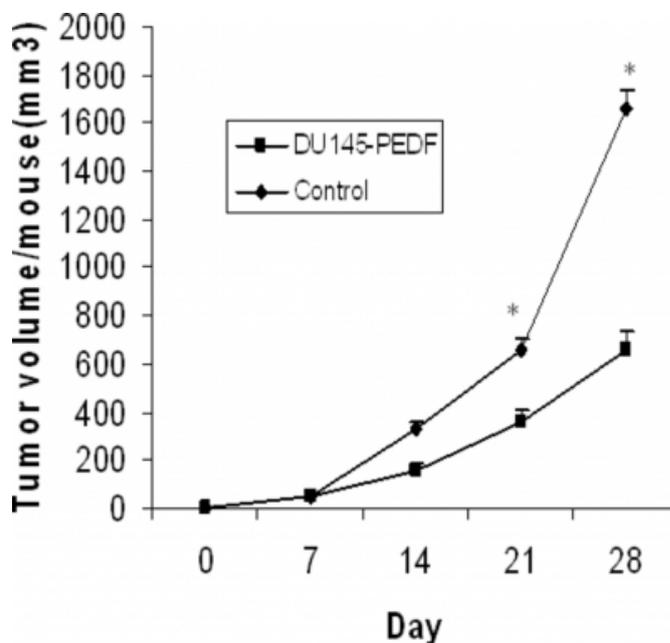

PEDF INHIBITS TUMOR LUNG METASTASIS

Interestingly, PEDF expression was inversely correlated to metastasis and outcome in pancreas cancer ([11]). Strong suppression of the growth of primary prostate cancer by PEDF compelled us to study its effect on the prostate cancer metastases. Lung of SCID mouse was injected with ink solution and fixated in Fekete's solution, and lung metastases (white dots) were counted. It was found that PEDF re-expression completely inhibited the generation of macroscopic lung metastases (Fig. 2). Thus, our data strongly suggested that re-expression of PEDF inhibit tumor growth and lung metastasis.

Figure 2

Figure 2: India Ink Staining of Macrometastases of Lung. Lung of SCID mouse was injected with ink solution and fixated in Fekete's solution, and lung metastases (white dots) were counted. It was found that PEDF re-expression completely inhibited the generation of macroscopic lung metastases

DU145-PEDF   Control

DU145-PEDF   Control
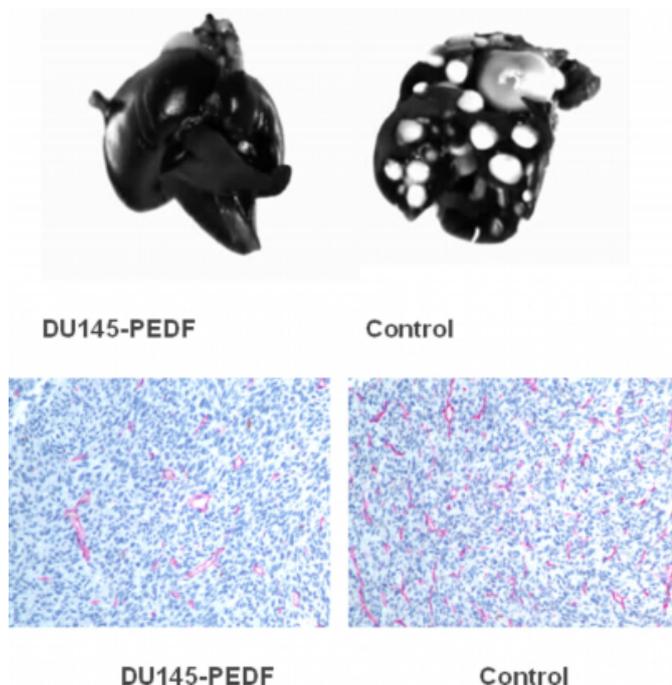
PEDF DECREASES TUMOR ANGIOGENESIS

Forced PEDF expression delays the growth and invasion of lung carcinoma, hepatocellular carcinoma, melanoma, and glioblastoma, where it blocks neovascularization ($_{12}$). Given that angiogenic factors, which were secreted by tumor cells, recruits vascular endothelial cells for newvascularation in order to provide oxygen and nutrient for supporting tumor growth and development, and PEDF exhibited potent anti-angiogenic activity, we addressed whether PEDF tumor-suppressor activity was due to anti-angiogenesis. Fig. 3 showed that re-expression of PEDF did decrease microvessel density in tumor tissue. Compared to controls, MVD decreased significantly in the mice transfected with PEDF [(31 ± 3.25) versus (14.25 ± 3.40) (p < 0.01)]. Furthermore, conditioned media from DU145-PEDF cells decreased the ability of HMVEC plated onto Matrigel-coated wells to form capillary-like tube structures in comparison to control (Fig. 4). Collectively, these data indicate that PEDF inhibits tumor vascularization in vivo and vascular differentiation in vitro.

Figure 3

Figure 3: Tumor Average Microvessels Density x 200 . CD31 staining of primary . tumors of mice. Compared to controls, Microvessel density (MVD) decreased significantly in the mice transfected with PEDF [(31 Â± 3.25) versus (14.25 Â± 3.40). Error bars, . of 6 random visual fields. *.
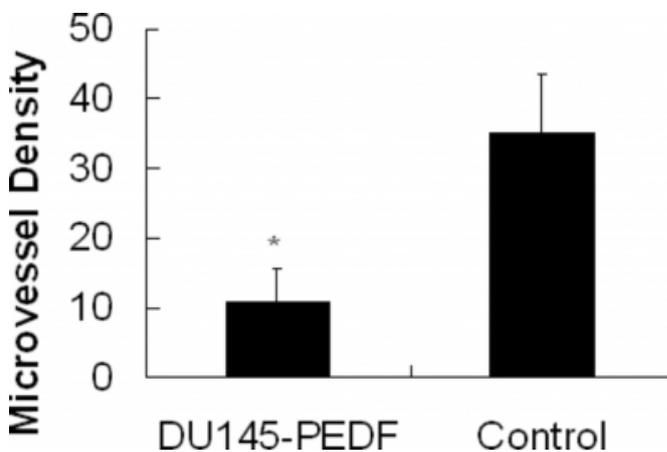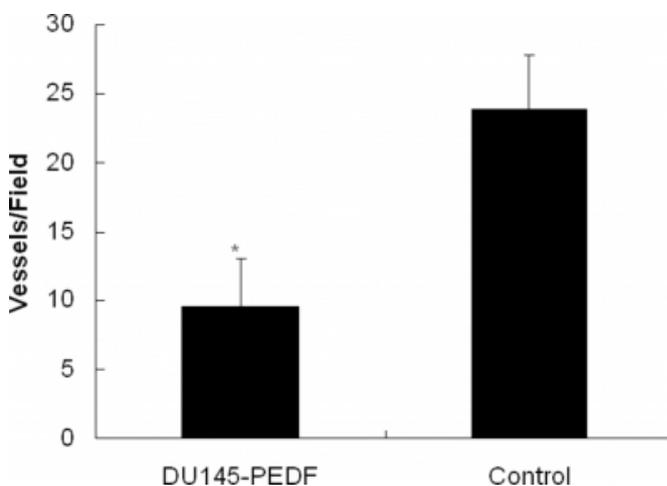

Figure 4

Figure 4: Tube formation assay . Tube formation by HMEC plated onto Matrigel in the presence of 40% CM from DU145-PEDF cells or control cells. Average number of tubes per visual field (6 fields) from three independent experiments. Error bar, ; * <.


PEDF DECREASES PRODUCTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN DU145 CELLS

We next investigated the effects of PEDF on VEGF production in DU145 cells. As shown in Fig. 5, secreted VEGF transcript levels were decreased more than 3-fold following the induction of PEDF over-expression in DU145 cells.

Figure 5

Figure 5: Suppression of vascular endothelial growth factor (VEGF) expression by PEDF. The level of secreted VEGF in conditioned media was determined from DU145-PEDF and control prostate cancer cells by enzyme-linked immunosorbent assay (ELISA). The data presented were obtained from three independent experiments. * <.
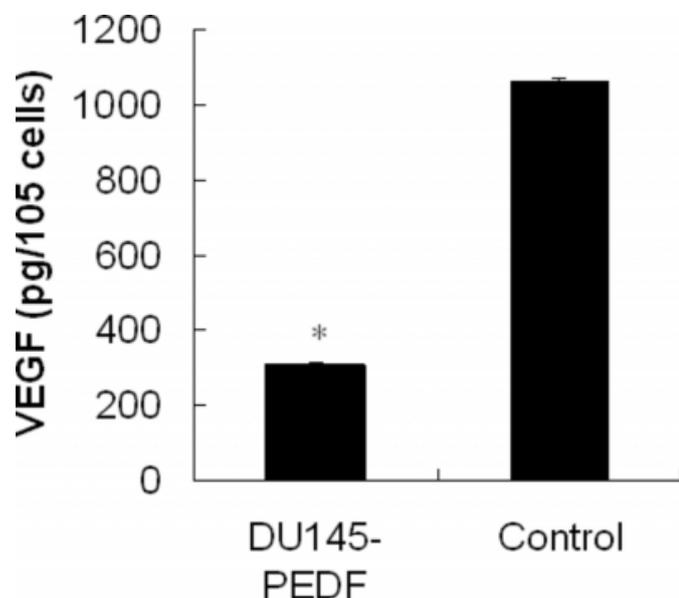
DISCUSSION

Currently, PEDF is a candidate tumor suppressor in neuroectodermal tumors, mouse melanoma, and ovarian cancer ([13,14]). In addition, by Western blot, real-time quantitative reverse transcription-PCR, and immunostaining, Halin et al ([15]) found that PEDF was detected in highly differentiated, nonmetastatic, androgen-sensitive Dunning tumors and in the anaplastic, androgen insensitive but nonmetastatic Dunning tumors. In contrast, the metastatic Dunning tumor sublines showed very low PEDF expression levels. In both the rat model and in the human tumors, the vascular count, as determined by factor VIII-related antigen staining, inversely correlated with pigment epithelium-derived factor mRNA levels. Together, these data suggest that decreased PEDF expression may contribute to tumor progression, possibly through increasing tumor cell proliferation and increased angiogenesis.

To assess a role of PEDF on prostate cancer cells, we developed an experimental system using stable re-expression of PEDF in DU145 human prostate cancer cells, which normally express very low baseline PEDF level and high VEGF level, to demonstrated that PEDF significantly decreased growth of primary tumor at s.c. site, and PEDF re-expression completely inhibited the generation of macroscopic lung metastases. Thus, there data strongly suggested that re-expression of PEDF inhibits tumor growth and lung metastasis.

Angiogenesis plays a critical role in cancer growth and progression ([16]). Since tumors can not grow above about 1 mm^3 without development of a new blood supply, their growth and expansion are thought to be totally dependent on angiogenesis ([17]). The finding that blood vessel growth increases in PEDF knockout mice is one of the several lines of evidence that support an inhibitory role for PEDF in angiogenesis ([18]). Given the role of PEDF being a potent inhibitor of angiogenesis in the eye by inducing apoptosis in actively dividing endothelial cells, we addressed whether PEDF' tumor-suppressor and metastasis-suppressor activity was due to anti-angiogenesis. We showed that re-expression of PEDF did decrease microvessel density in tumor tissue, and decreased the ability of HMVEC plated onto Matrigel-coated wells to form capillary-like tube structures. The data indicate that PEDF inhibits tumor vascularization in vivo and vascular differentiation in vitro.

As above mentioned, there exits a loose inverse correlation between PEDF levels and the ability of prostate cancer cells to secrete VEGF. It was indicated that PEDF may function to counterbalance VEGF in prostate cancer cells. VEGF is a key mediator of angiogenesis and is essential for blood vessel formation, promoting the proliferation, survival, and migration of endothelial cells ([19]). The upregulation of expression of VEGF by tumor cells is considered a major contributor to tumor angiogenesis and is especially important to the formation of lesions at peripheral metastatic sites ([20]). Guan et al previously reported that PEDF decreased expression of VEGF in glioma U251([10]). Recently, Garcia M et al. ([21]) also reported similar results showing that PEDF overexpression in a melanoma induced changes in the angiogenic factor profile, including down-regulation of VEGF expression. It potentially means that PEDF decreases angiogenesis by suppressing VEGF expression in DU145 prostate cancer cells. We show that SSeCKS can inhibit secreted VEGF protein levels in DU145 cells. Furthermore, we will study whether the forced re-expression of VEGF in DU145 cells is sufficient to reverse the tumor-suppressor and metastasis-suppressor activity of PEDF in vivo.

Taken together, we demonstrated that PEDF inhibited prostate cancer growth and metastasis through decreasing angiogenesis of tumor and VEGF expression.

ACKNOWLEDGE

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