Overexpression of Pigment Epithelium-Derived Factor Decreases Angiogenesis and Inhibits the Growth of Human Malignant Melanoma Cells *in Vivo*

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Pigment epithelium-derived factor (PEDF) has recently been shown to be the most potent inhibitor of angiogenesis in the mammalian eye, and is involved in the pathogenesis of angiogenic eye disease such as proliferative diabetic retinopathy. However, a functional role for PEDF in tumor growth and angiogenesis remains to be determined. In this study, we have investigated both the *in vitro* and *in vivo* growth characteristics of human malignant melanoma G361 cell lines, stably transfected to overexpress human PEDF. Expression levels of PEDF proteins in melanoma cell lines G361 and A375 were comparable with that of human cultured melanocytes, whereas vascular endothelial growth factor levels in two tumor cell lines were much stronger than that in normal melanocytes. Overexpression of PEDF was found to significantly inhibit tumor growth and vessel formation in G361 nude mice xenografts. Furthermore, *in vitro* proliferation rates of G361 cells were decreased in PEDF-transfected cells. PEDF proteins showed dose-dependent induced growth retardation and apoptotic cell death in nontransfected G361 cells, which were completely prevented by treatment with antibodies against the Fas ligand. Our present study highlights two beneficial effects of PEDF treatment on melanoma growth and expansion; one is the suppression of tumor angiogenesis, and the other is induction of Fas ligand-dependent apoptosis in tumor cells. PEDF therefore might be a promising novel therapeutic agent for treatment of patients with melanoma. (Am J Pathol 2004, 164:1225–1232)

Angiogenesis, a process by which new vascular networks are formed from pre-existing capillaries, is required for tumors to grow, invade, and metastasize.^{1,2} Tumors are unable to grow beyond a volume of 1 to 2 mm³ without establishing a vascular supply because cells must be within 100 to 200 μ m of a blood vessel to survive.^{1,2} Tumor vessels are genetically stable, and less likely to accumulate mutations that allow them to develop drug resistance in a rapid manner.³ Therefore, targeting vasculatures that support tumor growth, rather than cancer cells, is considered the most promising approach to cancer therapy.

Pigment epithelium-derived factor (PEDF), a glycoprotein that belongs to the superfamily of serine protease inhibitors, was first purified from human retinal pigment epithelial cell-conditioned media as a factor with potent human retinoblastoma cell neuronal differentiating activity.⁴ Recently, PEDF has been shown to be a potent inhibitor of angiogenesis in both cell culture and animal models. Indeed, PEDF is reported to inhibit retinal endothelial cell growth and migration and suppress ischemia-induced retinal neovascularization.^{5,6} Furthermore, loss of PEDF was associated with angiogenic activity in proliferative diabetic retinopathy.⁷ However, a functional role for PEDF in tumor growth and angiogenesis remains to be elucidated.

In this study, we investigated both *in vitro* and *in vivo* growth characteristics of the human malignant melanoma cell line G361, stably transfected to overexpress human PEDF.

Materials and Methods

Cells and Mice

Two human malignant melanoma cell lines G361 and A375 (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin/streptomycin. Normal

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Figure 1. Immunofluorescence staining (**A**) and Western blot analysis (**B**) of G361, A375, and normal melanocytes. **A:** Immunofluorescence staining for PEDF was performed as described in Materials and Methods. The **inset** indicates negative staining without primary antibody. **B:** Expression levels of PEDF, VEGF, and α -tubulin in cell lysates were analyzed as described in Materials and Methods. Scale bar, 10 μ m.

human neonatal melanocytes were purchased from Kurabo (Osaka, Japan) and maintained according to the manufacturer's instructions.

BALB/c-nu/nu mice were purchased from Japan Clea (Tokyo, Japan) and maintained under specific pathogen-free conditions. All animal procedures were conducted according to guidelines provided by the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol.

Preparation of Polyclonal Antibodies against Human PEDF

Polyclonal antibody against 44-mer PEDF peptides (VLLSPLSVATALSALSGLAEQRTEIIHRALYYDLISSFDIHGT) was prepared as previously described.⁸ We confirmed that the polyclonal antibody actually bound to purified PEDF protein (data not shown).

Immunofluorescence Microscopy

Immunofluorescence staining was performed on G361, A375, or normal melanocytes cultured on glass cover-

slips. Each cell type was incubated with an anti-PEDF antibody at 4°C overnight, and then these primary antibodies were detected with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Fluorescence staining was detected using a confocal laser-scanning fluorescence microscope (Laser Scanning Confocal Imaging System MRC 1024; Bio-Rad, Richmond, CA).

Construction of PEDF Expression Vector

PEDF cDNA was originally cloned from a human placenta cDNA library (Clontech, Palo Alto, CA), and inserted into the mammalian expression vector pBK-CMV (Stratagene, La Jolla, CA) as described previously.⁸

Purification of PEDF Proteins

293T cells (American Type Culture Collection, Rockville, MD) were transfected with a PEDF expression vector using the FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Then PEDF proteins were purified

Figure 2. Western blot analysis of conditioned medium from G361 cells, stably transfected with the human PEDF expression vector (**lane 2**) or with the expression vector alone (**lane 3**). Purified PEDF proteins (0.2 μ g) were used as a positive control (**lane 1**).

from conditioned media by a Ni-NTA spin kit (Qiagen GmbH, Hilden, Germany) according to the manufacture's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified PEDF proteins revealed a single band with a molecular weight of ~ 50 kd, which showed reactivity with the previously described antibody against human PEDF.⁸

Selection of Stable Transfectants Overexpressing PEDF

Subconfluent G361 or A375 cultures were stably transfected either with a PEDF expression vector or with an expression vector alone using the FuGENE 6 transfection reagent. Twenty-four hours after transfection, cells were split 1:3 into their full growth medium containing 400 μ g/ml of Zeocin (Invitrogen, Carlsbad, CA) to select transfectants. Stably transfected clones were expanded, and the clones were characterized for PEDF production.

Western Blot Analysis

G361, A375, and normal melanocytes were grown to confluence in 100-mm dishes, washed with phosphate-buffered saline (PBS), and lysed as previously described.⁹ Conditioned medium was obtained from stably transfected G361 cells grown for 48 hours in serum-free culture medium, and then concentrated 20-fold using Microcon 10 MWCO filters (Amicon, Beverly, MA). Pro-

teins were electrophoresed on polyacrylamide gels under reducing conditions, and then blotted onto nitrocellulose filters. Filters were blocked with nonfat dried milk and followed by incubation with a primary antibody against human PEDF, human vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, CA), or α -tubulin (Santa Cruz Biotechnology). After incubation, the filters were treated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ), and the resultant immune complexes were visualized using an enhanced chemiluminescence detection system (ECL) (Amersham) as previously described.⁸

Growth of G361 Xenografts in Nude Mice

Confluent G361 cells, stably transfected with the human PEDF expression vector or with the expression vector alone, were trypsinized and resuspended in PBS at a density of 1×10^7 cells/ml. One million tumor cells were injected intradermally into the flanks of each 6-week-old female athymic nude mouse ($n = 5$). The smallest and largest diameters of the tumors were measured after a 5-day interval using a pair of digital calipers, and the tumor volumes were calculated using the following formula: volume (mm^3) = [(smallest diameter)² $\times$ (largest diameter)]/2.¹⁰

Immunofluorescence Staining of Tumor Vessels

To determine the degree of tumor-induced angiogenesis, cryostat sections were prepared from tumor xenografts 30 days after implantation. Five cryostat sections of each tumor xenograft were stained with fluorescein isothiocyanate-conjugated rat anti-mouse CD31 (PharMingen, San Diego, CA). Nuclei were stained with propidium iodide. Three different fields at $\times 60$ magnification were examined on each section using a confocal laser-scanning fluorescence microscopy. The percentage of fluorescent-positive areas in three different fields from each section was measured.

Cell Growth and Apoptosis Assays

G361 cells, stably transfected with the human PEDF expression vector or with the expression vector alone, were serum-starved for 24 hours, and cell numbers were counted at days 2, 4, and 6 using a dye exclusion method. To investigate effects of PEDF proteins on the growth and apoptosis of nontransfected G361 cells, the cells were incubated with or without various concentrations of PEDF proteins in the presence or absence of 10 μ g/ml of monoclonal antibody against Fas ligand (PharMingen). After 6 days of incubation, viable cell numbers were determined. Apoptosis was measured with an enzyme-linked immunosorbent assay for DNA fragments after a 16-hour incubation according to the manufactur-

Figure 3. Growth of G361 xenografts in nude mice. G361 cells, stably transfected with the human PEDF expression vector or with the expression vector alone, were injected intradermally into the flanks of five 6-week-old female athymic nude mice. The tumor volumes were calculated as described in Materials and Methods. *, $P < 0.0001$ compared with control cells.

Figure 4. Tumor vessels in G361 xenografts. Typical photomicrographs of immunofluorescent staining for CD31 (green) in G361 xenografts. Nuclei were stained with propidium iodide (red). G361 cells, stably transfected with the human PEDF expression vector (**A**) or with the expression vector alone (**B**). **Bottom:** The quantitative analysis of the fluorescent-positive area (per 1000 μm^2) in tumors. *, $P < 0.0001$ compared to control cells. Scale bar, 50 μm .

er's instructions (Cell Death Detection ELISA; Roche Molecular Biochemicals, Mannheim, Germany).

Assay for *in Situ* Apoptosis

To determine the degree of apoptosis, cryostat sections were prepared from tumor xenografts 30 days after implantation. Terminal dUTP nick-end labeling (TUNEL) assay was performed using *in situ* apoptosis detection kit (Boehringer Mannheim, Mannheim, Germany). The number of apoptotic cells was counted in 10 randomly selected fields at $\times 200$ magnification using an immunofluorescence microscope.

Statistical Analysis

All values were presented as means $\pm$ SEM (standard error of the mean). Statistical significance was evaluated using the Student's *t*-test for paired comparison; $P < 0.05$ was considered significant.

Results

PEDF and VEGF Expression by Melanoma Cells and Normal Melanocytes

The human malignant melanoma cell lines G361 and A375 were chosen for their endogenous expression of PEDF and VEGF. As shown in Figure 1, A and B, G361 and A375 cells were found to express substantial amounts of PEDF, and the expression levels of PEDF in these tumor cells were comparable with that of normal human cultured melanocytes. In contrast, expression levels of VEGF among these cells were quite different; G361 and A375 cells were characterized by a strong expression of VEGF, whereas little VEGF protein was detected in cell lysates from normal melanocytes (Figure 1B).

Next, we investigated the expression levels of PEDF proteins in the G361 cell-conditioned medium that were stably transfected with the human PEDF expression vector. As shown in Figure 2, PEDF-transfected G361 cells

secreted increased amounts of PEDF, whereas control vector-transfected cells expressed little or no supernatant PEDF proteins. PEDF-transfected A375 cells showed similar results (data not shown).

Growth of PEDF-Overexpressing G361 Cells *in Vivo*

Next, we examined whether PEDF overexpression influences the tumor growth of G361 cells *in vivo*. As shown in Figure 3, control vector-transfected cells formed rapidly growing tumors, reaching 300 to 400 mm³ after 30 days. In contrast, PEDF overexpression almost completely inhibited *in vivo* tumor growth of G361 cells throughout this observation period of up to 30 days (Figure 3).

Tumor Vessels in PEDF-Overexpressing G361 Xenografts

To determine the tumor-induced angiogenesis within PEDF-transfected and control vector-transfected tumors, five different frozen sections from each tumor were stained with an antibody against mouse CD31. As shown in Figure 4, morphometric analysis revealed decreased tumor vessels in 30-day-old tumors derived from the PEDF-overexpressing G361 cells, compared with control vector-transfected tumor cells.

Growth and Apoptosis of G361 Cells *in Vitro*

To determine whether PEDF overexpression influences tumor cell proliferation *in vitro*, we measured growth rates of cultured G361 cells, stably transfected with a human PEDF expression vector or with the expression vector alone. As shown in Figure 5A, growth rates of PEDF transfectants were significantly lower than that of control vector-transfected cells. Furthermore, PEDF proteins dose dependently retarded growth and induced apoptotic cell death in cultured nontransfected G361 cells, which was completely blocked by treatments with a neutralizing antibody against Fas ligand (Figure 5, B and C). These results suggest that PEDF elicits apoptosis in G361 cells indirectly, in a Fas ligand-dependent manner.

Apoptosis in PEDF-Overexpressing G361 Xenografts

Finally, we examined apoptosis in the PEDF-transfected and control vector-transfected tumors, frozen sections from each tumor were analyzed using TUNEL assay. As shown in Figure 6, apoptotic cells were significantly increased in the tumors derived from the PEDF-overexpressing G361 cells, compared with control vector-transfected tumor cells.

Discussion

A major event in tumor growth and expansion is the angiogenic switch, an alteration in the balance of proan-

giogenic and anti-angiogenic molecules that leads to tumor neovascularization.¹ Indeed, many tumors, including malignant melanoma not only overexpress multiple angiogenic factors such as VEGF, basic fibroblast growth factor, and interleukin-8, but also underexpress angiogenic inhibitors such as thrombospondin-1, thus favoring

Figure 5. Growth and apoptosis of G361 cells *in vitro*. **A:** Growth rates of G361 cells, stably transfected with the human PEDF expression vector or with the expression vector alone *in vitro*. Growth (**B**) and apoptosis (**C**) of nontransfected G361 cells. Cells were incubated with or without the indicated concentrations of PEDF proteins in the presence or absence of 10 μ g/ml of monoclonal antibody against Fas ligand. *, $P < 0.005$ compared to the value without treatments.

Figure 6. Apoptotic cells in G361 xenografts. Typical photomicrographs of apoptotic cells using TUNEL assay (green) in G361 xenografts. Nuclei were stained with propidium iodide (red). G361 cells, stably transfected with the human PEDF expression vector (**A**) or with the expression vector alone (**B**). **Bottom:** The quantitative analysis of the apoptotic cell percentage in tumors. *, $P < 0.005$ compared to control cells. Scale bar, 50 μm .

angiogenesis.^{11,12} In this study, we demonstrated for the first time that two human malignant melanoma cell lines, G361 and A375, expressed substantial amounts of PEDF protein and that the expression levels of PEDF in cell lysates were comparable with that of normal (nonmalignant) human melanocytes. The present observations suggest that a decrease or loss in PEDF production alone might not contribute to malignant melanoma development. To elucidate the physiological roles of PEDF derived from normal melanocytes in skin would be beyond the original scope of this study. However, recently, others and we have shown that PEDF protected retinal vascular and neuronal cells from oxidative stress-induced injury.^{8,13} Because H_2O_2 is formed as a byproduct of melanin synthesis, after ultraviolet irradiation in mammalian skin, PEDF secreted by melanocytes might be involved in the maintenance of normal skin homeostasis through its anti-oxidative properties.¹⁴ In this study we found that two melanoma cell lines express high levels of VEGF proteins, whereas normal melanocytes produced a minimal amount of VEGF. Melanocytes are transformed to malignant melanoma cells by constitutive signal activation via

a mitogen-activated protein kinase, a process that is associated with an increase in production of VEGF, supporting the hypothesis for a functional role of VEGF in melanoma transformation.¹⁵

In this present study, we found for the first time that overexpression of PEDF decreases tumor angiogenesis and almost completely inhibits the growth of G361 melanoma xenografts in nude mice. PEDF is also known to effectively suppress retinal and choroidal neovascularization caused by ischemia and age-related macular degeneration, respectively.^{6,16} Very recently, Doll and colleagues¹⁷ reported that PEDF inhibits stromal vasculature and epithelial tissue growth, mediating an anticancer effect for pancreas cancer. Blocking tumor vascularization using PEDF may also be a promising approach for melanoma treatment. Previous studies have shown that malignant melanoma responds well to anti-angiogenic therapy using other endogenous angiogenic inhibitors such as angiostatin (plasminogen kringle 1 to 4) and endostatin.^{18,19} Because plasminogen kringle 5 was recently reported to inhibit ischemia-induced retinal neovascularization in a rat model by down-regulating

VEGF and up-regulating PEDF,²⁰ the anti-angiogenic and growth inhibitory effects of angiostatin on melanoma cells could be ascribed, at least in part, to this PEDF activity.

It is possible that the concentrations used in these present studies (up to 100 nmol/L) were beyond the normal physiological concentrations, and the inhibitory effect was nonspecific toxicity. However previous studies have shown, for example, complete inhibition of vessel formation that was obtained at a concentration of ~50 nmol/L.²¹ In addition, the estimated human blood concentration of PEDF is 100 nmol/L, which is a functionally significant concentration of PEDF in our studies, indicating that the blood circulation itself holds the capacity to inhibit angiogenesis in tissues throughout the entire body.²² Therefore the PEDF concentrations in our experiments may not be too high and remain within the parameters found under normal physiological conditions. Furthermore, we experimented the assay for *in situ* apoptosis to determine whether exogenous PEDF within tumor induces apoptosis. We could show that apoptotic cells were significantly increased in the tumors derived from the PEDF-overexpressing G361 cells, compared with control vector-transfected tumor cells (Figure 6). This result suggested that the endogenous PEDF in our animal model was not physiologically at too high a level to induce nonspecific toxicity, because nonspecific toxicity resulted in necrosis.

Here, we have been the first to identify a PEDF-induced growth retardation and induction of apoptotic cell death in melanoma cells that is dependent on the Fas ligand. PEDF up-regulates Fas ligand in endothelial cells, thereby specifically sensitizing tumor vessels to apoptosis.²³ PEDF could directly elicit apoptosis in G361 cells by inducing Fas ligand on these tumor cells. Recently, granulocytes have been shown to inhibit melanoma lung metastasis by inducing Fas ligand-associated apoptosis, further supporting this speculation.²⁴

Our present study has highlighted two beneficial aspects of the effects of PEDF on melanoma growth and expansion; one is the suppression of tumor angiogenesis, and the other is induction of Fas ligand-dependent apoptosis in tumor cells. PEDF is therefore a promising novel therapeutic agent for treatment of patients with certain types of cancer including melanoma.

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