Inhibition of Xenografted Human Melanoma Growth and Prevention of Metastasis Development by Dual Antiangiogenic/Antitumor Activities of Pigment Epithelium-Derived Factor

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ABSTRACT

Human melanoma mortality is associated with the growth of metastasis in selected organs including the lungs, liver, and brain. In this study, we examined the consequences of overexpression of pigment epithelium-derived factor (PEDF), a neurotrophic factor and potent angiogenesis inhibitor, on both melanoma primary tumor growth and metastasis development. PEDF overexpression by melanoma cells greatly inhibited subcutaneous tumor formation and completely prevented lung and liver metastasis in immunocompromised mice after tail vein injection of metastatic human melanoma cell lines. Whereas the effects of PEDF on primary tumor xenografts appear mostly associated with inhibition of the angiogenic tumor response, abrogation of melanoma metastasis appears to depend on direct PEDF effects on both migration and survival of melanoma cells. PEDF-mediated inhibition of melanoma metastases could thus have a major impact on existing therapies for melanoma.

INTRODUCTION

The incidence of melanoma has increased steadily during the last decades. The latest epidemiology data indicate that in the United States, around 50,000 new cases will be diagnosed (and almost 10,000 of them will have a lethal outcome) within a year’s time (1). The high mortality rates associated with advanced melanoma rely on the metastatic dissemination of tumor cells from their primary sites and generation of chemoresistance as a consequence of alteration of key molecules involved in the regulation of cell survival (2–4). Thus far, systemic therapies for metastatic cutaneous melanoma, the most aggressive human solid tumors, have proven disappointing. Use of modifiers of biological responses, alone or in combination with chemotherapy, aim at improving and building more durable responses. Among such modifiers, those targeting the metastasis-host tissue interactions, including antiangiogenic factors, remain promising therapeutic agents (5–7). Pigment epithelium-derived factor (PEDF), initially identified as neuronal differentiation factor produced by cultured human retinal pigment epithelial cells (8), has recently been shown to be a potent inhibitor of angiogenesis in the eye by inducing apoptosis in actively dividing endothelial cells (9). Conversely, however, PEDF has also been shown to promote the survival of certain neurons. The potential role of PEDF as an antiangiogenic agent in the context of solid tumors has recently begun to be explored, pointing to a more widespread angioinhibitory role for this multifunctional factor (10–12). Studies in PEDF knockout mice showed increased vessel density in several organs, combined with marked hyperplasia of the pancreas and prostate epithelium. Moreover, highly tumorigenic prostate cell lines showed reduced PEDF expression compared with less tumorigenic ones. Thus, it is likely that PEDF has an inhibitory effect on prostate tumor development (13).

In this study, we present evidence for a dramatic effect of PEDF on the growth of primary melanoma and metastases. We observed both a canonical antiangiogenic effect of PEDF, consistent with previous reports, and a newly discovered direct effect of PEDF on melanoma cells. Our results emphasize that PEDF is targeting both the tumor and tumor vasculature, leading to a more efficient blockade of tumor growth than that achieved by using purely antiangiogenic compounds aimed exclusively at destroying the tumor vasculature.

MATERIALS AND METHODS

Cells. Human umbilical vascular endothelial cells were obtained from umbilical cords as described previously (14) and cultured in M199 (Sigma, St. Louis, MO) supplemented with 20% fetal calf serum (FCS), 50 μg/ml endothelial cell growth supplement from bovine brain, 0.1 mg/ml heparin, and 1% antibiotic-antimycotic (GIBCO) in tissue culture flasks precoated with 0.5% gelatin. Cells between passages 4 and 6 were used for migration assays. Human melanoma cell line UCD-Mel-N was derived from a patient with multiple primary melanoma tumors (15, 16). The packaging cell line PA317 was obtained from American Type Culture Collection. BAEC, UCD-Mel-N, and PA317 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL) supplemented with 10% FCS and 1% antibiotic-antimycotic (GIBCO). Cells were cultured at 37°C in a humid atmosphere containing 5% CO₂.

Retroviral Vector Design and Production. Retroviral expression vectors were constructed using the LZRS backbone vector (17, 18). The human PEDF cDNA (a gift from Dr. Noel Bouck) was cloned into plLZR-ires-EGFP vector plasmid (19). Defective retroviruses were generated through transient transfection of 293T cells with packaging and retroviral vector plasmids (18).

Generation of a Stable Retroviral Packaging Cell Line. PA317-EGFP+ and PA317-PEDF-ires-EGFP. The packaging cell line PA317 (American Type Culture Collection) was used to generate a stable cell line to produce amphotropic retroviral particles containing the plLZR-ires-EGFP or plLZR-PEDF-ires-EGFP sequence. The sequence was integrated in the PA317 genome by infection with ecotropic particles obtained from transient transfection in 293T cells with packaging and retroviral vector plasmids (18).

Received 1/23/04; revised 6/3/04; accepted 6/23/04.

Grant support: Grants SAF-2001-1349 (B. Jimenez) and BMC-2001-1018 from Ministerio de Ciencia y Tecnología (J. Jorcano), Grant FIS 010556 from Ministerio de Sanidad y Consumo (F. Larcher), and Grant CAM 08 6/0004 (M. Del Rio). M. Garcia and N. Fernandez were supported by Comunidad Autonoma de Madrid fellowships.

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[cancer research 64, 5632–5642, August 15, 2004]
medium was replaced overnight with fresh medium. Infected PA317-EGFP- and PA317-PEDF-IRE5-EGFP-expressing cells were selected by fluorescence-activated cell sorting on a FAC-Star PLUS flow cytometer (Becton Dickinson, San Jose, CA).

**Generation of PEDF-Overexpressing Human Melanoma Cell Lines.** UCD-Mel-N and A375 cell lines were genetically modified by retroviral gene transfer. Briefly, the day before the infection, UCD-Mel-N cells were seeded in a T75 flask (Falcon). A supernatant from the PA317-GFP+ or PA317-PEDF+ confluent flask was recovered at the moment of the infection and filtered through a 45-μm filter. The supernatants were added, together with 8 μg/ml Polybrene (Sigma), to UCD-Mel-N cells in two cycles of 12 h. In between the two cycles, the melanoma cell infection medium was replaced overnight with fresh medium. The transfected melanoma cells [green fluorescent protein (GFP)] were selected by fluorescence-activated cell sorting (FAC-Star PLUS/flow cytometer; Becton Dickinson; Ref. 20). PEDF expression was analyzed in conditioned media using anti-PEDF antibodies (a gift from Dr. Noel Bouck).

**Tumor and Metastasis Models.** For tumor studies, cells (1.5 × 106 UCD-Mel-N-GFP or UCD-Mel-N-PEDF cells, resuspended in 100 μl of PBS) were injected subcutaneously into the flanks of NOD/SCID mice. Tumors were measured periodically with a caliper, and the volume was calculated as length × width2 × 0.52 (12). At different time points, the mice were killed, and the tumors were harvested and analyzed.

For metastasis studies, 5 × 105 UCD-Mel-N and A375 cells transduced with retrovirus, resuspended in 100 μl of PBS, were injected intravenously into the tail vein. To evaluate the evolution of the metastases at different organs, the animals were sacrificed, and an autopsy was performed. Critical organs (lung, liver, and brain) were illuminated (490 nm wavelength) to detect fluorescent metastases under a stereomicroscope using a GFP filter. Organs were dissected and fixed in buffered formalin for routine histology and immunohistochemistry.

**Detection of Melanoma Cell Arrival at Host Organs.** To assess for melanoma cell homing to specific organs after tail vein injection, PCR amplification of GFP sequences (present in both control and PEDF-transduced cells) was performed using DNA extracted from organs at 16 and 40 h after injection. The presence of a 300-bp band was indicative of GFP-transduced cells.

**Metalloproteinase Activity.** Melanoma cells were cultured to near confluency in DMEM-10% FCS. The cell monolayers were washed with PBS and cultured with serum-free DMEM for 24 h. Supernatants were collected and centrifuged to remove floating cells and cell debris. Gelatin zymography was realized according to Heussen and Dowdle (21). Twenty μl of the supernatants were separated on nonreducing 10% SDS-polyacrylamide gels containing 0.1% (w/v) gelatin (Bio-Rad). After electrophoresis, the gel was washed twice in 50 mM Tris (pH 7.5) containing 2.5% Triton X-100 for 30 min to remove SDS, washed with 50 mM Tris (pH 7.5) twice for 10 min, and then incubated for 24–48 h at room temperature in enzyme substrate buffer [50 mM Tris (pH 7.5), 0.15 M NaCl, 10 mM CaCl2, 0.1% (w/v) Triton X-100, and 0.02% (w/v) NaN3]. Gels were stained with 2.5% Coomassie Blue R-250 for 2–3 h and de-stained with methanol:acetic acid:water (5:1:5).

**Migration Assays.** Conditioned media were prepared from 80% confluent melanoma cultures; the cells were rinsed briefly three times with PBS and rinsed with serum-free DMEM for 4 h. Cells were then incubated in fresh serum-free DMEM for 48 h, and the media were collected, centrifuged to remove cell debris, concentrated, and dialyzed using Millipore Ultrafree centrifugal filters with a Mw 10,000 cutoff.

Endothelial or melanoma cell migration was assessed using modified Boy-
den chamber migration assays as described previously (22). Briefly, 5 × 10^5 melanoma cells or human umbilical vascular endothelial cells per cm^2 were seeded on gelatin-covered filters, and migration was performed for 4 h for endothelial cells and overnight for melanoma cells. Migration of endothelial cells toward conditioned media from UCD-Mel-N-GFP or UCD-Mel-N-PEDF and migration of melanoma cells toward 10% FCS were tested. Filters were stained using the Diff Quik staining kit, and migrated cells were counted in 10 high-powered fields. Each condition was tested in quadruplicate, and results were confirmed in three independent experiments.

**In Vitro Invasion Assay.** Tumor cell invasiveness was tested using gelatin-coated 8-μm pore polycarbonate membranes in transwells inserted in 24-well plates (Costar). Cells (1.65 × 10^5) were resuspended in 200 μl of DMEM and loaded in the upper compartment. The lower compartment was filled with 600 μl of DMEM and 10% FCS. After 16 h, cell migration was analyzed by detection of enhanced green fluorescent protein (EGFP) using a Leica TCS-SP2 confocal microscope with a ×20 oil immersion lens (0.70 NA). Fluorescence images were acquired each 2 μm along the z axis in the transwell and represented by the fluorescence intensity profiles obtained by image analysis using Leica confocal software version 2.5 built 1227. Filter position was determined using reflected light and appears as a white stripe in the projected image. Maximum projections of horizontal (XY) sections corresponding to cells binding to the upper side of the filter, cells that migrated inside the filter, and cells that crossed the filter and bound to the opposite side of the filter were obtained by confocal analysis and quantified using Leica confocal software. Basal migration towards 0.1% bovine serum albumin/DMEM was determined.

**In Vivo Angiogenesis Assay.** A corneal neovascularization assay was performed as described previously (23). Sulforafate/Hydropent solutions were prepared including the indicated substances and implanted into the avascular cornea of anesthetized C57B16 mice. Where indicated, the pellets contained 0.1 ng/μl basic fibroblast growth factor, 5 ng/ml anti-PEDF antibody (Chemicon), and 30 μg/ml conditioned media from UCD-Mel-N-GFP or UCD-Mel-N-PEDF cells. Results are shown as the number of positive corneas of the total number of corneas implanted. Photos were taken 7 days after implantation.

**Vascular Permeability Assay.** Vascular permeability was studied as described by Blazquez et al. (24). Briefly, tumor-bearing animals were anesthetized, and Evans Blue (1% in PBS; 100 μl/mouse) was injected into the tail vein. Dye leakage was subsequently detected as spots on the tumor surface.

**Anoikis Assays.** For cell cycle analysis by flow cytometry, 80% confluent melanoma cultures were serum-deprived for the indicated time periods in DMEM. In flow cytometry, cells were trypsinized, washed with PBS, and fixed in 70% ethanol pre-cooled at 4°C. Fixed cells were washed with PBS before treatment with 100 μg/ml RNase A and staining with 50 μg/ml propidium iodide for 30 min at 37°C. Results of a representative experiment, confirmed in three independent experiments, are shown.

**A.** Effect of PEDF overexpression on xenografted subcutaneous human melanomas. A, proliferation curves of PEDF-transduced or control melanoma cells growing in the presence of serum. B, tumor volume of PEDF and control melanomas at 30 and 90 days after cell injection. Results (mean ± SE) are from six mice in each group. ***, P < 0.01.
ysis. Data are expressed as the mean number of CD31-positive vessels per field. For double immunofluorescence detection of CD31 and SMA, 10-μm frozen sections were fixed in 1:1 methanol-acetone and incubated with combined primary antibodies. Fluorescent secondary antibodies against mouse (Texas Red-conjugated) and rat (FITC-conjugated) immunoglobulins were used. A total of 100 vessels in nonoverlapping fields were scored for quantitative analysis of CD31/SMA labeling.

To determine the tumor proliferative index, mice received intraperitoneal...
injection with 100 ng/kg 5-bromo-2′-deoxyuridine (BrdUrd) in 0.9% NaCl and were sacrificed 1 h later for tissue harvesting. To detect BrdUrd incorporation, deparaffinized tumor sections were incubated in 2N HCl for 1 h at room temperature. After washing in PBS, the sections were incubated with mouse anti-BrdUrd antibody for 1 h at room temperature. Anti-BrdUrd (Roche) primary antibody was diluted 1:50.

Different methods were used to determine the EGFP reporter gene expression. Green fluorescence was readily visualized in the intact xenograft in vivo with a fluorescence stereomicroscope (Olympus) under blue light. Formaldehyde-fixed sections were subjected to immunohistochemistry (see above) with anti-GFP polyclonal antibodies (A-11122; Molecular Probes) at a 1:200 dilution.

RESULTS

PEDF-Transduced Melanoma Cells Secrete Biologically Active PEDF. To determine the possible effects of PEDF on melanoma growth, we generated populations overexpressing human PEDF through retroviral gene transfer. The bicistronic retroviral construct included human PEDF cDNA followed by an internal ribosomal entry site (IRES) coupled to EGFP cDNA (Fig. 1A). UCD-Mel-N [a human melanoma cell line derived from a patient with multiple primary melanoma tumors (27)] melanoma cells were transduced and sorted for EGFP expression. Control cells were transduced with the same retroviral construct lacking the PEDF cDNA. Conditioned media from both control and PEDF-transduced cells were tested for PEDF expression by Western blot. The presence of a robust, specific M, 55,000 band in the PEDF-transduced cells (UCD-Mel-N-PEDF) indicated that PEDF was appropriately synthesized and processed (Fig. 1B). The biological activity of PEDF secreted by transduced melanoma cells was determined by an endothelial cell migration assay (22). Consistent with the effects reported for recombinant PEDF (28), conditioned medium from UCD-Mel-N-PEDF cells failed to stimulate endothelial cell chemotaxis above basal levels (Fig. 1C), whereas conditioned medium from UCD-Mel-N-GFP control melanoma cells induced migration at high levels. This result was confirmed in vivo in a corneal neovascularization assay. Media from UCD-Mel-N-GFP cells were highly angiogenic, whereas media conditioned by PEDF-positive cells were no longer stimulatory. The lack of angiogenic response to conditioned media from PEDF-transduced cells was due to PEDF because angiogenic activity was restored by adding the blocking anti-PEDF antibodies. These results indicate that PEDF is able to counterbalance the angiogenic stimulus produced by melanoma cells (Fig. 1D).

PEDF Impaired Subcutaneous Melanoma Growth. To rule out possible toxic effects of PEDF on melanoma cells, we compared the proliferation rates of GFP control and PEDF-transduced cells in vitro and in vivo. Typically, under standard culture conditions (10% serum), PEDF-positive cells remained attached to the plastic substrate and proliferated at rates similar to those of control cells (Fig. 2A).

We subsequently evaluated the growth of subcutaneous tumors formed by the retrovirally modified melanoma cell lines in immunodeficient NOD/SCID mice at different time points. Initially, both control and PEDF melanoma tumors grew at a similar rate and reached similar volumes in the first 4 weeks. However, the growth of PEDF-positive melanomas became highly inhibited thereafter (Fig. 2B). Histological examination at 12 weeks revealed extensive necrotic areas in the PEDF-positive tumors, but not in control tumors (Fig. 3A). To determine whether a growth impediment at later stages resulted from angiogenic switch inhibition by PEDF, we assessed both functional and quantitative markers of tumor vasculature at earlier time points, when size differences between control and PEDF tumors were not yet statistically significant. Thus, tumors were harvested at 18 and 30 days after injection. At 30 days, the Evans Blue permeability assay revealed severely reduced vascular leakage (Fig. 3B), whereas CD31 staining demonstrated a dramatic decrease in neovascularization in the
UCD-Mel-N-PEDF compared with control GFP tumors (Fig. 3C). Vascular density differences were also apparent (although not statistically significant) at 18 days (Fig. 3C).

Also consistent with angiogenic switch reversal, SMA, a marker of periendothelial cells indicative of vascular maturity, was observed in the vessels found at 12 weeks in residual UCD-Mel-N-PEDF tumors (likely the preexisting capillaries that favor initial tumor growth). In contrast, control UCD-Mel-N-GFP tumors showed highly dilated vessels not decorated by SMA-positive cells, consistent with immature, remodeling vessels (Fig. 3D). This feature was also evident in early tumors. In fact, the majority of vessels in the control tumors (GFP tumors) showed a SMA-negative/CD31-positive phenotype at both 18 and 30 days after injection. Conversely, tumors generated from PEDF-transduced cells exhibited a greater proportion of SMA-positive/CD31-positive vessels (Fig. 3, D and E).

We also determined the effect of PEDF on proliferation and apo...
ptosis in the subcutaneous tumors. UCD-Mel-N-PEDF tumors showed highly reduced BrdUrd incorporation compared with GFP-only controls 30 days after injection (Fig. 4A). As in the case of vascular density, differences in cell proliferation were not statistically significant at 18 days (Fig. 4A). These results indicate that tumor growth collapse, likely due to impaired vascularization, occurs around 20–30 days after injection. In fact, massive tumor cell apoptosis is present at 30 days but only slightly increased at 18 days in the PEDF tumors (Fig. 4B). The increase in TUNEL-positive cells in tumors at 30 days ranged from moderate to high, depending on the tumor areas inspected, whereas control tumors presented few homogeneously distributed apoptotic cells (Fig. 4B). A progressive increase in overall apoptosis and necrosis was found throughout tumor development in PEDF-positive melanomas (data not shown).

PEDF Abrogated Melanoma Metastasis Potential in the Tail Vein Model. Tumor dissemination in critical organs such as the lung, brain, and liver is the main cause of death in melanoma patients. Strong suppression of the growth of subcutaneous (primary) melanoma by PEDF compelled us to study its effect on the growth and spread of melanoma metastases. To that end, UCD-Mel-N-GFP control and UCD-Mel-N-PEDF cells were injected intravenously in immunodeficient NOD/SCID mice, and metastases were analyzed macroscopically in different organs by GFP fluorescence. Remarkably, 3 months after injection, 100% of the animals injected with UCD-Mel-N-GFP cells (n = 6; two experiments) developed large, visible lung nodules, which were visible under both white and blue light (GFP fluorescence), whereas none of the animals that received UCD-Mel-N-PEDF cells showed visible signs of metastases (Fig. 5, A and C). Histological examination and immunostaining for S100, a melanoma marker of serial sections of lungs, failed to reveal the presence of micrometastases in UCD-Mel-N-PEDF-injected mice even at the longest times analyzed, whereas UCD-Mel-N-GFP-injected mice all had metastases (Fig. 5, B and C). This fact led us to speculate whether melanoma cell homing to the target organ could be compromised. However, this was not the case because PCR analysis detected GFP DNA sequences in two of three and three of four lungs of UCD-Mel-N-GFP- and UCD-Mel-N-PEDF-injected mice, respectively. (Fig. 5D).

PEDF Inhibits Melanoma Cell Migration and Survival. The complete prevention of metastasis formation suggested that, in addition to the canonical inhibition of angiogenesis, PEDF also exerted a direct effect on melanoma cells that may contribute to the blockade of metastases. One attractive possibility was that PEDF could influence the migration of the melanoma cells into the new host “soil.” Therefore, we assessed the effect of PEDF gene transduction on melanoma cell migration. PEDF-positive melanoma cells showed significantly lower chemotaxis in modified Boyden chamber assay (Fig. 6A). Invasive properties of PEDF-secreting melanoma cells were compared with control melanoma cells using a transwell assay. We found a significant reduction in the number of cells that penetrated the filter and traversed the pores in PEDF-positive cells compared with GFP control (Fig. 6, B and C). Quantitative data analysis showed a 3- to
5-fold reduction in migration and invasiveness of UCD-Mel-N-PEDF cells.

Seeking mechanisms underlying impaired migration, we investigated matrix metalloproteinase profiles of PEDF-positive and GFP control cells. We were able to detect a high matrix metalloproteinase-2 activity by gelatin zymography in conditioned media from melanoma cells; however, no clear differences have been found between PEDF-transduced and control cells (data not shown).

We next investigated whether attenuated migration of PEDF-transduced melanoma cells may result from a PEDF-dependent decrease in cell survival. We therefore tested apoptosis of PEDF-positive cells and control cells under different environmental challenges (Figs. 7 and 8). Although under standard culture conditions (full serum), both control and PEDF-transduced melanoma cells grew at similar rates (Fig. 2A), serum withdrawal resulted in a higher number of detached cells in the PEDF-transduced cultures (data not shown).

A viability assay showed that UCD-Mel-N-PEDF cells progressively lost viability in the absence of serum (Fig. 7A). Consistently, quantitative measurement of apoptosis using flow cytometry analysis of DNA content revealed a significant increase in programmed cell death in the absence of mitogenic stimuli in the PEDF-positive population (Fig. 7B). The percentage of apoptotic cells increased in a time-dependent manner in the absence of serum in UCD-Mel-N-PEDF cells, whereas it remained at a constant low level in control UCD-Mel-N-GFP cells.

We also tested whether the lack of attachment could differentially induce apoptosis in PEDF-transduced melanoma cells. This type of cell stress, known as anoikis, could be significant in a context in which metastasizing cells have to survive en route to the target organ (29). After 48–72 h of suspension culture in high serum, a high proportion of PEDF-transduced cells showed a DNA content consistent with apoptotic cell death due to loss of cell anchorage (Fig. 8). The kinetics of anoikis was further accelerated when PEDF-positive cells were grown in suspension in the absence of serum (data not shown).

**Angiogenic Factor Profile of PEDF-Transduced Melanoma Cells.** PEDF has been shown to exert antiangiogenic activity by antagonizing VEGF action at the level of endothelial cell responses, and the VEGF/PEDF ratio is altered in ischemia-induced neovascular growth (30). However, little is known about possible modulation of angiogenic/antiangiogenic factor expression by PEDF. We thus studied the expression of VEGF (a relevant proangiogenic factor) and angiopoietins 1 and 2 (two important regulators whose balance controls the onset of the angiogenic process).

Surprisingly, enzyme-linked immunosorbent assay for human VEGF revealed that the expression of VEGF protein secreted in the conditioned medium of UCD-Mel-GFP (control) cells was 5-fold higher than that of PEDF-transduced cells (313 versus 74 pg/10^6 cells/24 h, respectively; Fig. 9A). Northern blot analysis of angiopoietin expression revealed a 2.6-fold increase in angiopoietin-2 messenger RNA levels in the PEDF-transduced cells (Fig. 9B), whereas angiopoietin-1 levels remained unchanged. This result was further confirmed by Western blot analysis of melanoma cell conditioned media (data not shown).

**PEDF Blocks Melanoma Metastasis to a Broad Spectrum of Targets.** We finally investigated whether inhibition of metastatic growth by PEDF was restricted to a particular melanoma cell type/host microenvironment or whether, on the contrary, it exhibited a broader inhibitory activity. We studied the effect of PEDF on the metastatic growth of the A375 melanoma cell clone with liver tropism in NOD/SCID mice using the same tail vein injection protocol. A375 melanoma cells transduced with PEDF retroviral vector also secreted high levels of PEDF in conditioned medium (Fig. 10A). Remarkably, as was the case for UCD-Mel-N-PEDF...
cells in the lung, metastases were abolished in the livers of five of six mice that received injection with PEDF-transduced A375 cells (Fig. 10B). Moreover, the single micrometastasis found in the liver of the only metastasis-positive mouse that received injection with PEDF-transduced melanoma cells stained negative for GFP. This may indicate either silencing of the transgene or contamination with nontransduced cells, which accounts for the appearance of the metastasis in this mouse. In contrast, histological examination of liver sections from animals that received injection with control cells 3 months after tail vein injection revealed a high infiltration of melanoma cells (both macrometastases and micrometastases; Fig. 10B) throughout the hepatic parenchyma.

DISCUSSION

Combating melanoma metastasis remains a major clinical challenge, and thus far, the existing therapeutic protocols have proven disappointing. Novel approaches include the use of promising angiogenesis inhibitors. The armory of such agents includes PEDF, a pleiotropic factor initially identified as a neurotrophin (8). In this study, we show that PEDF potently inhibits primary melanoma growth and completely prevents the development of metastases.

According to evidence accumulated mostly from studies of vascular pathologies in the eye, PEDF antagonizes the proangiogenic effect of VEGF. Moreover, attenuation of constitutive PEDF antiangiogenic activity promotes the activation and remodeling of previously quiescent vasculature (30). In our system, the reduction of both vascular leakage and vessel counts early in the progression of PEDF-positive melanomas, as well as the existence of a majority of SMA-positive blood vessels in the tumors overexpressing PEDF, suggests that PEDF precludes the development of immature vessels capable of rapid remodeling. The persistence of pericytes in PEDF tumors may be indicative of preexisting mature vessels resistant to destabilization by PEDF. The strong reduction in VEGF levels concomitant with an increase in angiopoietin 2 expression observed in PEDF-transduced melanoma cells reverses the angiogenic balance determining an inhibition of tumor angiogenesis. Increased levels of angiopoietin 2 in the absence of VEGF trigger endothelial cell apoptosis and vessel regression (31, 32). PEDF has recently been shown to block angiogenesis through antioxidative pathways that negatively regulate VEGF (32, 33). While this manuscript was under revision, Guan et al. (34) reported similar results showing that PEDF overexpression in a glioma cell line induced changes in the angiogenic factor profile, including down-regulation of VEGF expression.

Signal transduction pathways mediating PEDF antiangiogenic actions remain almost unexplored. It has been reported that PEDF binds immobilized heparin, chondroitin sulfate, dextran sulfate, cell surface glycosaminoglycans (GAG), and collagen (35–37). PEDF binds specifically to the Y-79 retinoblastoma and cerebral granule cells, and its neurotrophic effects are presumably associated with a $M_r$, 80,000 surface protein (38, 39). The GAG binding region was identified by homology modeling with the structures of antithrombin III and ovalbumin. Two peptides from the center of b-sheet, A-strands 2 and 3, and helix F with a basic surface potential, densely populated with the exposed lysines, are available to interact with GAG and polyanions (35). Binding to the positively charged external collagen areas requires a negatively charged surface region rich in acidic residues. This PEDF domain has no neurotrophic effect and may convey antiangiogenic activity (37). PEDF neurotrophic function and the ability to block vascular leakage are replicated by a 44-amino acid peptide (44-mer, residues 58–101; Refs. 36 and 40). Interestingly, four amino acids within 44-mer (Glu$^{101}$, Ile$^{103}$, Leu$^{112}$, and Ser$^{115}$) are critical for the effect on vascular permeability (40), and the same residues may be responsible for PEDF angioninhibitory properties. Whereas the robust effect of PEDF on primary melanoma devel-

Fig. 8. Induction of anoikis by PEDF in melanoma cells. A, induction of anoikis by PEDF as evaluated by flow-cytometric determination of DNA content in UCD-Mel-N-GFP or UCD-Mel-N-PEDF cells after 48 and 72 h of suspension culture. Individual nuclear DNA content as reflected by fluorescence intensity of incorporated propidium iodide is plotted as a histogram. Histograms are derived from a representative experiment. Quantification of apoptotic cells (sub-G0 population) is also shown (bar graph).
opment could be explained solely by its bona fide potent antiangiogenic action (28), the ability of PEDF to induce apoptosis directly in melanoma cells, described herein, likely produces a synergistic effect when antisurvival challenges (such as hypoxia) are present and presumably contributes to the antitumor and antimetastatic activities of PEDF. Our data are consistent with the recently shown ability of PEDF to induce apoptosis in cultured prostate epithelium and to reduce angiogenesis in prostate tumor xenografts after intratumor injections (13). Also in accordance with our results, gene therapy approaches using adenoviral PEDF gene transfer to different cell lines, including those derived from lung, colon, and liver tumors, have proven efficient to inhibit xenografted tumor growth (12, 41). Additional experimental evidence for PEDF as a widespread antiangiogenic agent has been provided during the revision of this manuscript. These reports include two studies using PEDF overexpression in melanoma primary xenografts (42) and glioma cells (34) and a clinical study correlating PEDF expression and prognosis of patients with pancreatic adenocarcinoma metastasis to the liver (43).

During the metastatic process, as tumor cells detached from the primary tumor migrate through the surrounding tissue, enter the circulation, invade, and reattach, each such step represents a different type of selective pressure, which, for the majority of cells, results in failure to survive. Thus, each step in the metastatic cascade is potentially rate-limiting, and the failure to complete a single step is likely to render a tumor cell nonmetastatic. The tail vein injection model allows assessment of rate-limiting events (namely, the ability to survive in the bloodstream, to adhere to the vascular endothelium of capillary walls, to extravasate, and to grow in a different tissue at a distant site) at a late stage of the metastatic process. Because we were able to detect PEDF-transduced melanoma cells at the target organ, it appears that short-term cell survival in the circulation and adhesion to the endothelium were not compromised by PEDF. However, the stress suffered by loosely attached melanoma cells favored programmed cell death by anoikis in the presence of PEDF. Increased cell death at this step would hinder further events such as migration, extravasation, and growth at a remote secondary site. However, it is not evident that increased anoikis is the only reason for diminished migratory and invasive capacity by PEDF-producing cells. Inability to migrate alone could serve as a limiting factor for metastasis formation. The complete lack of metastatic foci in the lungs of mice that received injection with PEDF-transduced melanoma cells precluded analysis of tumor cell apoptosis as a possible mechanism underlying the antimetastatic effect of PEDF.

The induction of apoptosis by PEDF in tumor cells (the nonendothelial compartment of the tumor) may represent a broader class of events, provided that appropriate challenge takes place. In fact, PEDF enhancement of apoptosis due to hypoxia or mimicking conditions in prostate tumor cells has been demonstrated (13). It is possible that under stress conditions, PEDF induces apoptotic cascades in melanoma cells similar to apoptotic cascades induced in endothelial cells (23). It appears even more feasible because some highly invasive melanoma types are capable of the vascular mimicry phenomenon (44). PEDF could be exploiting this trait by inhibiting melanoma cell invasion or incorporation into tumor vessels among endothelial cells, thus decreasing the frequency of metastasis and diminishing the number of mosaic vessels. The direct effects of PEDF on melanoma cells described in this study broaden the plethora of targets and processes affected by this factor (8). The fact that neural crest progenitors give rise to both neural cells and melanocytes and that particular cranial neural crest cells can generate the endothelium of the aortic arteries suggests that seemingly contradictory effects of PEDF may converge at the level of the ancestral origin of the cells affected. For example, migration, one of the common features for neural crest progenitors and their derivatives, is most dramatically affected by PEDF. Notwithstanding the complexity of response to PEDF treatment and/or expression, the dual antitumor and antiangiogenic activity doubles the efficacy and promise of PEDF as an agent for the treatment of malignant melanoma. Studies are in progress to

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**Footnotes:**

1. For the treatment of malignant melanoma. Studies are in progress to...
2. Additional experimental evidence for PEDF as a widespread antiangiogenic agent has been provided during the revision of this manuscript. These reports include two studies using PEDF overexpression in melanoma primary xenografts (42) and glioma cells (34) and a clinical study correlating PEDF expression and prognosis of patients with pancreatic adenocarcinoma metastasis to the liver (43).
3. The tail vein injection model allows assessment of rate-limiting events (namely, the ability to survive in the bloodstream, to adhere to the vascular endothelium of capillary walls, to extravasate, and to grow in a different tissue at a distant site) at a late stage of the metastatic process. Because we were able to detect PEDF-transduced melanoma cells at the target organ, it appears that short-term cell survival in the circulation and adhesion to the endothelium were not compromised by PEDF. However, the stress suffered by loosely attached melanoma cells favored programmed cell death by anoikis in the presence of PEDF. Increased cell death at this step would hinder further events such as migration, extravasation, and growth at a remote secondary site. However, it is not evident that increased anoikis is the only reason for diminished migratory and invasive capacity by PEDF-producing cells. Inability to migrate alone could serve as a limiting factor for metastasis formation. The complete lack of metastatic foci in the lungs of mice that received injection with PEDF-transduced melanoma cells precluded analysis of tumor cell apoptosis as a possible mechanism underlying the antimetastatic effect of PEDF.
4. The induction of apoptosis by PEDF in tumor cells (the nonendothelial compartment of the tumor) may represent a broader class of events, provided that appropriate challenge takes place. In fact, PEDF enhancement of apoptosis due to hypoxia or mimicking conditions in prostate tumor cells has been demonstrated (13). It is possible that under stress conditions, PEDF induces apoptotic cascades in melanoma cells similar to apoptotic cascades induced in endothelial cells (23). It appears even more feasible because some highly invasive melanoma types are capable of the vascular mimicry phenomenon (44). PEDF could be exploiting this trait by inhibiting melanoma cell invasion or incorporation into tumor vessels among endothelial cells, thus decreasing the frequency of metastasis and diminishing the number of mosaic vessels. The direct effects of PEDF on melanoma cells described in this study broaden the plethora of targets and processes affected by this factor (8). The fact that neural crest progenitors give rise to both neural cells and melanocytes and that particular cranial neural crest cells can generate the endothelium of the aortic arteries suggests that seemingly contradictory effects of PEDF may converge at the level of the ancestral origin of the cells affected. For example, migration, one of the common features for neural crest progenitors and their derivatives, is most dramatically affected by PEDF. Notwithstanding the complexity of response to PEDF treatment and/or expression, the dual antitumor and antiangiogenic activity doubles the efficacy and promise of PEDF as an agent for the treatment of malignant melanoma. Studies are in progress to...
assess the efficacy of systemic PEDF application to combat melanoma metastases.

ACKNOWLEDGMENTS

We are indebted to Isabel de los Santos and Pilar Hernandez for histology, Jesus Martinez for animal care, Drs. C. Gamallo and A. Bravo for expert advice on pathology, Yajaira Suarez for advice in flow cytometry, and D. Megias for confocal analysis of migration assays.

REFERENCES

Inhibition of Xenografted Human Melanoma Growth and Prevention of Metastasis Development by Dual Antiangiogenic/Antitumor Activities of Pigment Epithelium-Derived Factor

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