Published in final edited form as:

Prostate. 2013 April ; 73(5): 549–561. doi:10.1002/pros.22595.

Positive correlation between PEDF expression levels and macrophage density in the human prostate

Thomas Nelius¹, Christina Samathanam², Dalia Martinez-Marin¹, Natalie Gaines¹, Jessica Stevens¹, Johnny Hickson¹, Werner de Riese¹, and Stéphanie Filleur^{1,3}

¹Texas Tech University Health Sciences Center, Department of Urology, Lubbock-TX

²The University of Texas Health Science Center at Houston, Medical School, Department of Pathology and Laboratory Medicine, Houston-TX

³Texas Tech University Health Sciences Center, Department of Immunology and Molecular Microbiology, Lubbock-TX

Abstract

BACKGROUND—In this study, we investigated the capacity of PEDF to modulate the recruitment and the differentiation of monocytes/macrophages both *in vitro* and in human prostate.

METHODS—Using Boyden chambers, we assessed PEDF effect on the migration of monocytes and chemically-activated RAW264.7 macrophages. Normal, prostatitis and prostate cancer specimens were retrospectively selected and examined by immunohistochemistry for PEDF expression and infiltration of immune CD68+macrophagic cells. PEDF expression and macrophage density were then correlated with each other and clinicopathological parameters. M1 and M2 differentiation markers were quantified by qRT-PCR, western blotting and ELISA.

RESULTS—In chemotaxis, PEDF induced the migration of monocytes/macrophages. In immunohistochemistry, macrophages were markedly increased in prostatitis and malignant compared to normal tissues. PEDF was expressed at variable levels in the stroma and epithelium. PEDF mRNA was down-regulated in both prostate cancer and prostatitis compared to normal tissues. In correlation studies, macrophage density and PEDF expression were respectively positively and negatively associated with prostate size. Most importantly, PEDF expression positively correlated with macrophage density. Finally, PEDF stimulated the expression of iNOS, IL12 and TNF α ; and inhibited IL10 and arginase 1 in mouse and human macrophages confirming a M1-type differentiation.

CONCLUSIONS—Our data demonstrate that PEDF acts directly on monocytes/macrophages by inducing their migration and differentiation into M1-type cells. These findings suggest a possible role of macrophages in PEDF anti-tumor properties and may support further development of PEDF-based anti-cancer therapy.

Keywords

Prostate gland; PEDF; CD68; M1 macrophages; iNOS

Requests for reprints: Dr. Stéphanie Filleur, Ph.D., Texas Tech University Health Sciences Center, Department of Urology, 3601 4th Street-MS 6591, Lubbock-TX-79430-6591, Stephanie.filleur@ttuhsc.edu, Phone: (+1)-806-743-4868, Fax: (+1)-806-743-2334.

DISCLOSURE/CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

INTRODUCTION

Inflammation has been previously described as a key player in many prostatic pathologies such as benign prostatic hyperplasia, prostatic intraepithelial neoplasia and prostate cancer (1). In many cases of abacterial prostatitis (the most common form of prostatitis from a pathological point of view), the cause of prostatic inflammation (chronic prostatitis) is unclear. Possible initiating factors may include infectious agents, urine reflux, dietary factors, estrogens, or a combination of two or more of these factors (1). Among various inflammatory cell populations present in the prostate gland, macrophages have been recognized as one of the major inflammatory components. However, their precise mechanisms of contribution in the inflammatory process still remain conflicting and require further investigation.

Recent studies indicate that chronic inflammation may contribute to high-grade prostatic intraepithelial neoplasia and lead subsequently to the development of prostate cancer. Increased numbers of macrophages have been found in chronic inflammatory infiltrates in the prostate. They have also been associated with tissue injuries and focal epithelial atrophy. Based on histomorphological, pre-clinical, clinical, genetic and molecular studies, this model has been designated as proliferative inflammatory atrophy and has been suggested as a possible precursor lesion to adenocarcinoma (1,2). In support of a direct link between inflammation and prostate cancer, men with chronic and/or acute inflammation of the prostate have an increased risk of developing prostate cancer (1,3). Regular administration of non-steroidal anti-inflammatory drugs decreased prostate cancer risks by 60–80% in older men (4–6). Accordingly, multiple inflammatory cytokines have been found to be over-expressed in prostate cancer compared to normal cells and tissues (7–9). On the other hand, the role of immune infiltrates in prostate cancer progression and prognosis still remain conflicting. Lissbrant, et al. linked the volume density of tumor-associated macrophages (TAMs) to a shorter survival time in prostate cancer (10). In contrast, Shimura, et al. reported high TAMs numbers to be an independent predictor of disease-free survival after radical prostatectomy for prostate cancer (11). In agreement with Lissbrant's study, the inhibition of macrophages' function or pro-inflammatory pathways in prostate cancer cells delayed tumor growth in experimental prostate cancer (12–14); a reduced number of macrophages decreased angiogenesis and prostate tumor growth in rats (15), and interactions between prostate cancer cells and macrophages mediate hormonal resistance in prostate cancer (16).

Pigment Epithelium-Derived Factor (PEDF) is a 50 kDa secreted angio-inhibitor which promotes neuron differentiation and, acts as an anti-oxidant and anti-tumor factor (17). PEDF is expressed and secreted in many tissues (17). In the prostate gland, PEDF is expressed by epithelial and stromal cells, including smooth muscle cells (18). In the same organ, PEDF expression is down-regulated in response to hypoxia and androgens. It is also down-regulated in prostate cancer (18) where its reduction has been linked to poor prognosis (19). Additionally, in PEDF $-/-$ mice, hypervascularization of the prostate is combined with hyperplasia emphasizing the critical role of PEDF in the control of cell growth and differentiation in this gland (18). Recently, PEDF was suggested as an immune-modulating factor. PEDF appears to be pro-inflammatory in the central nervous system where it increases the expression of various pro-inflammatory factors (20–22). Conversely, PEDF is anti-inflammatory in the eye where it contributes to the suppression of innate immunity in the subretinal space (23,24). PEDF also emerged as anti-inflammatory in multiple inflammation-related pathologies (25–27). In agreement with these results, we recently showed that PEDF modified the inflammatory signature of human prostate cancer cells (28). The role of PEDF in inflammation is reinforced by the following observations: (i) PEDF blocks the pro-inflammatory signaling of angiotensin II (29,30), and (ii) it represses

macrophage function in the endotoxin-induced inflammation mouse model (31). Therefore, an increasing body of evidence ascribes an inflammatory function to PEDF. However, the link between PEDF immunomodulatory activity and inflammation-related pathologies of the prostate is yet to be established.

Recently, PEDF has been shown to stimulate macrophages to become more tumoricidal (32). PEDF expression has also been associated with an increase in density of tumor-cytotoxic M1 macrophages in the orthotopic MatLyLu rat prostate tumor model (15). However, neither the direct effect of PEDF on the migration and differentiation of monocytes/macrophages nor the association between PEDF expression levels and macrophage density in human prostate specimens has ever been studied. In the present study, we evaluated the effect of PEDF on monocytes and macrophages using *in vitro* chemotaxis assay, quantitative Reverse Transcriptase-Polymerase Chain Reaction and western blotting, and immunohistochemistry on human prostate tissues. We found that PEDF enhances the migration of monocytes and chemically-activated macrophages *in vitro*, and that the expression levels of PEDF positively correlate with the density of macrophages in human prostate. We also demonstrated that PEDF acted on macrophages by inducing their differentiation toward the M1-type pathway.

MATERIALS AND METHODS

Cells

RAW 264.7 monocytes (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT). THP-1 monocytes (ATCC) were grown in RPMI 1640 medium with 10% FBS and 0.05 mM 2 β -mercaptoethanol. Monocytes were differentiated into macrophages in complete medium with 50 nM Phorbol Myristate Acetate (PMA, Sigma-Aldrich, Saint Louis, MO) for 48 hours. For PEDF treatment, macrophages were incubated for an additional 48 hours in serum-free medium with 50 nM PMA and 0–10 nM PEDF (BioProducts MD, Middletown, MD). Prostate cancer PC3-E13 (control) and PC3-P15 cells (expressing PEDF; Nelius et al., in preparation) were grown in RPMI 1640 medium with 10% FBS. Conditioned media were collected from PC3 cells incubated in serum-free medium for 48 hours.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNAs were extracted from PMA-treated macrophages using the RNeasy extraction kit (Qiagen, Valencia, CA) or from one-two 6 μ m-thick tissue sections using the High Pure FFPE RNA Micro Kit (Roche, Indianapolis, IN). 2 μ g RNAs were then converted into cDNA using the Thermo Scientific VersoTM cDNA synthesis kit. cDNA was amplified (FastStart Universel SYBR Master Mix, Roche) in an MyiQ amplifier (BioRad, Hercules, CA): 1x heating for 10' at 95°C; 40x denaturing for 20'' at 95°C, followed by annealing/extension, 1' at 60°C. Primers for mouse/human IL10, arginase 1 and TNF α (Solaris qPCR Gene Expression Assay) were from Thermo Scientific. As reference, the housekeeping gene S15 (5'-ACAACGGCAAG ACCTTCAAC-3'/5'-GGCTTGTAGGTGATGGAGAAC-3') was amplified. Cycle threshold (C_T) values were determined by automated threshold analysis with MyiQ version 1.0 software and the fold change for each gene calculated using the $\Delta\Delta$ Ct method. Each sample was tested in triplicate. All the amplicons were run on a 2% agarose gel and showed correct sizes.

Meso-Scale Discovery (MSD) Technology

Cytokines expression was measured using the MSD mouse multi-spot Cytokine Assays (Meso-Scale) as recommended. Briefly, conditioned media and serial dilutions of calibrators in blocker B buffer were incubated into each well under shaking for two hours. After

washes, the detection antibody solution was added and the plate was further incubated for two hours. The plate was then washed, the read buffer added and the plate read on a Meso-Scale Sector Imager 2400 (Meso-Scale). The data were analyzed using MSD Data Analysis Toolbox software. The software fits the standard curves using a 4 parameter logistic fit with $1/y^2$ weighting. The 4-PL equation is $y=b_2+(b_1-b_2)/(1+(x/b_3)^{b_4})$ with y = signal, x = concentration, b_2 = estimated response at infinite concentration, b_1 = estimated response at zero concentration, b_3 = mid-range concentration, and b_4 = slope factor. The samples had measurable levels for all of the assays and were above the Lower Limit of Detection, calculated as the concentration equivalent to the signal 2.5 standard deviations above the 0 pg/mL calibrator.

Western blots

Macrophage differentiation was validated on whole cell lysates (lysis buffer: 50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, pH 8.0) by 10% SDS-PAGE using the mouse monoclonal PKC- α antibody (BD Biosciences, San Jose, CA) as described in (33). For iNOS, whole cell lysates (RIPA buffer: 50 mM Tris-cl pH 7.5, 150 mM NaCl, 1% NP40, 1% Na-deoxycholate, 0.1% SDS and 10 mM EDTA) were separated by 8% SDS-PAGE using the mouse (abcam, Cambridge, MA) or human iNOS antibodies (Millipore, Billerica, MA). After stripping, membranes were re-probed with the arginase 1 antibody (Santa Cruz Biotechnology). β -actin antibody (Sigma-Aldrich) was used to assess loading.

Chemotaxis assay

In vitro migration assay was performed in 48-well Boyden chambers (Neuroprobe, Gaithersburg, MD) as described in (34). 2×10^6 /ml monocytes were seeded on top of the gelatinized microporous membrane (8 μ m pore size, Neuroprobe). Test substances (0.1% BSA or 0–10 nM of PEDF) were added to the bottom part of the well and incubated for 6 hours. Migrated cells were visualized using the Diff-Quick staining kit (Dade Behring, Deerfield, IL) and counted in 10 high-powered (400x) fields. 0.1% BSA and 10 nM PEDF were used respectively as negative and positive controls. Each substance was tested in quadruplicate within a single experiment; each experiment was repeated at least three times. Neutralization assays were performed using 5 μ g/ml α -PEDF or isotype antibodies (Chemicon International, Billerica, MA).

Patients and specimens

Formalin-fixed, paraffin-embedded specimens from normal (n=18), prostatitis (n=13) and prostate cancer (n=19, Gleason score: 6–7) specimens were selected retrospectively from a series of human prostate specimens diagnosed and scored either at the time of surgery (radical prostatectomy) by specialized pathologists at TTUHSC (Lubbock, TX) or at the time of the autopsy by specialized pathologists from the Cooperative Human Tissue Network (CHTN, Southern Division, Birmingham, AL). At the time of the study, two pathologists re-evaluated the haematoxylin and eosin-stained tissue sections to confirm the previous histological diagnosis. Representative tissues were then selected and classified as normal (prostatitis-free normal area adjacent to benign prostatic hyperplasia or prostate cancer area), tumoral (prostatitis-free cancer area) or with areas of abacterial prostatitis (localized in otherwise normal or tumor tissues). Tissues were classified as normal when they showed normal histology without prostatitis in an otherwise benign prostatic hyperplasia (n= 3) or prostate cancer-derived tissues (n= 8). Normal prostate tissues were also obtained by accident victims (n= 7, CHTN). Normal prostatic tissue areas showed small to fairly large glandular spaces lined by epithelium and glands lined by two layers of cells; a basal layer of low cuboidal epithelium. Tissues were classified as tumoral when they showed tumoral histology without evidence of prostatitis. In prostate cancer, features

suggestive of malignancy may have shown prominent nucleoli, marginated nuclei, multiple nucleoli, blue-tinged mucinous secretions, intraluminal crystalloids, intraluminal amorphous eosinophilic material, collagenous micronodules, glomerulations, peripheral invasion and retraction clefting. Areas of abacterial prostatitis (adjacent to histologically-defined normal and tumor tissues) which is the most common form of prostatitis were characterized by inflammatory reaction in the prostate seen as aggregation of numerous lymphocytes, plasma cells and macrophages as well as neutrophils within the prostatic substance. The histopathological diagnosis of prostatitis/inflammation should not be confused with a clinical syndrome of prostatitis. Detailed clinicopathological information (age at time of surgery, preoperative PSA, prostate weight/volume, diagnosis, surgical margins, and lymph node and nerve invasion status) were obtained from patient records. For patients diagnosed with prostate cancer, the prostate volume was obtained by transrectal ultrasound. The prostate weight for this patient population was determined after radical prostatectomy. For accident victims, the prostate weight was determined at the time of autopsy. Detailed protocol (L08-090) and waiver of informed consent were approved by the Institutional Review Board at TTUHSC.

Immunohistochemistry

6 µm-thick tissue sections from representative paraffin blocks were deparaffinized, rehydrated through graded ethanol and, subjected to endogenous peroxidase blocking and antigen retrieval (0.01 M Citrate buffer pH 6.0 by conventional microwave heating for 30 min). Immunohistochemistry was performed using the mouse monoclonal anti-human CD68 antibody (clone PG-M1, Dako, Carpinteria, CA) and the goat polyclonal anti-human PEDF (clone I-15, Santa Cruz Biotechnology, Santa Cruz, CA) in combination with the Avidin/Biotin blocking solution (0.001%/0.001% in PBS) and ABC staining kit (Santa Cruz Biotechnology). In addition, PEDF-stained sections were processed using the TSA Biotin System (PerkinElmer, Waltham, MA). Slides were developed in a 3,3'-diaminobenzidine/H₂O₂ solution and counter-stained with hematoxylin. Normal liver tissues were used as positive and negative (omission of the primary antibody) controls for PEDF staining. Prostate cancer tissues were used as negative control for CD68 staining. All controls gave appropriate results.

Evaluation of Immunoreactivity

Macrophage density (CD68-positive cells with morphological features of macrophages) and PEDF expression levels were evaluated independently by the two pathologists (C.S, T.N.) without knowledge of clinical information. For CD68 count, in each section of the prostate tissue that was examined either with a diagnosis of normal prostate, chronic prostatitis or prostatic cancer, areas with the specific diagnosis were identified. Within these areas 10 random high power fields (HPF) with positive staining for CD68 were counted at 200X. The averages of the 10 HPF counts were used in the analysis and were correlated to the PEDF expression levels. For PEDF evaluation, in each section of the prostate tissue (stroma and epithelium or both) that was examined either with a diagnosis of normal prostate, chronic prostatitis or prostate cancer, areas with the specific diagnosis were identified. Within these areas 10 random HPF with positive staining for PEDF were scored. The scorings were performed using a scale of +, ++, +++ based on the staining intensity at 200X and reported as follows: +, 1; ++, 2; +++, 3; ++, 4; +++/+, 5; +++/++, 6; +++, 7. The averages of the 10 HPFs were used in the final analysis of the data. For the correlation study, several tissues were removed from the analysis. Exclusion criteria included: 1) Lower quality of the tissue specimen and therefore of the IHC staining; 2) Limited availability of certain samples; some specimens could only be processed for either CD68 or PEDF staining; 3) Samples containing only stroma compartment could not be used for epithelial PEDF quantification; and 4) Previously established diagnoses were not confirmed by both pathologists.

Statistical analysis of the data

Data are expressed as mean $\pm$ standard deviation of three independent experiments, each done in triplicate or quadruplicate. Statistical evaluation of the data was done using Student's T-test, One-Way analysis of variance (ANOVA) or Repeated Measures ANOVA (SPSS 11.5 software for Windows). The Pearson correlation coefficient (R) test was used for correlation studies. Statistical significance was set at a P value <0.05 .

RESULTS

PEDF induced the migration of monocytes and macrophages *in vitro*

Based on the fact that PEDF has been recently identified as a factor that modulates inflammation, we evaluated its capacity to induce the migration of monocyte cells using Boyden Chambers. In this assay, RAW 264.7 monocytes were seeded in the top compartment of the chamber and increasing concentrations of human recombinant PEDF were added to the bottom compartment (Figure 1A, Left). The results showed that PEDF induces the migration of monocytes in a dose-dependent manner (Figures 1A, Right and 1B). This effect was specific to PEDF as it was completely reversed by the addition of a PEDF-neutralizing antibody (Figure 1C). To determine if similar effects could be seen on macrophages, monocytes were chemically differentiated into macrophages using PMA. As observed with monocytes, PEDF induced the migration of macrophages (Figure 2) emphasizing on the chemoattractive properties of PEDF on inflammatory cells.

Positive association between PEDF expression and macrophage recruitment in human prostate tissues

To validate our *in vitro* results, we retrospectively selected a total of 50 prostate tissue specimens (median age of the patients: 68; range: 18–83) from the existing databases present at TTUHSC and at the Cooperative Human Tissue Network. After verification of the previously established diagnosis, unstained section specimens were immunolabelled for macrophages (using the CD68 macrophage-specific marker) and PEDF (Figure 3). CD68-positive cells were detected in the stroma, nearby glandular structures, and inside the lumen of prostatic glands in all specimens tested. The majority of the CD68-positive cells had morphological features of macrophages. As previously described, hepatocytes in normal liver tissue were positively and uniformly stained for PEDF [Figure 3B–(35,36)]. In contrast, in prostate tissues, PEDF was expressed at different levels by epithelial and stroma cells (Figures 3H–J). No significant differences in localization and expression levels of PEDF, and in number of macrophages were found between the sub-categories of normal tissues (normal tissues adjacent to benign prostatic hyperplasia, prostate cancer or normal tissues obtained from accident victims) therefore validating our control group. After quantification, the total number of macrophages was found to be markedly increased in areas with prostatitis or in tumor regions compared to normal areas ($P<0.001$), (Figures 3K–M and 4). Similar to the study of Halin et al., PEDF staining was very variable with some cancer cell staining intensity considerably higher than in adjacent non-malignant epithelial cells (19). To determine in a quantitative way the expression levels of PEDF, total RNAs were extracted from paraffin sections with normal ($n=5$), abacterial prostatitis ($n=9$) and cancer ($n=4$) areas. PEDF mRNA was then measured by qPCR. As shown in figure 5, we demonstrated that PEDF mRNA is markedly reduced in tumor compared to normal tissues ($p=0.045$). PEDF mRNA was also found down-regulated in specimens with prostatitis. However, we noticed that PEDF protein levels remain heterogeneous in close proximity of prostatitis areas. As a next step, we performed correlation studies. No correlation between macrophage density and PEDF expression levels with PSA, surgical margins or lymph node invasion was found. A positive trend was found between PEDF protein expression in glandular structures and nerve invasion emphasizing PEDF neuron differentiation activity;

however, the difference was not significant. Of note, while the total number of macrophages was positively associated with prostate volume and weight ($R=0.708$ (34), P (two-tailed) <0.01 ; and $R=0.72$ (33), P (two-tailed) <0.01 , respectively), total PEDF protein was negatively associated with prostate weight ($R=0.603$ (25), P (two-tailed) <0.01 -data not shown). In accordance, PEDF $^{-/-}$ mice develop prostatic hyperplasia (18). Most importantly, both total and epithelial PEDF expression levels and macrophage density were positively associated (Figure 6, $R=0.709$ (31), P (two-tailed) <0.001 ; and $R=0.609$ (34), P (two-tailed) <0.001). These results were highly significant ($P<0.001$) corroborating our *in vitro* findings.

PEDF induced the differentiation of macrophages *in vitro*

To determine if PEDF could induce the differentiation of macrophages, green fluorescent RAW 264.7 macrophages were treated with or without PEDF in the presence of PMA. After 48 hours, the cells were fixed, mounted, and observed by fluorescence microscopy. Cells treated with PEDF showed an increased size of their cell body (Figure 7). The number of dendrite like structures and branches were also greater in PEDF-treated cells compared to PMA-treated or non-treated cells. Similarly, media conditioned by human prostate cancer PC3 cells that expressed PEDF stimulated dendrite like formation compared to control conditioned media.

PEDF stimulated the expression of markers-specific to M1 macrophages

To characterize the phenotype of PEDF-treated macrophages, we measured the expression of M1 (iNOS, IL12, TNF α) and M2 (IL10, arginase 1) specific markers in RAW 264.7 cells. While IL10 mRNA was expressed in high amount in PMA-treated macrophages, its expression was reduced by up to 66% in the presence of PEDF (Figure 8A, left). IL10 was also reduced at the protein levels (Figure 8A, right). Inversely, iNOS was up-regulated in response to PEDF treatment (Figure 8B). As for iNOS, PEDF increased the expression level of IL-12 by 45% (Figure 8C) suggesting a M1-type phenotype. In agreement, arginase 1, a M2-type marker, could not be detected at both mRNA and protein levels (Data not shown). To confirm these results, further experiments were performed using the human THP-1 cell line. Similar to RAW 264.7 cells, PEDF treatment was concomitant to a 57% decrease in IL10 (Figure 9A) and a 30% increase in iNOS (Figure 9B) expression levels. PEDF also stimulated by 60% the expression of TNF α (Figure 9C). In contrast to RAW 264.7 cells, arginase 1 was detected in the protein cell lysate but was down-regulated by PEDF (Figure 9B) validating PEDF effect on the differentiation of macrophages towards a M1-type phenotype. To validate our *in vitro* data, iNOS mRNA levels were quantified in human specimens. Similarly to PEDF, iNOS mRNA was down-regulated in both prostatitis and prostate cancer compared to normal tissues (Figure 10).

DISCUSSION

The prostate gland is constituted at the cellular level by two main compartments: the epithelium and the surrounding stroma. Prostatic stroma is pivotal for normal prostate development and homeostasis. It is primarily composed of smooth muscle and extracellular matrix, but also consists of mesenchymal cells (fibroblasts and myofibroblasts), endothelial cells, pericytes and inflammatory cells (37). Among inflammatory cells, macrophages have been involved as key players in multiple inflammation-related pathologies such as benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and prostate cancer. However, their precise mechanisms of action and their prognostic significance still remain unclear; emphasizing the need to identify additional markers. PEDF is a natural angio-inhibitor which induces the normal differentiation of neurons and was recently suggested as an inflammation-modulating factor. In the present study, we demonstrated for the first time that PEDF induces the migration of monocytes and macrophages *in vitro* and that PEDF

expression levels strongly correlate with macrophage density in human prostate specimens. We also showed that PEDF stimulated the expression of several M1-specific markers emphasizing its inflammatory-modulating activity in the prostate gland.

PEDF expression has been shown to be down-regulated in colorectal (38), breast (39), lung (40,41), pancreatic (42), and prostate cancer (43) compared to corresponding non-malignant tissues. In most of these cancers, a higher expression of PEDF was also associated with a better patient outcome/prognostic (19,39–42,44). In contrast to PEDF, the biological significance of TAMs still remains controversial. While associated with poor outcomes in thyroid cancer (45), lung adenocarcinoma (46) and breast cancer (47), TAMs have been linked to good prognosis in gastric (48), non-small cell lung (49) and colon cancers (50). In prostate cancer, Lissbrant, et al. and Nonomura, et al. found that patients with higher macrophage density had a significant shorter cancer-specific survival (10,51). In our study, we showed that macrophage density was significantly higher in tumor areas compared to benign prostatic areas. However, we could not demonstrate any significant association between macrophage density and the status of the surgical margins or extraprostatic invasion. On the other hand, Shimura et al. demonstrated a positive correlation between total number of macrophages and recurrence-free survival after radical prostatectomy (11). The discrepancy of these results can be explained by the existence of two different phenotypes for macrophages, designated as M1-type (« classically activated ») and M2-type (« alternatively activated »). Although M1-type macrophages classically express inducible nitric oxide synthase (iNOS), they are predominately located at sites of inflammation and are tumor-cytotoxic. Tumor progression promotes a phenotypic switch to M2-type macrophages that express arginase 1 and promote tumor growth, survival and metastasis (52). Accordingly, Shimura, et al. showed a reduction in the density of iNOS-positive macrophages that infiltrate the stroma in highly aggressive prostate cancers compared with less aggressive disease suggesting that the cytotoxic activities of these cells become attenuated during the acquisition of increasing malignant potential of the cancer (11). Over-expression of PEDF in orthotopic MatLyLu rat prostate tumors increased the recruitment of iNOS-positive tumor-associated macrophages (15) suggesting that PEDF could enhance anti-tumor immunity. Our study demonstrates that PEDF increased the expression of iNOS, IL12 and TNF α M1-specific markers, and decreased the expression of IL10 and arginase 1 M2 markers. We have also shown that PEDF concomitantly reduces cell proliferation and up-regulates iNOS in highly metastatic prostate cancer cells (Filleur, personal unpublished data) implying that PEDF could regulate the expression of iNOS in various cell types. These data could be of interest as iNOS over-expression has been recently described as increasing cytotoxicity in androgen-dependent and -independent prostate cancer cell lines (53,54). iNOS gene therapy also enhances the toxicity of cisplatin in prostate cancer cells (55). iNOS activity is also essential for inhibition of prostate tumor growth by interferon-beta gene therapy (56).

CONCLUSIONS

The results of our study are of importance as they show that PEDF acts directly on macrophages to induce their migratory properties and their differentiation toward the M1 inflammatory pathway. These findings also suggest that macrophages may play a key role in PEDF anti-tumor effects. A better understanding of PEDF, specifically as it relates to its anti-tumor modes of action, may support further development of PEDF-based anti-cancer therapy.

Acknowledgments

We thank the Tobacco Settlement, the School of Medicine at TTUHSC, the South Plains Foundation (Lubbock, TX) and the NIH grant R15 CA161634 for their financial support.

Source of support: This project was performed with the financial support of the Tobacco Settlement, the School of Medicine at TTUHSC, the South Plains Foundation (Lubbock, TX) and the NIH grant R15 CA161634.

References

1. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. Inflammation in prostate carcinogenesis. *Nat Rev Cancer*. 2007; 7(4):256–269. [PubMed: 17384581]
2. Wang W, Bergh A, Damber JE. Morphological transition of proliferative inflammatory atrophy to high-grade intraepithelial neoplasia and cancer in human prostate. *The Prostate*. 2009; 69(13):1378–1386. [PubMed: 19507201]
3. MacLennan GT, Eisenberg R, Fleshman RL, Taylor JM, Fu P, Resnick MI, Gupta S. The influence of chronic inflammation in prostatic carcinogenesis: a 5-year followup study. *The Journal of urology*. 2006; 176(3):1012–1016. [PubMed: 16890681]
4. Jacobs EJ, Rodriguez C, Mondul AM, Connell CJ, Henley SJ, Calle EE, Thun MJ. A large cohort study of aspirin and other nonsteroidal anti-inflammatory drugs and prostate cancer incidence. *Journal of the National Cancer Institute*. 2005; 97(13):975–980. [PubMed: 15998950]
5. Palapattu GS, Sutcliffe S, Bastian PJ, Platz EA, De Marzo AM, Isaacs WB, Nelson WG. Prostate carcinogenesis and inflammation: emerging insights. *Carcinogenesis*. 2005; 26(7):1170–1181. [PubMed: 15498784]
6. Platz EA, Rohrmann S, Pearson JD, Corrada MM, Watson DJ, De Marzo AM, Landis PK, Metter EJ, Carter HB. Nonsteroidal anti-inflammatory drugs and risk of prostate cancer in the Baltimore Longitudinal Study of Aging. *Cancer Epidemiol Biomarkers Prev*. 2005; 14(2):390–396. [PubMed: 15734963]
7. Lu Y, Cai Z, Xiao G, Keller ET, Mizokami A, Yao Z, Roodman GD, Zhang J. Monocyte chemotactic protein-1 mediates prostate cancer-induced bone resorption. *Cancer research*. 2007; 67(8):3646–3653. [PubMed: 17440076]
8. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood*. 1996; 87(8):3336–3343. [PubMed: 8605350]
9. Savarese DM, Valinski H, Quesenberry P, Savarese T. Expression and function of colony-stimulating factors and their receptors in human prostate carcinoma cell lines. *The Prostate*. 1998; 34(2):80–91. [PubMed: 9465939]
10. Lissbrant IF, Stattin P, Wikstrom P, Damber JE, Egevad L, Bergh A. Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. *International journal of oncology*. 2000; 17(3):445–451. [PubMed: 10938382]
11. Shimura S, Yang G, Ebara S, Wheeler TM, Frolov A, Thompson TC. Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. *Cancer research*. 2000; 60(20):5857–5861. [PubMed: 11059783]
12. Vukanovic J, Isaacs JT. Linomide inhibits angiogenesis, growth, metastasis, and macrophage infiltration within rat prostatic cancers. *Cancer research*. 1995; 55(7):1499–1504. [PubMed: 7533663]
13. Krishnan AV, Moreno J, Nonn L, Swami S, Peehl DM, Feldman D. Calcitriol as a chemopreventive and therapeutic agent in prostate cancer: role of anti-inflammatory activity. *J Bone Miner Res*. 2007; 22(Suppl 2):V74–80. [PubMed: 18290727]
14. Nonn L, Duong D, Peehl DM. Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells. *Carcinogenesis*. 2007; 28(6):1188–1196. [PubMed: 17151092]

15. Halin S, Rudolfsson SH, Doll JA, Crawford SE, Wikstrom P, Bergh A. Pigment epithelium-derived factor stimulates tumor macrophage recruitment and is downregulated by the prostate tumor microenvironment. *Neoplasia* (New York, NY;). 12(4):336–345.
16. Zhu P, Baek SH, Bourk EM, Ohgi KA, Garcia-Bassets I, Sanjo H, Akira S, Kotol PF, Glass CK, Rosenfeld MG, Rose DW. Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. *Cell*. 2006; 124(3):615–629. [PubMed: 16469706]
17. Filleur S, Nelius T, de Riese W, Kennedy RC. Characterization of PEDF: a multi-functional serpin family protein. *Journal of cellular biochemistry*. 2009; 106(5):769–775. [PubMed: 19180572]
18. Doll JA, Stellmach VM, Bouck NP, Bergh AR, Lee C, Abramson LP, Cornwell ML, Pins MR, Borensztajn J, Crawford SE. Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas. *Nature medicine*. 2003; 9(6):774–780.
19. Halin S, Wikstrom P, Rudolfsson SH, Stattin P, Doll JA, Crawford SE, Bergh A. Decreased pigment epithelium-derived factor is associated with metastatic phenotype in human and rat prostate tumors. *Cancer research*. 2004; 64(16):5664–5671. [PubMed: 15313905]
20. Takanohashi A, Yabe T, Schwartz JP. Pigment epithelium-derived factor induces the production of chemokines by rat microglia. *Glia*. 2006
21. Zhang SX, Wang JJ, Gao G, Shao C, Mott R, Ma JX. Pigment epithelium-derived factor (PEDF) is an endogenous antiinflammatory factor. *Faseb J*. 2006; 20(2):323–325. [PubMed: 16368716]
22. Sanagi T, Yabe T, Yamada H. The regulation of pro-inflammatory gene expression induced by pigment epithelium-derived factor in rat cultured microglial cells. *Neurosci Lett*. 2005; 380(1–2): 105–110. [PubMed: 15854760]
23. Yoshida Y, Yamagishi SI, Matsui T, Nakamura K, Imaizumi T, Yoshimura K, Yamakawa R. Elevated levels of pigment epithelium-derived factor (PEDF) in aqueous humor of patients with uveitis. *Br J Ophthalmol*. 2006
24. Eichler W, Yafai Y, Wiedemann P, Fengler D. Antineovascular agents in the treatment of eye diseases. *Curr Pharm Des*. 2006; 12(21):2645–2660. [PubMed: 16842164]
25. Jenkins AJ, Zhang SX, Rowley KG, Karschikus CS, Nelson CL, Chung JS, O’Neal DN, Januszewski AS, Croft KD, Mori TA, Dragicevic G, Harper CA, Best JD, Lyons TJ, Ma JX. Increased serum pigment epithelium-derived factor is associated with microvascular complications, vascular stiffness and inflammation in Type 1 diabetes. *Diabet Med*. 2007; 24(12): 1345–1351. [PubMed: 17971181]
26. Park K, Jin J, Hu Y, Zhou K, Ma JX. Overexpression of pigment epithelium-derived factor inhibits retinal inflammation and neovascularization. *The American journal of pathology*;. 178(2):688–698.
27. Ueda S, Yamagishi S, Matsui T, Jinnouchi Y, Imaizumi T. Administration of pigment epithelium-derived factor inhibits left ventricular remodeling and improves cardiac function in rats with acute myocardial infarction. *The American journal of pathology*;. 178(2):591–598.
28. Hirsch J, Johnson CL, Nelius T, Kennedy R, Riese W, Filleur S. PEDF inhibits IL8 production in prostate cancer cells through PEDF receptor/phospholipase A2 and regulation of NFkappaB and PPARgamma. *Cytokine*. 2011; 55(2):202–210. [PubMed: 21570865]
29. Yamagishi S, Matsui T, Nakamura K, Yoshida T, Shimizu K, Takegami Y, Shimizu T, Inoue H, Imaizumi T. Pigment-epithelium-derived factor (PEDF) inhibits angiotensin-II-induced vascular endothelial growth factor (VEGF) expression in MOLT-3 T cells through anti-oxidative properties. *Microvasc Res*. 2006; 71(3):222–226. [PubMed: 16624340]
30. Yamagishi SI, Kikuchi S, Nakamura K, Matsui T, Makino T, Norisugi O, Shimizu T, Inoue H, Imaizumi T. Pigment epithelium-derived factor (PEDF) blocks angiotensin II-induced T cell adhesion to endothelial cells by suppressing intercellular adhesion molecule-1. *Horm Metab Res*. 2006; 38(8):546–548. [PubMed: 16941283]
31. Zamiri P, Masli S, Streilein JW, Taylor AW. Pigment epithelial growth factor suppresses inflammation by modulating macrophage activation. *Invest Ophthalmol Vis Sci*. 2006; 47(9): 3912–3918. [PubMed: 16936104]
32. Ho TC, Chen SL, Shih SC, Chang SJ, Yang SL, Hsieh JW, Cheng HC, Chen LJ, Tsao YP. Pigment epithelium-derived factor (PEDF) promotes tumor cell death by inducing macrophage membrane

- tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *The Journal of biological chemistry*. 286(41):35943–35954.
33. von Knethen A, Brune B. PKC alpha depletion in RAW264. 7 macrophages following microbial/IFNgamma stimulation is PC-PLC-mediated. *Antioxidants & redox signaling*. 2005; 7(9–10): 1217–1222. [PubMed: 16115026]
 34. Filleur S, Volz K, Nelius T, Mirochnik Y, Huang H, Zaichuk TA, Aymerich MS, Becerra SP, Yap R, Veliceasa D, Shroff EH, Volpert OV. Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer. *Cancer research*. 2005; 65(12):5144–5152. [PubMed: 15958558]
 35. Tombran-Tink J, Mazuruk K, Rodriguez IR, Chung D, Linker T, Englander E, Chader GJ. Organization, evolutionary conservation, expression and unusual Alu density of the human gene for pigment epithelium-derived factor, a unique neurotrophic serpin. *Mol Vis*. 1996; 2:11. [PubMed: 9238088]
 36. Browne M, Stellmach V, Cornwell M, Chung C, Doll JA, Lee EJ, Jameson JL, Reynolds M, Superina RA, Abramson LP, Crawford SE. Gene transfer of pigment epithelium-derived factor suppresses tumor growth and angiogenesis in a hepatoblastoma xenograft model. *Pediatric research*. 2006; 60(3):282–287. [PubMed: 16857775]
 37. Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. *The Journal of urology*. 2001; 166(6):2472–2483. [PubMed: 11696814]
 38. Wagsater D, Lofgren S, Zar N, Hugander A, Dimberg J. Pigment epithelium-derived factor expression in colorectal cancer patients. *Cancer investigation*. 28(8):872–877.
 39. Zhou D, Cheng SQ, Ji HF, Wang JS, Xu HT, Zhang GQ, Pang D. Evaluation of protein pigment epithelium-derived factor (PEDF) and microvessel density (MVD) as prognostic indicators in breast cancer. *Journal of cancer research and clinical oncology*. 136(11):1719–1727.
 40. Chen J, Ye L, Zhang L, Jiang WG. The molecular impact of pigment epithelium-derived factor, PEDF, on lung cancer cells and the clinical significance. *International journal of oncology*. 2009; 35(1):159–166. [PubMed: 19513563]
 41. Zhang L, Chen J, Ke Y, Mansel RE, Jiang WG. Expression of pigment epithelial derived factor is reduced in non-small cell lung cancer and is linked to clinical outcome. *International journal of molecular medicine*. 2006; 17(5):937–944. [PubMed: 16596284]
 42. Uehara H, Miyamoto M, Kato K, Ebihara Y, Kaneko H, Hashimoto H, Murakami Y, Hase R, Takahashi R, Mega S, Shichinohe T, Kawarada Y, Itoh T, Okushiba S, Kondo S, Katoh H. Expression of pigment epithelium-derived factor decreases liver metastasis and correlates with favorable prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer research*. 2004; 64(10):3533–3537. [PubMed: 15150108]
 43. Qingyi Z, Lin Y, Junhong W, Jian S, Weizhou H, Long M, Zeyu S, Xiaojian G. Unfavorable prognostic value of human PEDF decreased in high-grade prostatic intraepithelial neoplasia: a differential proteomics approach. *Cancer investigation*. 2009; 27(7):794–801. [PubMed: 19637042]
 44. Jiang Z, Fang Z, Ding Q. Prognostic role of pigment epithelium-derived factor in clear cell renal cell carcinoma. *Urologia internationalis*. 84(3):334–340.
 45. Ryder M, Ghossein RA, Ricarte-Filho JC, Knauf JA, Fagin JA. Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer. *Endocrine-related cancer*. 2008; 15(4):1069–1074. [PubMed: 18719091]
 46. Zhang, BC.; Gao, J.; Wang, J.; Rao, ZG.; Wang, BC.; Gao, JF. *Medical oncology*. Northwood; London, England: Tumor-associated macrophages infiltration is associated with peritumoral lymphangiogenesis and poor prognosis in lung adenocarcinoma.
 47. Mukhtar RA, Nseyo O, Campbell MJ, Esserman LJ. Tumor-associated macrophages in breast cancer as potential biomarkers for new treatments and diagnostics. *Expert review of molecular diagnostics*. 11(1):91–100.
 48. Ohno S, Inagawa H, Dhar DK, Fujii T, Ueda S, Tachibana M, Suzuki N, Inoue M, Soma G, Nagasue N. The degree of macrophage infiltration into the cancer cell nest is a significant predictor of survival in gastric cancer patients. *Anticancer research*. 2003; 23(6D):5015–5022. [PubMed: 14981961]

49. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. *BMC cancer*. 10:112. [PubMed: 20338029]
50. Zhou Q, Peng RQ, Wu XJ, Xia Q, Hou JH, Ding Y, Zhou QM, Zhang X, Pang ZZ, Wan DS, Zeng YX, Zhang XS. The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. *Journal of translational medicine*. 8:13. [PubMed: 20141634]
51. Nonomura N, Takayama H, Nakayama M, Nakai Y, Kawashima A, Mukai M, Nagahara A, Aozasa K, Tsujimura A. Infiltration of tumour-associated macrophages in prostate biopsy specimens is predictive of disease progression after hormonal therapy for prostate cancer. *BJU international*. 107(12):1918–1922.
52. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A. Macrophage polarization in tumour progression. *Seminars in cancer biology*. 2008; 18(5):349–355. [PubMed: 18467122]
53. Coulter JA, Page NL, Worthington J, Robson T, Hirst DG, McCarthy HO. Transcriptional regulation of inducible nitric oxide synthase gene therapy: targeting early stage and advanced prostate cancer. *The journal of gene medicine*. 12(9):755–765.
54. McCarthy HO, Coulter JA, Worthington J, Robson T, Hirst DG. Human osteocalcin: a strong promoter for nitric oxide synthase gene therapy, with specificity for hormone refractory prostate cancer. *The journal of gene medicine*. 2007; 9(6):511–520. [PubMed: 17471586]
55. Adams C, McCarthy HO, Coulter JA, Worthington J, Murphy C, Robson T, Hirst DG. Nitric oxide synthase gene therapy enhances the toxicity of cisplatin in cancer cells. *The journal of gene medicine*. 2009; 11(2):160–168. [PubMed: 19062185]
56. Olson MV, Lee J, Zhang F, Wang A, Dong Z. Inducible nitric oxide synthase activity is essential for inhibition of prostatic tumor growth by interferon-beta gene therapy. *Cancer gene therapy*. 2006; 13(7):676–685. [PubMed: 16470211]

Figure 1.

PEDF induces the migration of monocytes *in vitro*. (A) Left: Schematic representation of the mounted Boyden chamber. Right: RAW 264.7 cell chemotaxis to PEDF. *: $P < 0.05$. (B) Representative pictures of migrated monocytes. (C) Neutralization assay.

Figure 2. PEDF effect on the migration of chemically-induced macrophages. Left: Total protein extracts from PMA-treated RAW 264.7 cells tested for PKC- α by western blotting. Right: migration of PMA-activated macrophages in response to PEDF treatment.

Figure 3. PEDF and CD68 immunohistochemistry analysis. (A–C) Human liver sections stained for PEDF (B, 10X) with corresponding H&E staining (A, 10X). (C) Human liver immuno-

stained with omission of the primary antibody for PEDF (10X). (D) Human prostate specimen immunostained with omission of the primary antibody for CD68 (10X). E, H, K, Representative slide from a normal specimen (E, H&E staining, 10X) showing high glandular PEDF expression (H, 10X) and low macrophage density (K, 20X). F, I, L, Representative slide from a tissue specimen of a patient with histopathological features of prostatitis (F, H&E staining, 10X) showing intermediary glandular PEDF expression (I, 10X) and increased macrophage density without evidence of granulocytic tissue infiltration (L, 20X). G, J, M, Representative slide from a tissue specimen of a patient with prostate cancer (G, H&E staining, 10X) illustrating low PEDF expression levels (J, 10X) and high macrophage density (M, 20X).

Figure 4. Quantification of CD68 staining in normal (n=15), prostatitis (n=12) or prostate cancer (n=18) tissues.

Figure 5. PEDF mRNA levels in normal (n=5), prostatitis (n=9) and tumor (n=4) tissues.

Figure 6. Expression levels of total (A) and epithelial (B) PEDF positively correlates with numbers of macrophages in human prostate specimens.

Figure 7. Macrophage differentiation in response to PEDF. Green-fluorescent RAW 264.7 macrophages were treated with either increasing concentrations of PEDF or media conditioned by prostate cancer PC3 cells that express (PC3-P19) or do not (PC3-E13) express PEDF. Cell morphology was then observed under fluorescence microscopy.

Figure 8.

PEDF induced the expression of M1-specific markers in RAW 264.7 cells treated with PMA +/- PEDF. IL10 (A), iNOS (B) and IL12 (C) expression levels were measured by qRT-PCR (A left), western blotting (B) or multiple-spot ELISA (A right, C).

Figure 9. Expression levels of IL10 mRNA (A), iNOS (B), arginase 1 (B) and TNFα mRNA (C) in PEDF-stimulated THP-1 macrophages.

Figure 10.
iNOS mRNA levels in normal (n=5), prostatitis (n=9) and tumor (n=4) tissues.