Pigment epithelium-derived factor inhibits T24 bladder cancer cell growth in vitro and in vivo

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Abstract

Aim Pigment epithelium-derived factor (PEDF) is a multifunctional inhibitor on both angiogenesis and tumor growth and has not been studied yet in bladder cancer. We therefore investigated the therapeutic effects of PEDF on bladder cancer in vitro and in vivo.

Methods Exogenous PEDF cDNA was delivered into T24 bladder cancer cells with recombinant adenovirus vector (AdvPEDF). Virus without target cDNA (AdvCon) was used as control. A series of cell assays and animal experiment were conducted using AdvPEDF and AdvCon for comparison of inhibitive effects on cell viability and tumor growth. Real-time RT-PCR was used for mRNA detection of angiogenesis related factors.

Results PEDF induced retardation in cell viability, proliferation and invasiveness. Increased cell apoptosis and cell cycle arrest in G1 phase were also induced by PEDF. AdvPEDF induced down-regulation of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) and up-regulation of thrombospondin-1 (TSP-1). Injection of AdvPEDF significantly reduced tumor volume and microvessel density (MVD) in nude mice with transplantation tumor of bladder cancer.

Conclusion These results contributed to our understanding of the molecular mechanism of treatment strategies of PEDF for bladder cancer.

Keywords: Adenovirus; Angiogenesis; Bladder cancer; Gene therapy; Pigment epithelium-derived factor

Introduction

Bladder cancer, majorly referred to as urothelial carcinoma, is one of the most common malignancies in urinary system and is characterized by multicentricity, high recurrence and potent invasiveness. Conventional treatments including surgical removal and chemotherapy lack the effectiveness either in enhancement of quality of life or restriction in tumor progression. Angiogenesis has recently been demonstrated playing considerable roles in bladder tumor growth. Anti-angiogenic therapies for bladder cancer have been proven effective in tumor
inhibition and are therefore being developed to a wide extent.

Pigment epithelium-derived factor (PEDF) is a multifunctional glycoprotein, belonging to serine proteinase inhibitor superfamily and is extensively distributed in human tissues. PEDF has been reported to be inhibitive in both angiogenesis and tumor growth. Over-expression PEDF, delivered by recombinant virus vectors, has been demonstrated to inhibit cell growth and invasiveness in pancreatic cancer [1], melanoma[2, 3], hepatocellular carcinoma [4] and prostate cancer[5]. Nonetheless, no report on the inhibitive effect of PEDF on bladder cancer has yet been found.

Thus, we conducted the study with recombinant adenovirus for delivery of exogenous PEDF into T24 bladder tumor cells. Realtime RT-PCR and immunohistochemistry were performed to confirm correct expression and translation of PEDF. Transfected cells were studied as regards cell viability and proliferation with tetrazolium salt (WST-1), apoptosis and cell cycle using flow cytometry, and invasiveness by Transwell assay. Expression change of vascular endothelial growth factor (VEGF), Matrix metalloproteinase-9 (MMP-9) and thrombospondin-1 (TSP-1) in PEDF treated cells were analyzed by real-time RT-PCR. Finally, inhibitive effect of PEDF on tumors in vivo was evaluated with nude mice transplantation tumor model.

Materials and methods

1. Cell lines.

Human T24 bladder urothelial cancer cells and 293A cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). T24 cells were cultured in RPMI 1640 (PAA, Germany) with 10% fetal bovine serum (FBS) (PAA). 293A cells were cultured in DMEM (High Glucose) (PAA) with 10% FBS.

2. Construction of PEDF-bearing adenovirus system.

A human full-length cDNA clone of PEDF on pCMV6-XL5 vector (Origene, Rockville, MD) was sequenced and ligated to pIRES2-EGFP vector (Invitrogen, Shanghai, China), in which a green fluorescent protein (GFP) was encoded. The product was subsequently inserted into pDONR221 vector with BP recombination system (Invitrogen). A pAD/CMV/V5-PEDF vector was then generated by LR recombination reaction. The recombinant vector was then digested with PacI and packaged using lipofectamine 2000 system (Invitrogen) by transfection into 293A cells, generating AdvPEDF. Similar procedure was performed without initial ligation of PEDF segment to provide pAD/CMV/V5-GFP (AdvCon) for control. Viral titer was detected by immunostaining with anti-adenovirus polyclonal antibody (Invitrogen) and horseradish peroxidase (HRP) method. Approximately $2.5 \times 10^5$ cells were seeded in 24-well plates. After transfection with 50μl of viral fluid for 48 h at gradient dilution from $10^{-2}$ to $10^{-6}$, cells were performed immunostaining and the immunopositive cells were observed under microscope. Viral titer was calculated by the formula, $[(\text{infected cells/field}) \times (\text{fields/well})] / [\text{volume virus (ml)} \times (\text{dilution folds})]$ and was expressed in ifu/ml (infectious units per ml). Fluorescent distribution was also compared to immunopositive sites to verify the efficacy of GFP, which was evaluated in the study of transfective efficiency in T24 cells by observation of fluorescence coverage.
3. Viral transfection in T24 cells.

Approximately 1×10⁶ cells were cultured for 12 h for attachment. Cells were rinsed with PBS once and were added 10 ml of serum free medium. AdvPEDF or AdvCon was applied at 20 or 100 MOI (multiplicities of infection) respectively and cells were incubated for 24 h. Medium was then replaced by 10 ml of complete medium.

4. Immunohistochemistry on T24 cells.

Cells were seeded in 6-well flat bottom plates of 1.2×10⁵/well and were transfected with AdvPEDF or AdvCon at 100 MOI respectively. Fixation was performed with 10% formalin. Cells were then added 25μl/well of proteinase K and were kept at room temperature for 15 min. After that, 400μl of a mouse monoclonal antibody of PEDF (LL-J) (Santa Cruz, CA, USA) was applied per well at a dilution of 1:50 and cells were incubated at 37°C for 1 h, followed by application of a goat anti-mouse antibody (Dako Corp., CA, USA) for another 1 h. Development of color was conducted with DAB (diaminobenzidine tetrahydrochloride) solution for 10 min. Finally, cells were counterstained with Mayer’s Hematoxylin blue for 1 min. Cells treated with PBS instead of primary antibody were performed otherwise same procedures for negative control.


T24 cells were cultured in 96-well flat bottom plates for 12 h at the density of 2×10⁴/ml and were exposed to AdvPEDF or AdvCon respectively at 10, 20, 50 and 100 MOI. Cells treated with equivalent PBS were studied as control. The viability was investigated four days after exposure with WST-1 (Beyotime, Jiangsu, China), which could be reduced to orange fromazan in mitochondria of cells. The reagent was added 10μl/well in the medium and cells were incubated for 1 h at 37°C. The absorbance was detected on a BioTek Elx800 Microplate Reader at 450 nm for test length and 630 nm for reference. Percentage of survived cells was interpreted by absorbance related to PBS control.


Briefly 500 cells were seeded per well in 96-well plates and were infected with either AdvPEDF or AdvCon at 100 MOI for 4 consecutive days. Samples were detected absorbance each day after 1 h of WST-1 application.

7. Apoptosis assay.

Annexin V-fluorescein (BD Pharmingen, Pasig City, Philippines) and propidium iodide (PI) (BD) were reagents indicative of apoptosis and necrosis respectively. Approximately 1×10⁶ cells were transfected respectively with AdvPEDF or AdvCon at 100 MOI for 48 h in serum free medium. Cells were then harvested and washed 3 times with chilled PBS. An HEPES binding buffer (BD) containing Annexin V and PI was applied for 15 min at room temperature. Samples were then analyzed with BD FACSCanto flow cytometer to determine percentages of early apoptotic cells (Annexin V⁺/PI⁻). Untransfected cells treated with equivalent PBS were studied as control.


Briefly 1×10⁶ cells transfected with 100 MOI of AdvPEDF or AdvCon were seeded in 75 cm² flasks for 12 h. Cells were subsequently trypsinized and suspended in 1 ml of cell cycle staining buffer (MultiSciences Biotech, Hangzhou, China) for 15 min. Cell cycle profiles were studied with flow cytometry.

9. Real-time RT-PCR.
Total RNA of AdvPEDF or AdvCon treated cells was extracted with RNAiso reagent (TaKaRa, Dalian, China). After concentration was determined with Thermo Nanodrop 1000 spectrophotometer, RNAs were converted to cDNAs with PrimeScript™ RT Reagent Kit (TaKaRa) under the condition of 37℃, 15 min; 85℃, 5 sec. Forward and reverse primers of PEDF, VEGF, MMP-9, TSP-1 and internal control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were synthesized (Invitrogen) (Tab. 1) and were applied in real-time PCR procedure with SYBR Green Premix Ex Taq™ (TaKaRa) in 20μl system on ABI 7500n (Applied Biosystem, Forster City, CA). Samples were run at 95℃, 30 sec and were amplified for 40 cycles (95℃, 5 sec; 60℃, 34 sec). For each sample, the average value of threshold cycle was normalized to GAPDH level with the formula, \( 2^{-\Delta\Delta Ct} \). Results were thus presented by expressional fold over AdvCon control.

**Table 1.** Primers of PEDF, VEGF, MMP-9, TSP-1, and GAPDH

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEDF</td>
<td>5’-AGGCCCGAGGTGTACACGAC-3’</td>
<td>5’-CCTTGAAGTTGCCACACCG-3’</td>
</tr>
<tr>
<td>VEGF</td>
<td>5’-TACATATGGCAGAGATGAGAGAGGAG-3’</td>
<td>5’-TAGGACCTATACCCAGCTCCGTGTC-3’</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5’-CGTGAGATGACTGACAACTGC-3’</td>
<td>5’-GATTTCGACTCTCCAGTGGC-3’</td>
</tr>
<tr>
<td>TSP-1</td>
<td>5’-AACCGCATCCAGATCTGCTG-3’</td>
<td>5’-TTCACCAGGTTGTGTCAAAGGGT-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-GAAGGTGAAGGTCGGAGTCA-3’</td>
<td>5’-GAAGATGGTGATGGGATTTCC-3’</td>
</tr>
</tbody>
</table>


Inserts of Transwell 24-well plates (Corning Costar, NY, USA) were treated with 100μl/well of Matrigel (BD Bioscience, San Jose, CA). Supernatant was then carefully dispersed and inserts were left air dry for 30 min at room temperature. Rehydration was performed with 300μl/well of serum free medium for 1 hr at room temperature. Cells previously uninfected (PBS) or infected (AdvPEDF or AdvCon ) were resuspended at the density of 1×10^5/ml in 300μl of serum free medium. Samples were seeded into the interior of inserts when the rehydration medium was removed. The lower chambers were then filled with 500μl of complete medium. After 48 h of incubation the interior contents of inserts were gently removed with swabs. Cells invaded through the membrane were stained and observed at ×200 magnification.

11. In vivo experiments.

Fifteen male BALB/c athymic nude mice which were 6 weeks of age (Sino-British SIPPR/BK Lab. Animal Ltd, Shanghai, China) were bred in licensed SPF (special pathogen-free) grade laboratory (SYXK(HU)2009_0082). Mice were randomly divided into 3 groups. A total of 1.5×10^7 T24 cells in 100μl of PBS were injected subcutaneously at the left axilla of each mouse. Tumors became perceptible at approximately 4 mm in diameter on the 6th or 7th day after inoculation. Treatments with AdvPEDF or AdvCon were then offered to 2 groups by intratumoral injection at 2 sites at the dosage of 100 MOI in 100μl PBS. The third group was given 100μl PBS as mock control. Both injections and calibrations of tumors were conducted weekly. Tumor size was calculated with the formula, Length×Width^2×0.5236.

12. Immunohistochemistry on tumor sections.

Formalin fixed, paraffin embedded tumor samples were performed ordinary biotin-streptavidin procedures. Endogenous peroxidase of deparaffinized sections was
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blocked through incubation with 3% hydrogen peroxide. All sections were then treated with rabbit polyclonal vWF (von Willebrand Factor) antibody (Dako, Glostrup, Denmark) at a dilution of 1:400 to detect microvessels. VWF is a large glycoprotein confined within endothelial cells, megakaryocytes and platelets, commonly used for microvessel detection. DakoCytomation EnVision Doublestain System Kit (Dako Corp., CA, USA) and DAB solution was used thereafter for color development. PBS was applied instead of primary antibody for negative control. MVD (microvessel density) was studied in reference to Weidner [6].

A two-tailed t-test was used for comparison between results of distinctive assays. P value of <0.05 was accepted as statistically significant.

Results

Purchased cDNA clone encoding PEDF was identified as CDS NM_002615.4 by BLAST (NCBI). Real-time RT-PCR revealed significant difference in PEDF mRNA expression between AdvPEDF and AdvCon treated cells (>266.6-fold, \( p < 0.001 \)), as shown in figure 3C. Immunohistochemistry demonstrated concordant PEDF protein translation (Fig. 1A-B). Viral titer was determined \( 1 \times 10^{10} \)ifu/ml for AdvPEDF and \( 1.3 \times 10^{10} \)ifu/ml for AdvCon. Transfective efficiency in T24 cells was demonstrated 40% at 20 MOI and 100% at 100 MOI. The increased dosage up to 100 MOI induced no cytopathy.

![Figure 1](image1.png)

**Figure 1.** Analysis of PEDF by immunohistochemistry. (A) T24 cells of \( 1.2 \times 10^5 \)/well and was applied transfection of AdvPEDF at 100 MOI and displayed strong and diffuse brown staining in cytoplasm, at \( \times 400 \). (B) AdvCon treated cells hardly present PEDF, at \( \times 400 \).

2. Cell viability and proliferation.
Cell viability was significantly decreased in Adv\textsubscript{PEDF} group at both 50 and 100 MOI compared with Adv\textsubscript{Con} or PBS group ($p<0.001$ respectively) (Fig. 2A). A trend of dose dependent reduction in cell viability was observed concerning Adv\textsubscript{PEDF} in the figure. Proliferation curves were generated by absorbance at 450 nm (Fig. 2B). Adv\textsubscript{PEDF} induced significant retardation in proliferation on the 4\textsuperscript{th} day post-transfection compared with Adv\textsubscript{Con} and PBS ($p<0.05$, respectively).

3. Apoptosis assay and cell-cycle arrest.

To further investigate whether the reduced viability and retarded proliferation were due to over-expression of PEDF, we analyzed apoptosis with flow cytometry at 100 MOI of viral transfection. There was a significant increase in early apoptotic cells in Adv\textsubscript{PEDF} group in comparison with Adv\textsubscript{Con} ($p=0.011$) and PBS group ($p=0.005$) respectively (Fig. 4A-C). Adv\textsubscript{PEDF} also induced increased population in G1 phase and decreased percentage in S phase compared with Adv\textsubscript{Con}, as shown in figure 5A-B.

Figure 2. Cell viability and proliferation assay demonstrating: (A) significant viability reduction by Adv\textsubscript{PEDF} over Adv\textsubscript{Con} and PBS at 50 and 100 MOI. ($n=3$, $^*p<0.001$ respectively). (B) Proliferation was retarded at 100 MOI of Adv\textsubscript{PEDF} on the 4\textsuperscript{th} day post-infection ($n=3$, $^*p<0.05$). Results were presented as mean± SEM.
Figure 3. mRNA expression of VEGF, TSP-1, PEDF and MMP-9 were detected with real-time PCR. (A) Both VEGF and MMP-9 were down-regulated up to 6.33- and 3.92-fold respectively over AdvCon normalized by GAPDH (n=3, *p=0.001 and *p=0.013 accordingly). (B) TSP-1 1.69-fold up-regulated (n=3, *p<0.001). (C) AdvPEDF induced >266.6-fold of PEDF over-expression over AdvCon (n=3, *p<0.001). Results were presented as mean ± SEM.
Figure 4. T24 cells were treated with PBS, 100 MOI AdvPEDF or AdvCon, measured by flow cytometry with Annexin V and PI. Apparent deviation from Q3 (living cells, Annexin-/PI-) to Q4 (early apoptotic cells, Annexin+/PI-) was observed in AdvPEDF group (A) compared to AdvCon group (B). (C) Percentage of early apoptotic cells in each experiment was expressed as mean ± SEM. PEDF induced significantly higher portion of cell apoptosis (n=3, *p<0.05 respectively)
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Figure 5. Cell cycle arrest was detected with PI in T24 cells infected 100 MOI of AdvPEDF or AdvCon. Cells infected by AdvPEDF presented prominent arrest in G1 phase and less population in S phase (A) compared with by AdvCon (B). (C) Invasiveness was studied with Transwell assay carried out in cells exposed to PBS, 100 MOI AdvPEDF or AdvCon. The overall data was shown as mean ± SD (n=3, *p<0.05, respectively).

Expression of relevant genes and invasiveness assay.
Previously reported angiogenesis and invasiveness related genes [7] were detected with real-time RT-PCR. VEGF and MMP-9 expression were significantly down-regulated in AdvPEDF transfected cells (p=0.001 and p=0.013, respectively) up to 6.33-fold and 3.92-fold respectively (Fig. 3A). Expression of TSP-1 in AdvPEDF group was significantly up-regulated by 1.69-fold over AdvCon (p<0.001) (Fig. 3B). Invasiveness was studied using Transwell inserts and was interpreted by the amount of cells that protruded through the Matrigel covered membrane. Average count of penetrating cells were significantly lower in AdvPEDF group compared with AdvCon and PBS (p=0.034 and p=0.023, respectively), as shown in figure 5C.

In vivo tumor growth and MVD detection.
Therapeutic effect of over-expressed PEDF on tumor growth was investigated by injection with AdvPEDF, AdvCon or PBS in transplantation tumors. The tumors were calibrated
weekly until the 35th day postinjection when all 15 tumors were extracted. The average tumor volume in AdvPEDF group (374 mm³) were significantly less than AdvCon (1183 mm³) and PBS (1423 mm³) group (p<0.001 and p<0.001 respectively), and were statistically insignificant in AdvCon and PBS group (p=0.305) as shown in Fig. 6C. The MVD of AdvPEDF transfected tumors were apparently decreased compared with AdvCon group (Fig. 6A-B).

Figure 6. Suppression of tumor growth associated with decreased MVD on tumors inoculated in BALB/c nude mice. Immunohistochemistry with vWF antibody showed brown staining representing endothelium of microvessel, which was more sparsely distributed in AdvPEDF group (A) than AdvCon (B) at ×200. (C) Tumor volume was expressed as mean ± SD (n=5, *p<0.05 respectively). Arrow indicated schedule of injections of 100μl (100 MOI) AdvPEDF, AdvCon and PBS.

Discussion

Our preliminary study shows that PEDF expression is down-regulated in bladder tumor compared with healthy tissue. The results encourage us in the present study to further explore the inhibitive effects of PEDF on bladder cancer in vitro and in vivo. Dramatic outcomes are obtained, indicating that PEDF inhibited both cell growth and angiogenesis of bladder cancer potently.
Over-expression of PEDF mediated by viral vector has been studied in several malignancies and was demonstrated effective in tumor inhibition [1-5]. In concordance with reported studies, we have identified correct over-expression of PEDF mRNA and protein transduced by adenovirus. The viability and proliferation of T24 cells are significantly inhibited by PEDF expression. Cell assays also show that adenoviral vectored-PEDF exerts obvious inhibitive function on a 4-day basis post-transfection. The results we have attained in the study further support that PEDF can directly suppress bladder cancer cells. The hindered of cell proliferation and compromised viability may result from both increased apoptosis and cell cycle arrest induced by PEDF. Nevertheless, PEDF has been reported controversially to effect dually or oppositely on apoptosis of different phenotypes of endothelial cells [8]. In the case of Parkinson’s disease, PEDF has also been reported to be in positive correlation with expression of VEGF, the factor which inhibits tumor apoptosis, in striatum [9]. Thus, the pro-apoptotic effect of PEDF should be further studied in different cell lines of bladder cancer.

Local invasiveness of bladder cancer is suggestive of worsened prognosis and aggressive surgical strategy. Degradation of extracellular matrix (ECM) is prerequisite for tumor cells to exert further invasion. MMPs are most commonly studied among all the enzymes which degenerate the ECM due to the potent activity. MMP-9, which is one of the strongest in MMPs family, has been revealed to associate with both grade and stage of bladder cancer [10]. In our study, MMP-9 expression is down-regulated when PEDF is transduced in to T24 cells. Reduced invasiveness is also observed in the Transwell assay. Such findings indicate that invasiveness of bladder cancer may be compromised by decreased MMP-9 expression induced by over-expression of PEDF.

Not limited with direct inhibitive effects on viability and invasiveness of cancer cells, PEDF has majorly been characterized by anti-angiogenic effect on tumorous tissue [11] as well. VEGF has been proved to be regulating angiogenesis and be associating with MVD in bladder cancer[12]. Increased VEGF expression is in connection with progression of grade, stage and worsened prognosis in bladder cancer [10, 13]. On the contrary, TSP-1 is an anti-angiogenic factor, whose decrease has been reported to facilitate the growth of bladder cancer [10]. In the experimental treatment on murine models, the group treated with Adv_PEDF showed remarkable reduction of tumor growth with total tolerance given the dosage of 100 MOI. The MVD reduction in PEDF treated tumors is sufficient in demonstrating the efficacy of PEDF-bearing adenoviral injection. The findings in the present study indicate that exogenous over-expression of PEDF can induce down-regulation of VEGF expression and up-regulation of TSP-1 expression, which may contribute integrally in presenting the anti-angiogenic effect. Taking note that MMP-9 is also recognized as an pro-angiogenic factor, the anti-angiogenic mechanism of PEDF may involve VEGF, TSP-1 and MMP-9 in a shared pathway similar to what REN et al. [14] have postulated. Furthermore, as the adenoviral vector-delivered PEDF has already been used in phase I clinical trial in neovascular age-related macular degeneration treatment [15], experimental treatment with adenovirus-mediated PEDF in human bladder cancer may prove promising.

**Conclusion**

Collectively, our study has demonstrated that adenovirus-delivered PEDF induces retardation in cell viability and proliferation in T24 cells. PEDF also suppresses expression of
VEGF and MMP-9, yet promoting TSP-1 expression. Injection with PEDF-bearing adenovirus causes decrease in tumor volume and reduction of MVD. All the findings may contribute to a promising treatment strategy against bladder cancer.

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