

Pigment epithelium-derived factor inhibits caveolin-induced interleukin-8 gene expression and proliferation of human prostate cancer cells

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Abstract. Caveolin-1 (Cav), a primary protein component of caveolae, is overexpressed in prostate cancer, thereby promoting growth and metastasis of this tumor. By contrast, pigment epithelium-derived factor (PEDF) has been shown to inhibit tumor growth and metastasis, including that of prostate cancer, via its anti-angiogenic and anti-inflammatory effects. Although it was recently demonstrated that PEDF binds to Cav and blocks its pro-inflammatory actions in endothelial cells, it remains unclear whether PEDF also inhibits the tumor-promoting effects of Cav in cultured prostate cancer cells. The present study examined the effects of PEDF on cell growth, in addition to the gene expression of interleukin-8 (IL-8), which is involved in prostate cancer progression, in the PC-3 human prostate cancer cell line. Exogenous Cav led to a dose-dependent upregulation of the mRNA expression of IL-8 in PC-3 cells, which was blocked by treatment with 1 or 10 nM PEDF, or following the overexpression of small interfering RNAs directed against Cav. Cav (10 nM) increased DNA synthesis in PC-3 cells, which was again suppressed by the administration of 10 nM PEDF. The results of the present study indicated that PEDF may inhibit Cav-induced increases in IL-8 gene expression and proliferation of PC-3 cells. Therefore, the suppressive effects of PEDF in prostate cancer may, in part, be ascribed to its inhibitory actions on Cav.

Introduction

Caveolin-1 (Cav) is an important protein component of caveolae, a specialized type of membrane lipid raft, which are involved

in multiple cellular processes, such as molecular transport, cell adhesion and signal transduction (1,2). Recently, Cav has been shown to be overexpressed in prostate cancer, and to promote the growth and metastasis of this tumor (3-6). Cav has been demonstrated to activate oncogenic pathways involving Akt and to promote the proliferation of prostate cancer, in addition to stimulating tumor-associated angiogenesis and inflammation (3-6). Furthermore, in a large population study involving men with a serum prostate-specific antigen of >10 ng/ml, high pre-treatment serum levels of Cav were a prognostic marker for prostate cancer recurrence (7). Increased circulating levels of Cav were also shown to be associated with the histological grade and progression of prostate cancer (8,9). These observations suggest the involvement of Cav in tumor growth and aggressiveness of prostate cancer.

Pigment epithelium-derived factor (PEDF) is a glycoprotein that belongs to the superfamily of serine protease inhibitors, which exhibit potent neuronal differentiating activity (10). PEDF has been identified as a highly effective inhibitor of angiogenesis in cell culture models as well as in animal models (11,12). Furthermore, studies have demonstrated that PEDF blocks cytokine-induced and vascular endothelial growth factor-induced angiogenesis and inflammatory reactions, inhibits tumor growth and induces apoptosis in tumors, including prostate cancer (11-24). Our group has recently demonstrated that PEDF binds to Cav and blocks its pro-inflammatory effects in endothelial cells (25). It is therefore possible that PEDF may exert its antitumor effects in prostate cancer by modulating the actions of Cav. However, it remains unclear whether PEDF inhibits the tumor-promoting effects of Cav in cultured prostate cancer cells. The present study examined the effects of PEDF on prostate cancer cell growth and on the expression of the interleukin-8 (IL-8) gene, which contributes to prostate cancer progression in PC-3 cells, a human hormone-refractory (HRPC) cell line (17).

Materials and methods

Materials. Polyclonal anti-human rabbit antibodies (Abs) directed against Cav (cat no. 3238) and polyclonal biotin-conjugated Abs against PEDF (cat no. BAF 1177) were purchased

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from Cell Signaling Technology Japan K.K. (Tokyo, Japan) and R&D Systems (Minneapolis, MN, USA), respectively. Monoclonal mouse anti-human Abs raised against GAPDH were obtained from Santa Cruz Biotechnology, Inc., Dallas, TX, USA (cat no. sc-32233).

Expression and purification of Cav. Full-length human Cav (residues 1-178) was amplified using polymerase chain reaction (PCR), sub-cloned into the *Nde*I and *Xho*I sites of the pET21b vector, incorporating pentahistidine-tag into the C-terminus of the protein and purified as previously described (26). Briefly, the PCR primers for Cav were as follows: Forward, 5'-CCGGTCCATATGTCTGGGGGCAAATAC-3' and reverse, 5'-CCCCTCGAGTATTTCTTTCTGCAAGTT-3'. PCR reactions were performed using PrimeSTAR[®] HS (Takara Bio Inc., Shiga, Japan) and the PCR conditions were as follows: 35 cycles of 98°C for 10 sec, 55°C for 10 sec and 72°C for 120 sec.

Preparation of PEDF proteins. Hexahistidine-tagged PEDF proteins were purified from conditioned media using a Ni-NTA spin kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions as described previously (27). SDS-PAGE analysis of the purified PEDF proteins identified a single band with a molecular mass of ~50 kDa, which exhibited positive reactivity with monoclonal mouse Abs against human PEDF (cat. no. KM037; 1:1,000 dilution; Transgenic, Kumamoto, Japan).

Construction and transfection of small interfering RNAs (siRNAs). The following siRNAs to Cav (siCavs) were used: Sense, 5'-GCUUCCUGAUUGAGAUUCAtt-3' and antisense, 5'-UGAAUCUCAAUCAAGGAAGCtc for siCav#1; and sense, 5'-GCCGUGUCUAUCCAUCUAtt-3' and antisense, 5'-UAGAUGGAAUAGACACGGCtg for siCav#2. The siCavs were obtained from Life Technologies Japan, Ltd. (Tokyo, Japan). Non-silencing control siRNAs (siCon) were also obtained from Life Technologies Japan, Ltd. (Silencer Negative Control #1 siRNA). The siRNA duplexes were transfected into PC-3 cells using Lipofectamine 2000[™] (Invitrogen Life Technologies, Carlsbad, CA, USA), as previously described (28). Following 2 days of transfection, Cav and GAPDH protein levels were analyzed using western blotting.

Cells. PC-3 human prostate cancer cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin (Life Technologies Japan, Ltd.). PEDF or Cav treatment was conducted in a medium containing 1% fetal bovine serum.

Reverse transcription-quantitative PCR (RT-qPCR). Transfected or non-transfected PC-3 cells were treated with or without the indicated concentrations of Cav (0.1, 1 or 10 nM), in the presence or absence of 10 nM PEDF for 4 h. Total RNA was then extracted using a NucleoSpin RNA kit (Takara Bio Inc.), according to the manufacturer's instructions. qRT-PCR was performed using Assay-on-Demand and TaqMan 5 fluorogenic nuclease chemistry (Life Technologies Japan Ltd.), according to the manufacturer's instructions. The IDs of the primers for human interleukin-8 (IL-8) and the β -actin gene (Applied

Biosystems Life Technologies, Foster City, CA, USA) were Hs99999034_m1 and Hs01060665_g1, respectively. RT-qPCR conditions were as follows: 50°C for 2 min and 95°C 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 2 min. The results were quantified using the $2^{-\Delta\Delta CT}$ method (29).

Serum preparation. Serum was obtained from 3 individuals: A healthy 38 year-old male [body mass index (BMI), 21.1]; a 59 year-old diabetic man with cardiovascular disease (BMI, 31.1; HbA1c, 8.6%), as PEDF levels have been shown to increase in diabetic patients (24); and a 64 year-old patient with stage II prostate cancer (BMI unknown). All prostate cancer patient serum specimens were purchased from Tissue Solutions Ltd., Glasgow, UK.

Western blotting analysis. Proteins were extracted from siCon- or siCav-transfected PC-3 cells, using lysis buffer as previously described (28). The samples or serum were then separated by SDS-PAGE and transferred to nitrocellulose membranes (Life Technologies Japan, Ltd.). Membranes were probed with Abs against Cav (1:1,000 dilution), PEDF (1:1,000 dilution) or GAPDH (1:200 dilution) for 12 h at room temperature, and then incubated with peroxidase-conjugated polyclonal donkey anti-rabbit IgG Abs (1:20,000 dilution; cat no. NA934-100UL; GE Healthcare UK Ltd., Little Chalfont, UK), streptavidin-conjugated peroxidase (1:5,000) or polyclonal sheep anti-mouse Abs (1:20,000 dilution; cat no. NA931-100UL; GE Healthcare UK Ltd.), respectively. Immune complexes were visualized using an enhanced chemiluminescence detection system (Amersham Bioscience, Buckinghamshire, UK). The protein signals were quantified using ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA). The Cav protein levels of each sample were normalized to those of GAPDH.

Measurement of [³H] thymidine incorporation into PC-3 cells. PC-3 cells were treated with or without 10 nM Cav, in the presence or absence of 10 nM PEDF for 20 h. Subsequently, [³H] thymidine was added, to produce a final concentration of 1 mCi/ml, and cells were incubated for a further 4 h. Following incubation, PC-3 cells were fixed with ice-cold 10% (w/v) trichloroacetic acid for 20 min. [³H] thymidine incorporation into PC-3 cells was measured as described previously (30). Briefly, [³H] thymidine incorporation into PC-3 cells was measured by processing the resultant acid-insoluble materials for liquid scintillation counting (AccuFLEX LSC 7200; Hitachi Aloka Medical, Ltd., Tokyo, Japan).

Statistical analysis. Data are presented as the mean \pm standard error. Student's t-test was performed for statistical comparisons. All statistical analyses were performed using PASW Statistics version 18.0 software (SPSS Japan Inc., Tokyo, Japan) and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cav leads to upregulation of IL-8 expression in PC-3 cells and this effect is abrogated by PEDF. The effect of PEDF on IL-8 gene expression in Cav-exposed PC-3 cells was

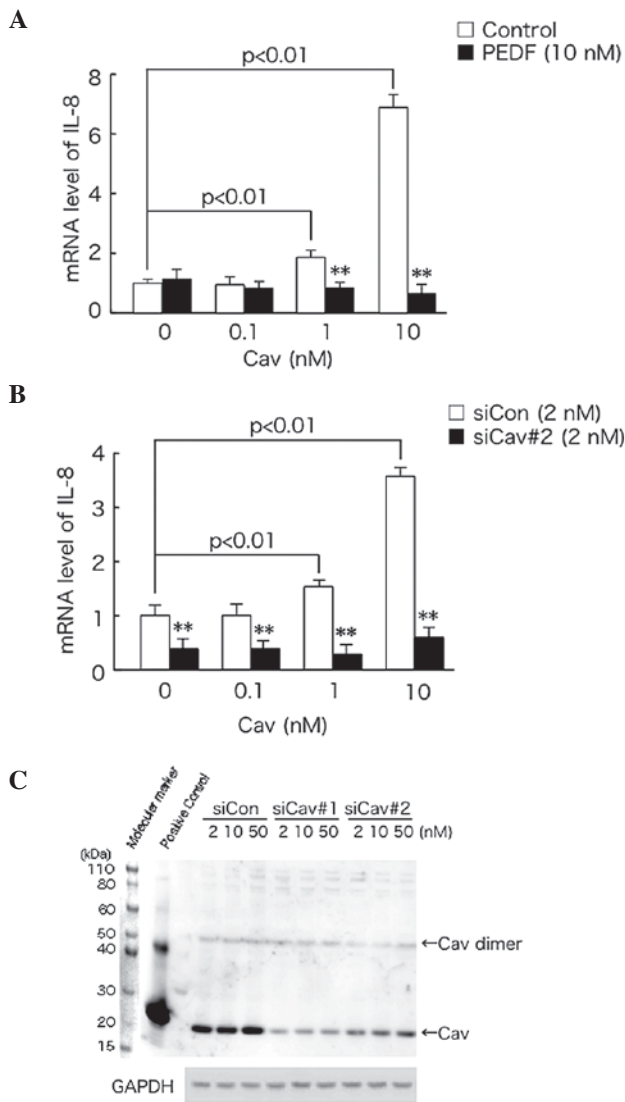


Figure 1. Effect of PEDF on IL-8 gene expression in PC-3 cells. (A) Non-transfected or (B) transfected PC-3 cells were treated with or without the indicated concentrations of Cav, in the presence or absence of 10 nM PEDF for 4 h. (A and B) Total RNA was transcribed and amplified by reverse transcription-quantitative polymerase chain reaction. Data were normalized to the level of β -actin, and then compared with the control value without PEDF (A) or to the value obtained with siCon treatment alone (B). ** $P<0.01$, compared with the group without PEDF (A) or with the siCon group (B). n=4. (C) Proteins were extracted from transfected PC-3 cells. Western blot analysis was performed using anti-Cav antibodies. PEDF, pigment epithelium-derived factor; IL-8, interleukin-8; Cav, caveolin-1; siCon, small interfering control RNA; siCav, siRNA to Cav.

investigated. As shown in Fig. 1A, exogenously administered Cav upregulated the mRNA expression of IL-8 in PC-3 cells, in a dose-dependent manner. This effect was abrogated by treatment with 1 or 10 nM PEDF.

Cav increased IL-8 mRNA expression in siCon-transfected cells, in a dose-dependent manner and this effect was suppressed by siCav#2 transfection (Fig. 1B). Furthermore, transfection with siCav#1 or siCav#2, reduced Cav expression in PC-3 cells to 1/5-1/10 of that of the siCon-transfected control cells (Fig. 1C).

PEDF leads to decreased DNA synthesis in PC-3 cells. The effect of PEDF on DNA synthesis in PC-3 cells was also

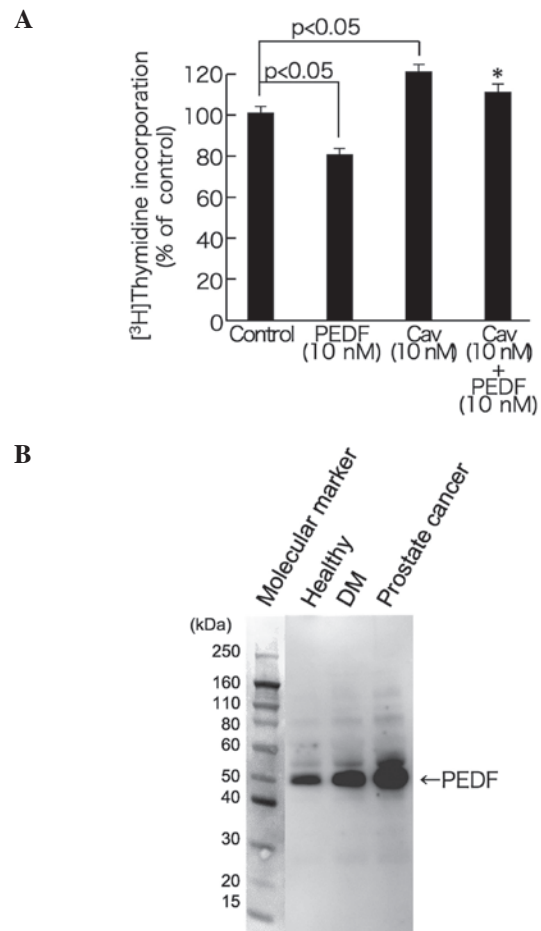


Figure 2. Effect of PEDF on DNA synthesis in PC-3 cells (A) and circulating PEDF levels in three subjects (B). (A) PC-3 cells were treated with or without 10 nM Cav, in the presence or absence of 10 nM PEDF for 20 h. Subsequently, [³H]thymidine was added, to produce a final concentration of 1 μ Ci/ml, and cells were further incubated for 4 h. Following incubation, PC-3 cells were fixed, and [³H]thymidine incorporation into PC-3 cells was measured. * $P<0.05$, compared with the 10 nM Cav alone group. n=4. (B) Serum was separated using SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then probed with antibodies against PEDF. PEDF, pigment epithelium-derived factor; Cav, caveolin-1; DM, diabetes mellitus.

examined. As shown in Fig. 2A, 10 nM Cav significantly increased DNA synthesis in PC-3 cells. This effect was inhibited by treatment with 10 nM PEDF. PEDF (10 nM) also significantly decreased DNA synthesis in PC-3 cells when administered alone.

Finally, the circulating levels of PEDF in the serum of the patient with prostate cancer were higher than those of the healthy control or the diabetic subjects (Fig. 2B).

Discussion

There is accumulating evidence that overexpression of Cav promotes tumor growth and metastasis of prostate cancer via diverse pathways (3-6), while PEDF may protect against the progression of prostate cancer, via anti-angiogenic, anti-inflammatory and proapoptotic effects (17-19). To the best of our knowledge, the present study demonstrated for the first time that treatment with 1 or 10 nM PEDF significantly inhibited the Cav-induced increase in IL-8 mRNA expression in PC-3

cells. Transfection with siCav#2 reduced endogenous Cav expression in PC-3 cells to ~1/5 of that of siCon-transfected cells, and decreased IL-8 mRNA expression in Cav-exposed PC-3 cells. Our group has recently demonstrated that PEDF directly binds to Cav at a K_D value of 7.36×10^{-7} M; that exogenously administered Cav is taken up into endothelial cells, resulting in increases in the membrane levels of Cav and promoting inflammatory reactions; and that siCav#1 treatment reduces Cav expression and suppresses inflammatory gene expression in endothelial cells (25). These observations indicate that PEDF may reduce Cav-induced IL-8 gene expression by binding to exogenously administered Cav and modulating its membrane levels in PC-3 cells, a type of hormone-refractory prostate cancer (HRPC) cell. IL-8 may promote the transition of prostate cancer to HRPC by upregulating androgen receptor expression and activation, and may stimulate the proliferation and migration of this cell type (17). Circulating Cav levels have been shown to be increased in patients with HRPC, while they were not increased in those with hormone-sensitive cancer (9). These findings further support the hypothesis that PEDF may inhibit the growth and metastasis of HRPC cells, such as PC-3 cells, by blocking Cav-evoked IL-8 overexpression.

The present study also demonstrated that PEDF inhibited the Cav-induced increase in DNA synthesis in PC-3 cells. Treatment with PEDF alone also decreased [3 H] thymidine incorporation into PC-3 cells. Therefore, PEDF may directly inhibit the proliferation of PC-3 cells via modulation of the growth-promoting activity of Cav.

To the best of our knowledge, the current study also demonstrated for the first time that circulating PEDF levels were higher in a patient with prostate cancer, compared with levels in the healthy control or diabetic subjects. As reported previously (31), PEDF levels in diabetic subjects were higher than those in healthy controls. The antitumor effects of PEDF that were observed in the present study, suggest that circulating PEDF levels may be elevated as a counter-system against Cav overexpression in prostate cancer. Treatment with Cav antisera reduced the development and growth of primary tumors and metastases in mouse models of prostate cancer (32). Since it has been demonstrated that the laminin receptor mediates the anti-inflammatory and antithrombotic effects of PEDF in malignant myeloma cells (33), blockade of the effects of Cav using a PEDF-laminin receptor system may be a novel therapeutic approach for prostate cancer.

It has previously been reported that the human blood concentration of PEDF is 100-200 nM (31,34). However, the majority of circulating PEDF in serum may exist as a protein-bound and inactive form, and the free active form of PEDF comprises only a small portion of the total level of this protein (31). In addition, a 4 nM-increase in serum PEDF levels has been shown to exert anti-inflammatory effects in animal models (35,36). Therefore, it is unlikely that PEDF at 10 nM would produce toxic effects on PC-3 cells. Recently, serum levels of Cav were demonstrated to be increased in patients with prostate cancer compared with healthy controls. Serum levels of Cav were highest in those in the advanced stages of prostate cancer (8). Furthermore, epithelial cell hyperplasia in the prostate, in association with

increased angiogenesis, was observed in PEDF-deficient mice (16). The present results support the involvement of an interaction between PEDF and Cav in prostate cancer progression *in vivo*.

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